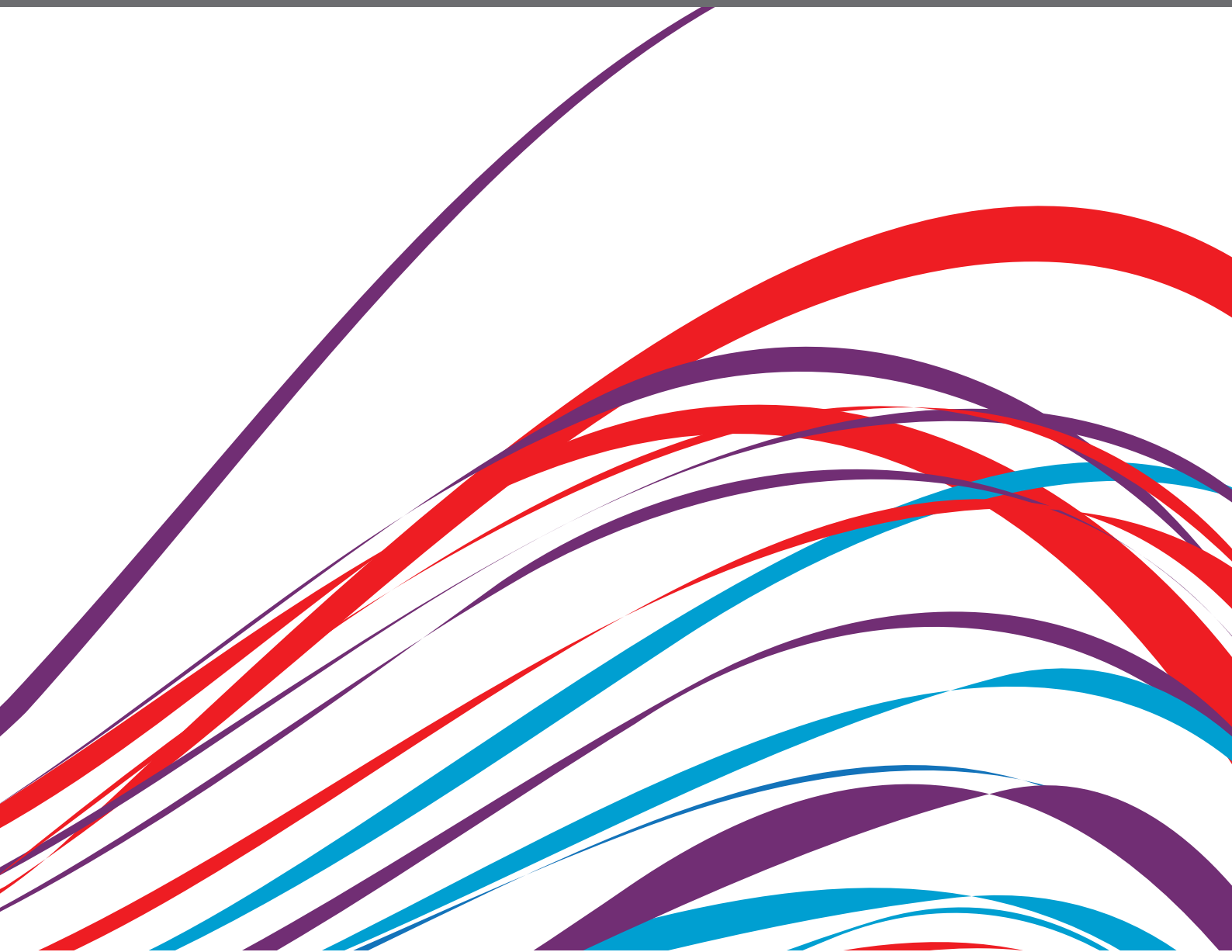


ESTABLISHED AND NOVEL ROLES OF PLATELETS IN HEALTH AND DISEASE

EDITED BY: Marie Lordkipanidzé, Vera Ignjatovic and Paul Jurasz
PUBLISHED IN: Frontiers in Cardiovascular Medicine





frontiers

Frontiers eBook Copyright Statement

The copyright in the text of individual articles in this eBook is the property of their respective authors or their respective institutions or funders. The copyright in graphics and images within each article may be subject to copyright of other parties. In both cases this is subject to a license granted to Frontiers.

The compilation of articles constituting this eBook is the property of Frontiers.

Each article within this eBook, and the eBook itself, are published under the most recent version of the Creative Commons CC-BY licence.

The version current at the date of publication of this eBook is CC-BY 4.0. If the CC-BY licence is updated, the licence granted by Frontiers is automatically updated to the new version.

When exercising any right under the CC-BY licence, Frontiers must be attributed as the original publisher of the article or eBook, as applicable.

Authors have the responsibility of ensuring that any graphics or other materials which are the property of others may be included in the CC-BY licence, but this should be checked before relying on the CC-BY licence to reproduce those materials. Any copyright notices relating to those materials must be complied with.

Copyright and source acknowledgement notices may not be removed and must be displayed in any copy, derivative work or partial copy which includes the elements in question.

All copyright, and all rights therein, are protected by national and international copyright laws. The above represents a summary only. For further information please read Frontiers' Conditions for Website Use and Copyright Statement, and the applicable CC-BY licence.

ISSN 1664-8714

ISBN 978-2-88974-552-4

DOI 10.3389/978-2-88974-552-4

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.

Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

ESTABLISHED AND NOVEL ROLES OF PLATELETS IN HEALTH AND DISEASE

Topic Editors:

Marie Lordkipanidzé, Université de Montréal, Canada

Vera Ignjatovic, Murdoch Childrens Research Institute, Australia

Paul Jurasz, University of Alberta, Canada

Citation: Lordkipanidzé, M., Ignjatovic, V., Jurasz, P., eds. (2022). Established and Novel Roles of Platelets in Health and Disease. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88974-552-4

Table of Contents

- 05 Editorial: Established and Novel Roles of Platelets in Health and Disease**
Paul Jurasz, Vera Ignjatovic and Marie Lordkipanidzé
- 09 Platelet Function During Extracorporeal Membrane Oxygenation in Adult Patients: A Systematic Review**
Camilla Mains Balle, Anni Nørgaard Jeppesen, Steffen Christensen and Anne-Mette Hvas
- 19 Inherited Thrombocytopenia: Update on Genes and Genetic Variants Which may be Associated With Bleeding**
Ibrahim Almazni, Rachel Stapley and Neil V. Morgan
- 28 Platelet-Neutrophil Interplay: Insights Into Neutrophil Extracellular Trap (NET)-Driven Coagulation in Infection**
Amanda Z. Zucoloto and Craig N. Jenne
- 36 Circadian Rhythm of Cardiovascular Disease: The Potential of Chronotherapy With Aspirin**
Marleen Buurma, Jeske J. K. van Diemen, Abel Thijs, Mattijs E. Numans and Tobias N. Bonten
- 43 Comparative Analysis of Microfluidics Thrombus Formation in Multiple Genetically Modified Mice: Link to Thrombosis and Hemostasis**
Magdolna Nagy, Johanna P. van Geffen, David Stegner, David J. Adams, Attila Braun, Susanne M. de Witt, Margitta Elvers, Mitchell J. Geer, Marijke J. E. Kuijpers, Karl Kunzelmann, Jun Mori, Cécile Oury, Joachim Pircher, Irina Pleines, Alastair W. Poole, Yotis A. Senis, Remco Verdoodt, Christian Weber, Bernhard Nieswandt, Johan W. M. Heemskerk and Constance C. F. M. J. Baaten
- 58 Platelet Function in Aging**
Jessica Le Blanc and Marie Lordkipanidzé
- 66 Platelet Function During Extracorporeal Membrane Oxygenation in Adult Patients**
Camilla Mains Balle, Anni Nørgaard Jeppesen, Steffen Christensen and Anne-Mette Hvas
- 78 Regenerative Effect of Platelet Concentrates in Oral and Craniofacial Regeneration**
Faez Saleh Al-Hamed, Mohammed Mahri, Haider Al-Waeli, Jesus Torres, Zahi Badran and Faleh Tamimi
- 92 Platelets and Complement Cross-Talk in Early Atherogenesis**
Hugh Kim and Edward M. Conway
- 103 Platelets are at the Nexus of Vascular Diseases**
Héloïse Lebas, Katia Yahiaoui, Raphaël Martos and Yacine Boulaftali
- 122 Advances in Platelet Subpopulation Research**
Gabriela Lesyk and Paul Jurasz
- 130 Effect on Platelet Function of Metal-Based Nanoparticles Developed for Medical Applications**
Nadhim Kamil Hante, Carlos Medina and Maria Jose Santos-Martinez

- 140 Platelet Phenotype and Function in the Setting of Pediatric Extracorporeal Membrane Oxygenation (ECMO): A Systematic Review**
Hui Ping Yaw, Suelyn Van Den Helm, Graeme MacLaren, Matthew Linden, Paul Monagle and Vera Ignjatovic
- 147 Shear-Dependent Platelet Aggregation: Mechanisms and Therapeutic Opportunities**
Akshita Rana, Erik Westein, Be'eri Niego and Christoph E. Hagemeyer
- 168 Role of Platelet Mitochondria: Life in a Nucleus-Free Zone**
Hannah Melchinger, Kanika Jain, Tarun Tyagi and John Hwa
- 179 Stratified Approaches to Antiplatelet Therapies Based on Platelet Reactivity Testing**
Matgorzata Ostrowska, Jacek Kubica, Piotr Adamski, Aldona Kubica, Ceren Eyileten, Marek Postula, Aurel Toma, Christian Hengstenberg and Jolanta M. Siller-Matula
- 193 Procoagulant Phosphatidylserine-Exposing Platelets in vitro and in vivo**
Emily C. Reddy and Margaret L. Rand
- 204 Platelet Pathogen Reduction Technologies Alter the MicroRNA Profile of Platelet-Derived Microparticles**
Idrissa Diallo, Abderrahim Benmoussa, Jonathan Laugier, Abdimajid Osman, Walter E. Hitzler and Patrick Provost
- 213 Platelet Measurements and Type 2 Diabetes: Investigations in Two Population-Based Cohorts**
Benjamin A. T. Rodriguez and Andrew D. Johnson



Editorial: Established and Novel Roles of Platelets in Health and Disease

Paul Jurasz^{1,2,3,4}, Vera Ignjatovic^{5,6} and Marie Lordkipanidze^{7,8*}

¹ Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, AB, Canada, ² Department of Pharmacology, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, AB, Canada, ³ Cardiovascular Research Centre, University of Alberta, Edmonton, AB, Canada, ⁴ Mazankowski Alberta Heart Institute, University of Alberta, Edmonton, AB, Canada, ⁵ Department of Haematology Research, Murdoch Children's Research Institute, Melbourne, VIC, Australia, ⁶ Department of Paediatrics, The University of Melbourne, Melbourne, VIC, Australia, ⁷ Research Center, Montreal Heart Institute, Montreal, QC, Canada, ⁸ Faculty of Pharmacy, Université de Montréal, Montreal, QC, Canada

Keywords: platelets, thrombosis and haemostasis, non-traditional roles, special populations, platelet function

Editorial on the Research Topic

Established and Novel Roles of Platelets in Health and Disease

Platelets have been long recognized for their role in maintaining hemostasis, with defects in their number or function leading to bleeding. This has led many to simply think of platelets as “band-aids” of the vascular system (1). Conversely, as appreciated by cardiologists, neurologists, and hematologists alike, platelet hyperactivity is itself a predisposition to thrombosis. Beyond the recognized Yin and Yang dualism of regulating bleeding and thrombosis, platelets are increasingly emerging as key regulators of an array of diverse physiological and pathological processes. Technological advancements have allowed researchers to identify the contribution of platelets to vascular and lymphatic development (2–4), maintenance of vascular integrity and maturation of the circulatory system (5, 6), as well in formation of new blood vessels (7–9). The fact that platelets also participate in inflammation and immunity, in part, via their surface expression of P-selectin, toll-like receptors (10, 11), complement receptors (12), CD40L (13), and programmed death—ligand 1 (14) is now well recognized. Correspondingly, platelets respond to both viral and bacterial infections (15–17), and have even been caught in the act of migration to scavenge bacteria (18). Platelets also contribute to wound healing and bone formation (19). Thus, platelets and their-derived products are also frequently used as therapeutic agents, increasing the healing efficiency in maxillo-facial and plastic surgery (20), as well as in sports medicine (21).

On the other hand, platelets are involved in pathological conditions including but not limited to atherosclerosis and related diseases, cancer (22), and diseases of the central nervous system including Alzheimer's disease, depression and multiple sclerosis (23–25). Moreover, platelets participate in the pathophysiology of auto-immune diseases, such as allergies, skin diseases, rheumatoid arthritis and liver disease (26–28).

This extraordinary capability of platelets to regulate such an extensive array of physiological and pathophysiological processes largely stems from their ability to store and release a wide range of biologically active substances via their granules and microparticles (29, 30). Platelets also express biochemical and functional heterogeneity, with numerous platelet subpopulations having been identified, including the procoagulant platelet type (31, 32). This potential specialization of function is likely to contribute to the diverse roles of platelets. It is therefore unsurprising that platelets are no longer viewed as simply “band-aids” of the vascular system. In an effort to raise awareness of the ever-increasing scope of platelet functions, this Research Topic aims to increase our current understanding of the wider contribution of platelets in physiological and pathological conditions.

OPEN ACCESS

Edited and reviewed by:

Masanori Aikawa,
Brigham and Women's Hospital and
Harvard Medical School,
United States

*Correspondence:

Marie Lordkipanidze
marie.lordkipanidze@umontreal.ca

Specialty section:

This article was submitted to
Atherosclerosis and Vascular
Medicine,
a section of the journal
Frontiers in Cardiovascular Medicine

Received: 14 December 2021

Accepted: 10 January 2022

Published: 31 January 2022

Citation:

Jurasz P, Ignjatovic V and
Lordkipanidze M (2022) Editorial:
Established and Novel Roles of
Platelets in Health and Disease.
Front. Cardiovasc. Med. 9:835615.
doi: 10.3389/fcvm.2022.835615

Identifying novel genetic determinants of platelet disorders, Almazni et al. summarize advancements in diagnosis of inherited thrombocytopenia, covering the spectrum from phenotypic approaches, high-throughput sequencing, to targeted panels and bioinformatics. The authors conclude that a combination of the genomics approaches with high throughput data handling pipelines is essential in ensuring transformational advancements in patient diagnosis and personalized care.

Rana et al. review our current knowledge of how shear forces impact aggregation and thrombosis. With a major focus on the shear-sensitive protein von Willebrand Factor, the shear-induced activation, conformational changes, interaction with GPIIb α and role in platelet arrest is summarized. The authors also discuss platelet aggregation under shear gradients and novel therapeutic strategies that aim to reduce shear-induced platelet activation and thrombus formation.

Kim and Conway review the complement system and describe its interaction with platelets and contribution to early-stage atheroma formation. They describe how complement proteins activate platelets and that, in turn, activated platelets in conjunction with complement proteins may propagate vascular inflammation. Understanding the complex cross-talk between platelet, vascular endothelium, and the complement system in early atheroma formation may allow for development of novel anti-complement therapies to treat atherosclerosis.

In their article Melchinger et al. discuss how a lack of nucleus allows platelets to efficiently travel through small blood vessels and rapidly undergo extreme morphological changes that enable to platelets to efficiently respond to disruptions in hemostasis, infection, and inflammation. Hence, in the absence of a nucleus, the authors describe how mitochondria are key to powering these platelet responses and as well as regulating platelet lifespan.

The ability of platelets to interact with vascular cells and leukocytes at sites of vascular injury and inflammation carves out an important role for platelets as modulators of vascular diseases. In their review, Héloïse Lebas et al. describe how this crosstalk influences vascular diseases in different vascular beds. In their review of the platelet-neutrophil crosstalk, Zucoloto and Jenne explore in more detail how neutrophil extracellular trap (NET)-driven coagulation influences outcomes in the context of infectious diseases, and propose targeting of NETs as a potential therapeutic avenue to uncouple immunity and coagulation. Conversely, this ability of platelets to interact with circulating elements can also be deleterious, an aspect that is discussed by Hante et al. in their review of metal-based nanoparticles developed for medical applications.

Platelet subpopulations are an important area of research. For instance, the ability of platelets to engage with the coagulation system is explored by Reddy and Rand in their review of procoagulant platelets. Phosphatidylserine (PS) exposure, a key event in the platelet-coagulation interplay, is dissected from the perspective of inherited and acquired bleeding disorders, as well as a possible antithrombotic target. Lesyk and Jurasz further dive into platelet subpopulation diversity focusing on their physical, biochemical, and functional heterogeneity, the dynamic nature of platelet characteristics in health and disease, and how differences between individuals can influence platelet responsiveness to

antiplatelet therapy. In their review, Le Blanc and Lordkipanidzé look at how age-related changes in platelet biology could lead to thrombotic disease. In the context of global aging populations, it is important to investigate how platelets from older subjects differ in their function and structure from their younger counterparts. Evidence showing platelets from the elderly to be more prone to activation and less sensitive to inhibition highlight important areas for future work.

Ostrowska et al. discuss the current role and future perspectives of platelet function testing to optimize antiplatelet therapy. With evidence of high on-treatment platelet reactivity being associated with thrombotic outcomes and low on-treatment platelet reactivity being associated with bleeding, the idea of a therapeutic window for optimal platelet inhibition is presented. They further highlight that de-escalation of antiplatelet therapies may help balance the delicate bleeding/thrombotic risk. Targeting circadian rhythms in cardiovascular disease to improve the efficacy of antiplatelet agents is at the heart of the review by Buurma et al. The evidence for chronotherapy, especially with aspirin, is presented and discussed, and the authors call for randomized controlled trials with cardiovascular events and side-effects as endpoints, to help translate pharmacological findings to potential clinical benefits.

Extracorporeal membrane oxygenation (ECMO) presents important hemostatic challenges, in already vulnerable patients. Balle et al. summarize the state of knowledge in the setting of adult ECMO patients, and identify a significant knowledge gap in our understanding of platelet function in this critical clinical setting. Specifically, the authors point out the limitations of methods such as platelet aggregation in the setting of low platelet count, as well as in the lack of studies focusing on association between platelet function and clinical outcomes such as bleeding and thrombosis. This is clearly a field with excellent potential for future research, particularly due to the fact that ECMO is a high complication, high cost setting. In addition, the pediatric population is often under-studied, and the very limited platelet phenotype and function data in the pediatric ECMO population is further explored by Yaw et al. The systematic review highlights multiple research gaps in the setting of pediatric ECMO, highlighting the need for robust, well-designed studies in children.

In a different register, the review by Al-Hamed et al. provides an overview of platelet concentrates (PC) and the ability of platelets to enhance bone and soft tissue healing. Subsequently, the authors summarize the various clinical applications of platelet concentrates in the context of oral and craniofacial regeneration, and discuss some of the reasons for the inconsistencies in observed effects of PC on tissue regeneration.

This Research Topic also contains four original research articles. Using a standardized microfluidics and multi-parameter approach Nagy et al. quantitatively examine the changes in collagen-dependent thrombus formation for 38 different strains of genetically modified mice. Their novel approach can be used to determine the severity of the platelet defect and stage at which the altered thrombus function occurs. Diallo et al. investigate the impact of platelet pathogen reduction technologies on platelet microRNA content demonstrating that they may impact

microRNA loading into platelet microparticles. Balle et al. present an original prospective study of platelet function in 33 adult patients undergoing ECMO support utilizing impedance aggregometry and flow cytometry. The authors present a novel approach to data analysis and interpretation by accounting for platelet count when interpreting the aggregometry data, and suggest that platelets might not be as impaired during ECMO support as previously suggested. This study confirms the need for additional studies in this field. Rodriguez and Johnson show platelet parameters that segregate with diabetic status, using platelet function assessment collected in the Framingham Heart Study Offspring cohort and platelet indices available in the UK

BioBank cohort. The largest analysis to date, this study argues for the use of mean platelet volume as a biomarker to segment pre-diabetics and diabetics for risk prediction.

We hope that the articles within this Research Topic will spur further research into the diverse roles platelets play in physiological and pathological conditions within and beyond hemostasis and thrombosis.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

REFERENCES

- Leslie M. Beyond clotting: the powers of platelets. *Science*. (2010) 328:562–4. doi: 10.1126/science.328.5978.562
- Lowe KL, Finney BA, Deppermann C, Hägerling R, Gazit SL, Frampton J, et al. Podoplanin and CLEC-2 drive cerebrovascular patterning and integrity during development. *Blood*. (2015) 125:3769–77. doi: 10.1182/blood-2014-09-603803
- Bertozzi CC, Schmaier AA, Mericko P, Hess PR, Zou Z, Chen M, et al. Platelets regulate lymphatic vascular development through CLEC-2–SLP-. *Signaling*. 73:661–70. doi: 10.1182/blood-2010-02-270876
- Carramolino L, Fuentes J, Garc  a-Andr  s C, Azcoitia V, Riethmacher D, Torres M. Platelets play an essential role in separating the blood and lymphatic vasculatures during embryonic angiogenesis. *Circul Res*. (2017) 106:1197–201. doi: 10.1161/CIRCRESAHA.110.218073
- Kisucka J, Butterfield CE, Duda DG, Eichenberger SC, Saffaripour S, Ware J, et al. Platelets and platelet adhesion support angiogenesis while preventing excessive hemorrhage. *Proceed Nat Acad Sci USA*. (2006) 103:855–60. doi: 10.1073/pnas.0510412103
- Echtler K, Stark K, Lorenz M, Kerstan S, Walch A, Jennen L, et al. Platelets contribute to postnatal occlusion of the ductus arteriosus. *Nat Med*. (2010) 16:75–82. doi: 10.1038/nm.2060
- Jurasz P, Alonso D, Castro-Blanco S, Murad F, Radomski MW. Generation and role of angiotatin in human platelets. *Blood*. (2003) 102:3217–23. doi: 10.1182/blood-2003-02-0378
- Brill A, Dashevsky O, Rivo J, Gozal Y, Varon D. Platelet-derived microparticles induce angiogenesis and stimulate post-ischemic revascularization. *Cardiovasc Res*. (2005) 67:30–8. doi: 10.1016/j.cardiores.2005.04.007
- Amano H, Hackett NR, Rafi S, Crystal RG. Thrombopoietin gene transfer-mediated enhancement of angiogenic responses to acute ischemia. *Circ Res*. (2005) 97:337–45. doi: 10.1161/01.RES.0000179534.17668.f8
- Shiraki R, Inoue N, Kawasaki S, Takei A, Kadotani M, Ohnishi Y, et al. Expression of Toll-like receptors on human platelets. *Thrombosis Res*. (2004) 113:379–85. doi: 10.1016/j.thromres.2004.03.023
- Andonegui G, Kerfoot SM, McNagny K, Ebbert KJ, Patel KD, Kubes P. Platelets express functional Toll-like receptor-4. *Blood*. (2005) 106:2417–23. doi: 10.1182/blood-2005-03-0916
- Peerschke EIB, Yin W, Grigg SE, Ghebrehiet W. Blood platelets activate the classical pathway of human complement. *J Thrombosis Haemost*. (2006) 4:2035–42. doi: 10.1111/j.1538-7836.2006.02065.x
- Henn V SJ, Gr  fe M, Anagnostopoulos I, F  rster R, M  ller-Berghaus G, Kroczeck RA. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature*. (1998) 391:591–4. doi: 10.1038/35393
- Rolfes V, Idel C, Pries R, Pl  tze-Martin K, Habermann J, Gemoll T, et al. PD-L1 is expressed on human platelets and is affected by immune checkpoint therapy. *Oncotarget*. (2018) 9:27460–70. doi: 10.18632/oncotarget.25446
- Clark SR, Ma AC, Tavener SA, McDonald B, Goodarzi Z, Kelly MM, et al. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nature Med*. (2007) 13:463–9. doi: 10.1038/nm1565
- Wang J, Zhang W, Nardi MA, Li Z. HIV-1 Tat-induced platelet activation and release of CD154 contribute to HIV-1-associated autoimmune thrombocytopenia. *J Thrombosis Haemostasis: JTH*. (2011) 9:562–73. doi: 10.1111/j.1538-7836.2010.04168.x
- Onlamoon N, Noisakran S, Hsiao H-M, Duncan A, Villinger F, Ansari AA, et al. Dengue virus-induced hemorrhage in a nonhuman primate model. *Blood*. (2010) 115:1823–34. doi: 10.1182/blood-2009-09-242990
- Gaertner F, Ahmad Z, Rosenberger G, Fan S, Nicolai L, Busch B, et al. Migrating platelets are mechano-scavengers that collect and bundle bacteria. *Cell*. (2017) 171:1368–82. doi: 10.1016/j.cell.2017.11.001
- Li Q, Reed DA, Min L, Gopinathan G, Li S, Dangaria SJ, et al. Lyophilized platelet-rich fibrin (prf) promotes craniofacial bone regeneration through runx2. *Int J Mol Sci*. (2014) 15:5. doi: 10.3390/ijms15058509
- Del Corso M, Vervelle A, Simonpieri A, Jimbo R, Inchingolo F, Sammartino G, et al. Current knowledge and perspectives for the use of platelet-rich plasma (PRP) and platelet-rich fibrin (PRF) in oral and maxillofacial surgery part 1: Periodontal and dentoalveolar surgery. *Curr Pharmaceutical Biotechnol*. (2012) 13:1207–30. doi: 10.2174/138920112800624391
- Randelli PS, Arrigoni P, Cabitza P, Volpi P, Maffulli N. Autologous platelet rich plasma for arthroscopic rotator cuff repair: a pilot study. *Disabil Rehabil*. (2008) 30:1584–9. doi: 10.1080/09638280801906081
- Yan M, Jurasz P. The role of platelets in the tumor microenvironment: from solid tumors to leukemia. *Biochimica et Biophysica Acta (BBA)*. (2016) 1863:392–400. doi: 10.1016/j.bbamer.2015.07.008
- Donner L, F  lker K, Gremer L, Klinker S, Pagani G, Ljungberg LU, et al. Platelets contribute to amyloid-   aggregation in cerebral vessels through integrin   IIb  3-induced outside-in signaling and clusterin release. *Science Signalling*. (2016) 9:1937–9145. doi: 10.1126/scisignal.aaf6240
- Sonia D'Souza C, Li Z, Luke Maxwell D, Trusler O, Murphy M, Crewther S, et al. Platelets drive inflammation and target gray matter and the retina in autoimmune-mediated encephalomyelitis. *Electronic*. 9:1554–6578. doi: 10.1126/electronic.aaf6240
- Starosom SC, Veremeyko T, Yung AW, Dukhinova M, Au C, Lau AY, et al. Platelets play differential role during the initiation and progression of autoimmune neuroinflammation. *Circ Res*. (2015) *Electronic*. 117:1524–4571. doi: 10.1161/CIRCRESAHA.115.306847
- Boilard E, Nigrovic Peter A, Larabee K, Watts Gerald FM, Cobyln Jonathan S, Weinblatt Michael E, et al. Platelets amplify inflammation in arthritis via collagen-dependent microparticle production. *Science*. (2010) 327:580–3. doi: 10.1126/science.1181928
- Boilard E, Blanco P, Nigrovic PA. Platelets: active players in the pathogenesis of arthritis and SLE. *Nat Rev Rheumatol*. (2012) 8:534–42. doi: 10.1038/nrrheum.2012.118
- Chauhan A, Adams DH, Watson SP, Lalor PF. Platelets: no longer bystanders in liver disease. *Hepatology*. (2016) 64:1774–84. doi: 10.1002/hep.28526

29. Golebiewska EM, Poole AW. Platelet secretion: from haemostasis to wound healing and beyond. *Blood Rev.* (2015) 29:153–62. doi: 10.1016/j.blre.2014.10.003
30. Joshi S, Whiteheart SW. The nuts and bolts of the platelet release reaction. *Platelets.* (2017) 28:129–37. doi: 10.1080/09537104.2016.1240768
31. Dachary-Prigent J, Freyssinet JM, Pasquet JM, Carron JC, Nurden AT. Annexin V as a probe of aminophospholipid exposure and platelet membrane vesiculation: a flow cytometry study showing a role for free sulfhydryl groups. *Blood.* (1993) 81:2554. doi: 10.1182/blood.V81.10.2554.2554
32. Alberio L, Safa O, Clemetson KJ, Esmon CT, Dale GL. Surface expression and functional characterization of α -granule factor V in human platelets: effects of ionophore A23187, thrombin, collagen, and convulxin. *Blood.* (2000) 95:1694. doi: 10.1182/blood.V95.5.1694.005k24_1694_1702

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Jurasz, Ignjatovic and Lordkipanidzé. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Platelet Function During Extracorporeal Membrane Oxygenation in Adult Patients: A Systematic Review

Camilla Mains Balle¹, Anni Nørgaard Jeppesen², Steffen Christensen² and Anne-Mette Hvas^{1,3*}

¹ Department of Clinical Biochemistry, Aarhus University Hospital, Aarhus, Denmark, ² Department of Anesthesiology and Intensive Care Medicine, Aarhus University Hospital, Aarhus, Denmark, ³ Department of Clinical Medicine, Aarhus University, Aarhus, Denmark

OPEN ACCESS

Edited by:

Vera Ignjatovic,
Murdoch Childrens Research Institute,
Australia

Reviewed by:

Daniel Duerschmied,
Albert-Ludwigs-Universität Freiburg,
Germany
Benoît Ho-Tin-Noé,
Institut National de la Santé et de la
Recherche Médicale (INSERM),
France

*Correspondence:

Anne-Mette Hvas
am.hvas@dadlnet.dk

Specialty section:

This article was submitted to
Atherosclerosis and Vascular
Medicine,
a section of the journal
Frontiers in Cardiovascular Medicine

Received: 15 August 2018

Accepted: 15 October 2018

Published: 09 November 2018

Citation:

Balle CM, Jeppesen AN, Christensen S and Hvas AM (2018) Platelet Function During Extracorporeal Membrane Oxygenation in Adult Patients: A Systematic Review. *Front. Cardiovasc. Med.* 5:157. doi: 10.3389/fcvm.2018.00157

Background: Hemorrhagic and thromboembolic complications are common during treatment with extracorporeal membrane oxygenation (ECMO), resulting in considerable morbidity and mortality. This emphasizes the clinical relevance of understanding hemostatic changes occurring during ECMO treatment. As platelets are key players in hemostasis, detailed knowledge on how ECMO treatment affects platelet function is of great importance. We therefore aimed to systematically summarize and discuss existing knowledge on platelet function during ECMO treatment in adult patients.

Methods: Systematic review complying with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. Objectives and methods were specified in a PROSPERO protocol (ID no CRD42018084059). The MEDLINE/PubMed, EMBASE, and Web of Science databases were systematically searched on September 10, 2018. A standardized quality assessment tool was used to assess the risk of bias in included studies. Primary outcome was platelet function during ECMO treatment, measured as platelet adhesion, activation or aggregation. Secondary outcomes were thrombosis, bleeding, and mortality during ECMO treatment.

Results: A total of 591 studies were identified, of which seven were eligible for inclusion in the qualitative synthesis. Of these, one study investigated expression of platelet adhesion receptors and found them to be reduced during ECMO treatment; two studies reported a decrease in platelet activation markers during ECMO treatment; and five studies demonstrated reduced platelet aggregation during ECMO treatment. Three studies reported on thrombosis, mortality and/or bleeding during ECMO treatment; no thromboembolic events were reported; all three studies reported frequent bleeding episodes defined on basis of transfusion requirements. An in-hospital mortality of 35–40% and a 30-day mortality of roughly 30% were reported in three different studies.

Conclusions: The present systematic review reveals a substantial knowledge gap regarding platelet function during ECMO treatment in adult patients and underscores the demand for more and well-designed studies on this topic. There is suggested evidence of reduced platelet adhesion, decreased platelet activation, and reduced

platelet aggregation in adult patients during ECMO treatment. Importantly, platelet aggregation results need to be interpreted in the light of low platelet counts. The associations of platelet function and bleeding and/or thromboembolic complications during ECMO treatment remain to be fully elucidated.

Keywords: blood platelets, extracorporeal membrane oxygenation, extracorporeal life support, platelet activation, platelet aggregation, platelet function tests, systematic review

INTRODUCTION

Extracorporeal membrane oxygenation (ECMO) can be lifesaving in patients with respiratory and/or cardiac failure refractory to conventional treatment. In recent years, the use of ECMO has increased dramatically (1), most likely owing to studies reporting favorable outcomes for adult ECMO patients (2–4) in combination with advances in technology.

Hemorrhagic and thromboembolic complications are common during ECMO treatment, resulting in considerable morbidity and mortality (5–8). Bleeding is the most common complication, occurring in 29–33% of adult ECMO patients (7, 9). Intracranial hemorrhage, the most potentially devastating bleeding complication, is reported to occur in around 5% of adult ECMO patients (5, 6). Thromboembolic complications are generally less common; intracranial infarction is reported to occur in 2–5% (5, 6) and venous thrombosis in 10% of adult ECMO patients (9). Although some thromboembolic events are rapidly clinically apparent, many are subclinical and the true incidence of thromboembolic complications during ECMO is therefore likely underestimated (10). In addition, clot formation in the circuit can lead to oxygenator failure and exchange of the entire circuit, which is reported to occur in up to nearly one third of adult ECMO runs (5, 9).

Several mechanisms that could affect platelet function during ECMO treatment have been investigated *in vitro*, suggesting that platelets become both activated and impaired during ECMO treatment. Artificial surfaces, such as the extracorporeal circuit, induce platelet adhesion and activation (11). Furthermore, high shear stress, as in the ECMO circuit, has been shown to cause enhanced platelet activation (12, 13), which may lead to an increased thrombotic propensity. On the contrary, high shear stress has also been shown to cause loss of platelet surface receptors important for platelet adhesion (12, 13), as well as loss of high-molecular-weight von Willebrand factor multimers, resulting in decreased binding of von Willebrand factor to platelets (13, 14). Both mechanisms may result in impaired platelet adhesion and hence impaired activation, leading to an increased risk of bleeding. The risk of bleeding is further

augmented by ongoing consumption of platelets due to platelet adhesion to the surface of the circuit, and formation of microthrombi in the circulation.

The increased thrombotic propensity necessitates antithrombotic therapy to conserve patency of the ECMO circuit and to reduce thrombotic complications. Although anticoagulation guidelines vary widely among ECMO centers, unfractionated heparin (UFH) is the most widely administered anticoagulant (15). The use of UFH can, however, result in bleeding, posing an additional risk to the patient.

Limited data exist on platelet function during ECMO treatment in adult patients (16–22) and no systematic review has yet summarized the current knowledge. Consequently, the aim of the present systematic review was to summarize and discuss existing knowledge regarding platelet function during ECMO treatment in adult patients.

METHODS

The present systematic review complies with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (23). Prior to data extraction, the objectives and methods were specified in a PROSPERO protocol (ID no. CRD42018084059).

Search Strategy

The MEDLINE/PubMed, Embase, and Web of Science databases were systematically searched for relevant publications on September 10, 2018, without time boundaries. All searches were filtered to include English language only. A manual search of the references identified relevant articles supplementing the electronic search. Search terms were as follows: **PubMed:** (((((((extracorporeal membrane oxygenation (24)) OR extracorporeal life support) OR extracorporeal cardiopulmonary resuscitation) OR *ecmo*) OR *ecls*) OR *ecpr*)) AND (((((((platelet activation [MeSH]) OR platelet aggregation [MeSH]) OR platelet activity) OR platelet function) OR platelet dysfunction) OR platelet function tests [MeSH]) OR flow cytometry [MeSH]) OR aggregometry). **Embase:** (“extracorporeal membrane oxygenation”/exp OR “extracorporeal membrane oxygenation” OR “*ecmo*” OR “extracorporeal life support” OR “*ecls*” OR “extracorporeal cardiopulmonary resuscitation” OR “*ecpr*” OR “extracorporeal oxygenation”) AND (“thrombocyte activation”/exp OR “platelet activation” OR “thrombocyte activity” OR “platelet activity” OR “thrombocyte aggregation”/exp OR “platelet aggregation” OR “thrombocyte function”/exp OR “platelet function” OR

Abbreviations: ECMO, Extracorporeal membrane oxygenation; VV, Venovenous; VA, Veno-arterial; ARDS, Acute respiratory distress syndrome; COPD, Chronic obstructive pulmonary disease; ACT, Activated clotting time; aPTT, Activated partial thromboplastin time; UFH, Unfractionated heparin; LMWH, Low molecular weight heparin; VKA, Vitamin K antagonist; ASA, Acetylsalicylic acid; ELISA, Enzyme-linked immunosorbent assay; GPIV, Glycoprotein VI; GPIIb/IIIa, Glycoprotein IIb/IIIa; TRAP, Thrombin receptor-activating peptide-6; ADP, Adenosine diphosphate; AA, Arachidonic acid; PLC, Platelet count; RBC, Red blood cells; AUC, Area under the curve; IQR, Interquartile range.

“thrombocyte dysfunction”/exp OR “platelet dysfunction” OR “thrombocyte function analyzer”/exp OR “flow cytometry”/exp OR “flow cytometry” OR “aggregometry”) AND [English]/lim.

Web of Science: (extracorporeal membrane oxygenation OR extracorporeal life support OR extracorporeal cardiopulmonary resuscitation) AND (platelet activ* OR thrombocyte activ* OR platelet function OR thrombocyte function OR platelet dysfunction* OR thrombocyte dysfunction* OR platelet aggregation OR thrombocyte aggregation OR platelet function test* OR thrombocyte function test* OR flow cytometry OR aggregometry) AND language: (English).

Study Selection

Fifty abstracts were randomly chosen and screened independently by two authors (CB, AH). The abstracts were screened based on predefined in- and exclusion criteria and any divergences were discussed to reach consensus. One author (CB) screened the remaining 541 abstracts. Studies eligible for full-text reading were assessed by two authors (CB, AH). Publications were included if they met the following criteria: (a) original data; (b) adult patients (≥ 18 years) receiving ECMO treatment; (c) platelet function determined (platelet adhesion, platelet activation, or platelet aggregation); (d) English language. Exclusion criteria were: (a) reviews or guidelines; (b) letters, editorials or comments without original data; (c) case reports with < 5 cases; (d) *in vitro* or animal data; (e) posters or conference abstracts; (f) only platelet count determined.

Data Extraction

Data extraction was performed by CB and verified by AH. Platelet function analyses were categorized as measurements of platelet adhesion, activation, or aggregation. Platelet adhesion analyses were defined as measurements of adherence to foreign surfaces or measurements of adhesion potential (expression of surface receptors essential to platelet adhesion); platelet activation analyses as measurements of soluble markers of platelet activation or platelet activation potential (expression of activation-dependent platelet surface markers); and platelet aggregation analyses as functional analyses measuring platelet aggregation.

To assess the potential risk of bias in the included studies, two authors (CB, AH) independently evaluated each study using a standardized study quality assessment tool from the National Heart, Lung, and Blood Institute, US (25). Any discrepancies were discussed to reach consensus.

RESULTS

The systematic literature search returned a total of 591 studies. In total, 584 studies were excluded, resulting in seven studies to be included in the qualitative synthesis. **Figure 1**, modified from (23), shows a flow chart outlining the screening and selection process. During the process of full-text screening, the study by Nair et al. was included in the final synthesis despite their inclusion criterion of age > 16 years (21). The article contained no information specifying whether any patients aged < 18 years were included, and data could not be obtained from the investigators.

However, the median age of included patients was 41 years [interquartile range (IQR): 38–52 years] indicating that very few, if any, patients were below 18 years of age.

A summary of included studies is presented in **Table 1**. All seven studies were observational cohort studies. Using the study quality assessment tool, the study by Tauber et al. rated *good* (19), five studies rated *fair* (16–18, 20, 21), and one study rated *poor* (22).

Platelet Count

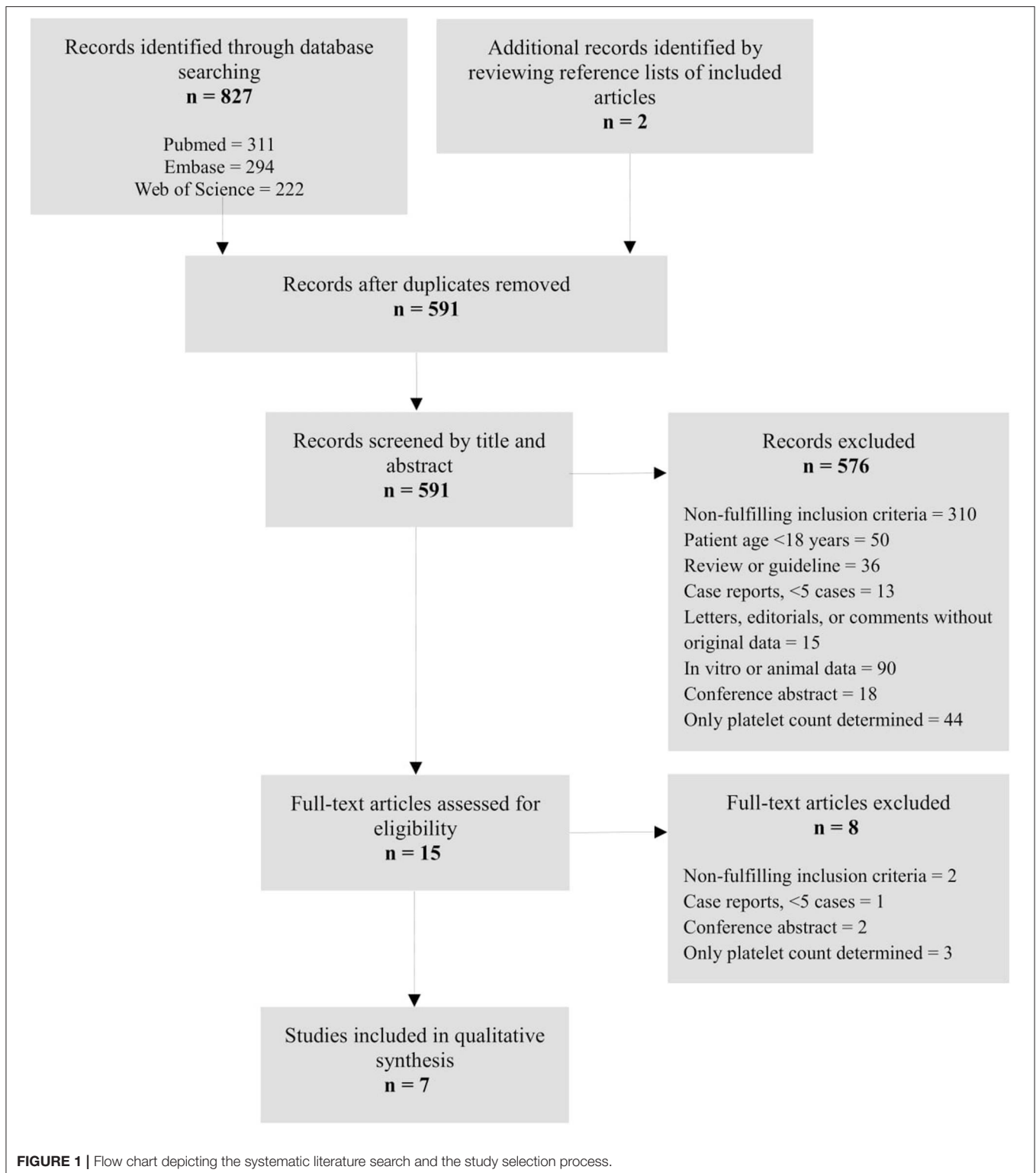
Five studies reported platelet counts at several time points during the study period. Of these, two studies measured platelet counts prior to and during ECMO treatment (18, 21); they reported lower platelet counts during ECMO treatment compared with before ECMO initiation, and persistently lower platelet counts during ECMO treatment compared with healthy individuals. Two studies measured platelet counts both prior to, during, and after ECMO treatment (19, 22); they demonstrated significantly lower platelet counts during ECMO treatment compared with before ECMO initiation; lower platelet counts during ECMO compared with healthy individuals; and after termination of ECMO, platelet counts did not differ from platelet counts before ECMO initiation. In contrast, Chung et al. reported widely diverging platelet counts during the course of ECMO treatment, demonstrating no uniform changes in platelet counts over time (17). The remaining two studies measured platelet counts at a single time point during the study period (16, 20); Mutlak et al. measured platelet counts prior to ECMO initiation and found them to be reduced compared with healthy individuals (20); similarly, Lukito et al. measured platelet counts at an unspecified time point during ECMO treatment and found the counts to be reduced compared with healthy individuals (16). The effects of administered platelet transfusions on platelet counts were not accounted for in any of the included studies.

Platelet Function

Lukito et al. reported reduced levels of adhesion receptors for collagen and vWF (GPVI and GPIIb/IIIa) on circulating platelets compared with healthy individuals, at a single, unspecified time point during ECMO treatment (16); furthermore, they reported an increase in the level of soluble GPVI in ECMO patients.

Chung et al. investigated platelet function by measuring soluble markers of platelet activation (17); they reported a decrease in platelet-activation markers, β -thromboglobulin and platelet factor 4, during the first 72 h of ECMO treatment compared with before ECMO initiation. On day seven of ECMO treatment, β -thromboglobulin levels remained low, while platelet factor 4 levels had returned to a level corresponding to before ECMO initiation. Kalbhenn et al. investigated platelet activation by measuring the expression of activation-dependent platelet surface markers: CD62 (P-selectin) and CD63; they reported reduced surface expression of CD62 and CD63 compared with healthy individuals, at a single, unspecified time point during ECMO treatment.

Five studies investigated platelet function by measuring platelet aggregation. Four studies employed whole blood impedance aggregometry (18–21) and demonstrated reduced



platelet aggregation during ECMO treatment compared with either before ECMO initiation (18–20) or with healthy individuals (21). Tauber et al. (19) also measured platelet aggregation after ECMO termination and found no difference in platelet aggregation 24 h after ECMO compared with before

ECMO initiation. Two studies demonstrated reduced platelet aggregation compared with healthy individuals even before initiation of ECMO (18, 19). Kalbhenn et al. employed light transmission aggregometry when measuring platelet aggregation at two unspecified timepoints during and after ECMO treatment

(22). They excluded all patients with platelet counts $<100 \times 10^9$ /L from platelet aggregation analyses. They reported a decrease in platelet aggregation (for ADP and ristocetin) or no difference in platelet aggregation (for collagen and epinephrine) during ECMO compared with after ECMO treatment.

Clinical Events

Four out of seven studies reported major clinical events: thrombosis, bleeding, and mortality (18, 21); bleeding alone (19); or mortality alone (22). No thromboembolic events were reported during ECMO treatment (18, 21). Patients frequently required transfusions of blood products; Laine et al. reported severe bleeding in 26% of patients (18) from either cannula insertion sites or postoperative chest tube drains; Nair et al. reported bleeding episodes in 50% of patients without reporting bleeding sites or severity (21); and Tauber et al. reported transfusion requirements of ≥ 2 units of red blood cells (RBC) per day in 26% of patients without reporting bleeding sites (19). In addition, Tauber et al. (19) found an increased transfusion requirement (≥ 2 units RBC/day) during ECMO to be associated with lower platelet aggregation values [using thrombin receptor activating peptide-6 (TRAP) as agonist] compared with a moderate transfusion requirement (<2 units RBC/day). In-hospital mortalities of 35% (22) and 40% (21), and a 30-day mortality of 30.4% (18) were reported.

DISCUSSION

This systematic review revealed that only a limited number of published studies investigate platelet function in patients during ECMO treatment; only seven studies investigating platelet function during ECMO treatment in adult patients were identified. They suggest evidence of a reduced potential for platelet adhesion, and decreased platelet activation during ECMO treatment. Furthermore, platelet aggregation is reduced during ECMO treatment compared with either before ECMO initiation, after ECMO termination, or with healthy individuals. The association between platelet function and bleeding/thromboembolic complications during ECMO treatment remains to be fully elucidated.

All included studies were rated using a study quality assessment tool (25). Tauber et al. (19) rated *good*, suggesting a low risk of bias, whereas *fair* ratings of five studies (16–18, 20, 21) insinuated a greater risk of bias of the estimates. Fair ratings were given due to small cohort sizes with no sample size justification and no *a priori* sample size estimate; retrospective data collection; and/or no statistical adjustment of estimates for potential confounding variables (e.g., platelet count). Justifying a *fair* rating of these studies, however, were detailed and structured descriptions of exposure and outcome measures together with transparent presentations of data. One study (22) was rated *poor*, due to insufficient information regarding the study population and methods employed.

Lukito et al. reported significant reductions in the expression of adhesion receptors (GPIb α and GPVI) on circulating platelets in ECMO patients compared with healthy individuals (16). Loss of the GPIb α and GPVI receptors reduces the binding

capacity of platelets to vWF and collagen, leading to impaired platelet adhesion and hence impaired platelet activation (26, 27). Recently, Chen et al. supported these findings by demonstrating loss of platelet receptors, GPIb α and GPVI, during *in vitro* conditions of high shear stress (12). The loss of receptors resulted in reduced adhesion of platelets to collagen and vWF (12). However, Chen et al. also measured adherence of the sheared platelets to fibrinogen, deposited on an artificial surface, demonstrating that high shear stress also induces platelet activation, thereby increasing thrombotic propensity (12). The authors proposed that these two concurrent mechanisms, leading to impaired *and* enhanced platelet activation, may explain why bleeding and thrombotic complications occur simultaneously in patients treated with ECMO (12).

The increase in soluble GPVI levels, measured by Lukito et al., indicates proteolytic shedding as the main mechanism for reduced adhesion-receptor levels (16). Their findings are supported by Al-Tamimi et al., who reported metalloproteinase-dependent shedding of surface-bound GPVI after exposure of platelets to high shear stress *in vitro* (28). In line with others, they proposed that this shear-induced GPVI shedding may be a protective mechanism for down-regulating platelet adhesiveness during elevated shear stress, thereby reducing the level of platelet activation and thrombus formation (28, 29). Shedding of platelet adhesion receptors has been shown to accompany platelet activation (30, 31). Therefore, reduced levels of GPIb and GPVI measured by Lukito et al. may be suggestive of enhanced platelet activation during ECMO treatment (16).

In contrast, Chung et al. reported reduced platelet activation during ECMO treatment, demonstrated by decreasing levels of plasma β -thromboglobulin and platelet factor 4 during the first 72 h on ECMO treatment compared with before ECMO initiation (17). This may be explained by two distinct mechanisms. β -thromboglobulin and platelet factor 4 are thought to be sensitive markers of platelet activation, as they are secreted from the α -granules upon platelet activation (32). Platelets are able to release 100% of their total α -granule content (33). It is therefore plausible that initial extensive or continuous platelet activation would lead to release of the entire β -thromboglobulin and platelet factor 4 content, resulting in exhausted platelets and a decrease in plasma β -thromboglobulin and platelet factor 4 levels. Chung et al. obtained the first post-cannulation blood sample at 24 h after ECMO initiation (17), and an initial rise in β -thromboglobulin and platelet factor 4 would therefore not have been detected due to fast clearance times; the half-life of β -thromboglobulin is reported to be 100 min and platelet factor 4 clearance is even faster (34). The other mechanism to be considered is adherence of β -thromboglobulin to the heparin-coated surface of the ECMO circuit. A recent study by Sagedal et al. (35) suggested absorption of β -thromboglobulin to heparin-coated surfaces, as both β -thromboglobulin and platelet factor 4 are heparin-binding proteins (32). If so, β -thromboglobulin might not be a reliable marker of platelet activation in heparin-coated medical devices including ECMO circuits.

Kalbhehn et al. (22) performed flow-cytometric analyses on platelet rich plasma revealing significantly reduced expressions of CD62 (impaired α -granule secretion) and CD63 (impaired

TABLE 1 | Studies included in the systematic review investigating platelet function during extracorporeal membrane oxygenation in adult patients.

Year, Author, Quality rating	Patients, n ECMO configuration ECMO duration ECMO indication	Antithrombotic therapy	Markers of platelet function (method)	Platelet count $\times 10^9/L$, mean $\pm$ SD	Platelet function	Results Clinical events (definition)
PLATELET ADHESION						
Lukito et al. (16) Rating Fair	n = 20 Configuration 14 VA + 6 VV Duration Median: 4 days (range 1–11) Indication Cardiomyopathy (n = 9) Respiratory failure (n = 7) Cardiac arrest (n = 2) Others (n = 2)	VKA + ASA + dipyridamole (n = 1) VKA + ASA (n = 1) VKA only (n = 1) Heparin [#] + antiplatelets (n = 3) Heparin [#] only (n = 9) No antithrombotics (n = 5)	Soluble GPVI (ELISA) Surface-bound GPIIb/IIIa and GPVI (flow cytometry) Integrin α_{IIb} subunit (flow cytometry)	Unspecified time point during ECMO: 132 (range 51–268)	Unspecified time point during ECMO compared with healthy individuals: $\downarrow$ GPIIb/IIIa $\downarrow$ GPVI $\uparrow$ sGPVI $\rightarrow$ Integrin α_{IIb} subunit	Thrombosis Not reported Bleeding Not reported Mortality Not reported
PLATELET ACTIVATION						
Chung et al. (17) Rating Fair	n = 13 Configuration VV Duration Median: 7 days (range 6–7) Indication Acute respiratory failure (n = 10) Acute-on-chronic lung disease (n = 2) Pulmonary embolism (n = 1)	UFH Target ACT = 140–160 s.	β -thromboglobulin (ELISA) Platelet factor 4 (ELISA)	During ECMO compared with before ECMO: Widely diverging platelet counts – no significant trend over time. At ECMO cannulation: 194 $\pm$ 74	72 h. on ECMO compared with before ECMO: $\downarrow$ β -thromboglobulin ($\downarrow$) Platelet factor 4	Thrombosis Not reported Bleeding Not reported Mortality Not reported
PLATELET AGGREGATION						
Laine et al. (18) Rating Fair	n = 23 Configuration 18 VA + 5 VV Duration Range 3–30 days Indication Cardiac surgery (n = 6) Dilated cardiomyopathy (n = 2) Acute myocardial infarction (n = 2) Cardiac failure/cardiogenic shock (not AMI) (n = 1) Myocarditis (n = 2) Respiratory failure (n = 3) Pulmonary artery hypertension (n = 1) Pneumonia (n = 3) Complications of pulmonary/cardiac transplantation (n = 3)	UFH Target ACT: PLC > 100 $\times 10^9/L$: 180–200 s. PLC < 100 $\times 10^9/L$: 160–180 s.	Platelet aggregation (Multiplate Analyzer, ADP, TRAP, AA as agonists) Agonist concentrations Not reported	Before ECMO, median: 130 During ECMO compared with before ECMO: $\downarrow$ PLC	Before and during ECMO compared with healthy individuals: $\downarrow$ Platelet aggregation (TRAP; AA, ADP) During ECMO compared with before ECMO: $\downarrow$ Platelet aggregation (TRAP) $\uparrow$ Platelet aggregation (AA) $\rightarrow$ Platelet aggregation (ADP)	Thrombosis No events (A venous or arterial thromboembolism leading to neurological complication or pulmonary embolism) Bleeding Severe bleeding: 26.1% (n = 6) (Two or more liters in the first 24 h following cannulation or administration of more than five units of RBC in 24 h) Mortality 30-day mortality: 30.4%

(Continued)

TABLE 1 | Continued

Year, Author, Quality rating	Patients, n ECMO configuration ECMO duration ECMO indication	Antithrombotic therapy	Markers of platelet function (method)	Platelet count $\times 10^9/L$, mean $\pm$ SD	Results Platelet function	Clinical events (definition)
Tauber et al. (19) Rating Good	n = 38 Configuration 26 VA + 12 WV Duration Not reported Indication Intractable cardiac or respiratory failure (n = 38)	UFH Target ACT = 150–180 s.	Platelet aggregation (Multiplate Analyzer, ADP 6.4 μ M, TRAP 32 μ M, AA 0.5 mM as agonists)	Before ECMO: 134 $\pm$ 95 During ECMO compared with before ECMO: $\downarrow$ PLC During ECMO compared with after ECMO: $\downarrow$ PLC Before ECMO compared with after ECMO: $\rightarrow$ PLC	Before ECMO compared with healthy individuals: $\downarrow$ Platelet aggregation (ADP, AA) $\rightarrow$ Platelet aggregation (TRAP) 24 h. on ECMO compared with before ECMO: $\downarrow$ Platelet aggregation (ADP, AA, TRAP) 48 h. on ECMO compared with before ECMO: $\downarrow$ Platelet aggregation (ADP, AA) ($\downarrow$) Platelet aggregation (TRAP) 24 h. after ECMO compared with before ECMO: $\rightarrow$ Platelet aggregation (TRAP, ADP, AA)	Thrombosis Not reported Bleeding Moderate (<2U RBC/d) n = 28 (74%) Increased ($\geq$ 2U RBC/d); n = 10 (26%) (Estimated indirectly by transfusion requirements) Mortality Not reported
Mutlak et al. (20) Rating Fair	n = 5 Configuration WV Duration Not reported Indication ARDS (n = 5)	UFH Target aPTT = 45–55 s.	Platelet aggregation (Multiplate Analyzer, ADP 0.4 mM, TRAP 32 mM, AA 0.5 mM as agonists)	Before ECMO: 136 $\pm$ 30	90 min. on ECMO compared with before ECMO: $\downarrow$ Platelet aggregation (ADP) ($\downarrow$) Platelet aggregation (AA) $\rightarrow$ Platelet aggregation (TRAP) 120, 150 and 180 min. on ECMO compared with before ECMO: $\downarrow$ Platelet aggregation (ADP) ($\downarrow$) Platelet aggregation (AA) $\rightarrow$ Platelet aggregation (TRAP)	Thrombosis Not reported Bleeding Not reported Mortality Not reported
Nair et al. (21) Rating Fair	n = 10 Configuration 7 VA + 3 WV Duration Median: 10 days (IQR 5–14) Indication Primary graft dysfunction (lung) (n = 2) Severe ARDS (n = 1) Primary graft dysfunction (heart) (n = 3) Cardiogenic shock (n = 2) Post cardiectomy (n = 2)	UFH Target aPTT = 1.5–2 x normal value	Platelet aggregation (Multiplate Analyzer, ADP, TRAP, collagen, ristocetin as agonists) Agonist concentrations Not reported	During ECMO compared with before ECMO: $\downarrow$ PLC	Unspecified time point during ECMO compared with healthy individuals: $\downarrow$ Platelet aggregation (ADP, TRAP, collagen, ristocetin) 120, 150 and 180 min. on ECMO compared with before ECMO: $\downarrow$ Platelet aggregation (ADP) ($\downarrow$) Platelet aggregation (AA) $\rightarrow$ Platelet aggregation (TRAP)	Thrombosis No events (Clinically apparent arterial or venous thrombosis or oxygenator failure) Bleeding 5 (50%) experienced bleeding episodes. (Any bleeding significant enough to trigger administration of blood products (excluding FBC) or coagulation factors) Mortality In-hospital mortality: 40%

(Continued)

TABLE 1 | Continued

Year, Author, Quality rating	Patients, n ECMO configuration ECMO duration ECMO indication	Antithrombotic therapy	Markers of platelet function (method)	Platelet count $\times 10^9/L$, mean $\pm$ SD	Platelet function	Clinical events (definition)
PLATELET ACTIVATION AND AGGREGATION						
Kalbhenn et al. (22) Rating Poor	n = 6 (flow cytometry) n = Not reported (aggregometry) Configuration W Duration Mean: 10 days (SD 9.5) Indication ARDS Acute interstitial pneumonia Exacerbated COPD Bridge to lung transplant Post-lung transplant Post-pneumectomy Tracheal rupture	UFH Target aPTT = 40 s, or anti-factor Xa activity ≤ 0.2 U/ml LMWH Target anti-factor Xa activity ≤ 0.18 IU/ml	Surface-bound CD62 and CD63 (flow cytometry) Platelet aggregation (light transmission aggregometry, collagen, ristocetin, ADP, epinephrine as agonists) Agonist concentrations Not reported	During ECMO compared with before ECMO: $\downarrow$ PLC During ECMO compared with after ECMO: $\downarrow$ PLC Before ECMO compared with after ECMO: $\rightarrow$ PLC	On ECMO compared with healthy individuals: $\downarrow$ CD62 $\downarrow$ CD63 On ECMO compared with after ECMO: $\downarrow$ Platelet aggregation (ADP, ristocetin) ($\downarrow$) Platelet aggregation (collagen, epinephrine)	Thrombosis Not reported Bleeding Not reported Mortality In-hospital mortality: 35%

$\uparrow$ Statistically significant increase; $\downarrow$ Statistically significant reduction; $\rightarrow$ No change; (t) Non-significant increase; ($\downarrow$) Non-significant reduction; Φ Significance not tested; $\#$ Type of heparin not specified. ECMO, Extracorporeal membrane oxygenation; VV, Veno-venous; VA, Veno-arterial; ARDS, Acute respiratory distress syndrome; COPD, Chronic obstructive pulmonary disease; ACT, Activated partial thromboplastin time; UFH, Unfractionated heparin; LMWH, Low molecular weight heparin; VKA, Vitamin K antagonist; ASA, Acetylsalicylic acid; ELISA, Enzyme-linked immunosorbent assay; GPIIb/IIIa, Glycoprotein IIb/IIIa; TRAP, Thrombin receptor-activating peptide-6; ADP, Adenosine diphosphate; AA, Arachidonic acid; PLC, Platelet count; RBC, Red blood cells; AUC, Area under the curve; IQR, Interquartile range.

δ -granule secretion) in ECMO patients compared with healthy individuals. Their results are suggestive of a reduced platelet activation potential during ECMO treatment. However, it is interesting to speculate whether the mechanism leading to impaired platelet activation *ex vivo* could be vast activation of platelets *in vivo* during ECMO treatment, resulting in impaired platelet reactivity to agonist stimulation *ex vivo*.

Four out of five studies investigating platelet aggregation employed whole blood impedance aggregometry. All four studies indicated reduced platelet aggregation in adult patients during ECMO treatment (18–21). An inherent limitation of this analysis is a strong association between platelet count and platelet aggregation both within and below the reference interval for platelet count (36–41). Nair et al. (21) excluded data on platelet aggregation from analysis if platelet counts were below $100 \times 10^9/L$. Tauber et al. (19) also considered the influence of thrombocytopenia on impedance aggregometry results as a limitation to the study but deemed the effects to be minimal. Employing light transmission aggregometry, Kalbhenn et al. (22) demonstrated reduced platelet aggregation during ECMO treatment (for two out of four agonists) compared with after ECMO termination. They excluded patients from analysis if platelet counts were below $100 \times 10^9/L$; this threshold might be set a little too low, as light transmission aggregometry is influenced by platelet counts below $150 \times 10^9/L$ (37). Thus, data from the included studies do not enable us to make firm conclusions regarding platelet aggregation during ECMO treatment, as they might very well be influenced by low platelet counts.

For analyses of platelet aggregation, the most widely used agonists were adenosine diphosphate (ADP), arachidonic acid (AA), and TRAP; only two studies provided information regarding concentrations used (19, 20). Agonist concentrations differed; both studies, however, compared platelet aggregation during ECMO treatment to before ECMO initiation, abating the need for comparable agonist concentrations between studies.

Whether the observed alterations in platelet count and function are caused by the ECMO circuit itself or rather the underlying disease is difficult to determine. However, two studies clearly demonstrated significantly reduced platelet counts during ECMO treatment compared with before ECMO initiation and, importantly, they demonstrated that platelet counts recovered to pre-ECMO values within 24 h after ECMO termination (19, 22). This indicates that the reduction in platelet count was caused by the ECMO treatment. With regard to platelet function, there is suggested evidence that ECMO treatment induces platelet dysfunction; three studies demonstrated a decrease in platelet aggregation compared with before ECMO initiation (18–20); Kalbhenn et al. found platelet aggregation to increase after ECMO termination; and Tauber et al. demonstrated that platelet aggregation increased to pre-ECMO values within 24 h after ECMO termination. Additional studies investigating ECMO treatment as a cause of thrombocytopenia and platelet dysfunction are needed to strengthen these proposed associations.

Inflammation has been shown to induce bleeding in thrombocytopenic mice (42). Only one of the included studies

provided information regarding inflammatory parameters measured during ECMO treatment (17); furthermore, the associations between bleeding episodes and platelet count or inflammatory parameters were not accounted for in any of the included studies. Laine et al. found no significant associations between platelet aggregation and the risk of bleeding or mortality (18), and no thromboembolic events were reported in any of the included studies; both of which might be attributed to the small cohort sizes. Whether there is an association between platelet function and clinical complications such as bleeding or thromboembolic events therefore remains to be established.

Strengths and Limitations

The systematic approach and a broad search string without time boundaries across three major databases make this systematic review comprehensive, minimizing the risk of overlooking relevant studies. Included studies are thoroughly presented in a transparent manner, including design, quality rating, and results found in each study.

However, some limitations must be considered. Only seven studies compiling with the aim and inclusion criteria were identified. The quality of included studies varied; only one study rated *good* (19), suggesting possible risk of bias in the remaining studies (16–18, 20–22). Furthermore, the patient cohorts were heterogeneous with respect to their underlying diseases (16, 18, 19, 21, 22), which could influence platelet count and function; e.g., patients treated with VV-ECMO, suffering from acute respiratory distress syndrome or severe sepsis, typically have bone marrow suppression, and hence platelet counts drop in these patients independently of platelet consumption in the ECMO circuit. Bone marrow involvement is not typical in the case of patients treated with VA-ECMO, however, these patients are frequently treated with platelet inhibitors, which alter platelet function. Any influences of these differences between ECMO cohorts on the results of this review cannot be assessed based on the data available from the studies included.

REFERENCES

- Gerke AK, Tang F, Cavanaugh JE, Doerschug KC, Polgreen PM. Increased trend in extracorporeal membrane oxygenation use by adults in the United States since 2007. *BMC Res Notes* (2015) 8:686. doi: 10.1186/s13104-015-1678-7
- Davies A, Jones D, Bailey M, Beca J, Bellomo R, Blackwell N, et al. Extracorporeal membrane oxygenation for 2009 influenza A(H1N1) acute respiratory distress syndrome. *JAMA* (2009) 302:1888–95. doi: 10.1001/jama.2009.1535
- Peek GJ, Mugford M, Tiruvoipati R, Wilson A, Allen E, Thalanany MM, et al. Efficacy and economic assessment of conventional ventilatory support versus extracorporeal membrane oxygenation for severe adult respiratory failure (CESAR): a multicentre randomised controlled trial. *Lancet* (2009) 374:1351–63. doi: 10.1016/s0140-6736(09)61069-2
- Linden V, Palmer K, Reinhard J, Westman R, Ehren H, Granholm T, et al. High survival in adult patients with acute respiratory distress syndrome treated by extracorporeal membrane oxygenation, minimal sedation, and pressure supported ventilation. *Intensive Care Med.* (2000) 26:1630–7. doi: 10.1007/s001340000697
- Thiagarajan RR, Barbaro RP, Rycus PT, McMullan DM, Conrad SA, Fortenberry JD, et al. Extracorporeal life support organization registry international report 2016. *ASAIO J* (2017) 63:60–7. doi: 10.1097/mat.0000000000000475
- Sutter R. Acute neurologic complications during extracorporeal membrane oxygenation—a systematic review. *Crit Care Med.* (2018) 1:3223. doi: 10.1097/CCM.00000000000003223
- Vaquer S, de Haro C, Peruga P, Oliva JC, Artigas A. Systematic review and meta-analysis of complications and mortality of veno-venous extracorporeal membrane oxygenation for refractory acute respiratory distress syndrome. *Ann Intensive Care* (2017) 7:51. doi: 10.1186/s13613-017-0275-4
- Aubron C, Cheng AC, Pilcher D, Leong T, Magrin G, Cooper DJ, et al. Factors associated with outcomes of patients on extracorporeal membrane oxygenation support: a 5-year cohort study. *Crit Care* (2013) 17:R73. doi: 10.1186/cc12681
- Zangrillo A, Landoni G, Biondi-Zoccai G, Greco M, Greco T, Frati G, et al. A meta-analysis of complications and mortality of extracorporeal membrane oxygenation. *Crit Care Resusc.* (2013) 15:172–8.

The majority of included studies measured platelet function using impedance aggregometry (18–21) entailing the limitation of a strong association between platelet count and platelet aggregation. Recently, our research group reported a model to derive a measure of platelet aggregation adjusted for platelet count (36, 43). This approach could be valuable in evaluating whether platelet aggregation is reduced due to dysfunctional or exhausted platelets, or merely due to a low number of circulating platelets during ECMO treatment.

Conclusions

The present systematic review reveals a substantial knowledge gap regarding platelet function during ECMO treatment in adult patients and underscores the demand for more and well-designed studies on this topic. At present, there is suggested evidence of reduced platelet adhesion and a decreased platelet activation potential in adult patients during ECMO treatment. Furthermore, platelet aggregation was found to be reduced during ECMO treatment. Importantly, measurements of platelet aggregation were hampered by low platelet counts, why these results need to be interpreted with caution. Whether platelet function is associated with clinical complications such as bleeding and thromboembolic events remains to be established.

AUTHOR CONTRIBUTIONS

CB and AH performed the systematic literature search, screened all abstracts and full-text articles independently, and performed the study quality assessment of all included articles. CB performed the data extraction, which was verified by AH; CB took the lead in writing the manuscript. All authors (CB, AH, AJ, SC) contributed to the qualitative analysis and provided critical revision of the manuscript.

FUNDING

The present systematic review was funded by a grant from Aarhus University, Denmark.

10. Rastan AJ, Lachmann N, Walther T, Doll N, Gradistanac T, Gommert JF, et al. Autopsy findings in patients on postcardiotomy extracorporeal membrane oxygenation (ECMO). *Int J Artif Organs*. (2006) 29:1121–31.
11. Yoshimoto Y, Hasebe T, Takahashi K, Amari M, Nagashima S, Kamijo A, et al. Ultrastructural characterization of surface-induced platelet activation on artificial materials by transmission electron microscopy. *Microsc Res Tech*. (2013) 76:342–9. doi: 10.1002/jemt.22172
12. Chen Z, Mondal NK, Zheng S, Koenig SC, Slaughter MS, Griffith BP, et al. High shear induces platelet dysfunction leading to enhanced thrombotic propensity and diminished hemostatic capacity. *Platelets* (2017) 2017:1–8. doi: 10.1080/09537104.2017.1384542
13. Chen Z, Mondal NK, Ding J, Koenig SC, Slaughter MS, Wu ZJ. Paradoxical effect of nonphysiological shear stress on platelets and von willebrand factor. *Artif Organs*. (2016) 40:659–68. doi: 10.1111/aor.12606
14. Heilmann C, Geisen U, Beyersdorf F, Nakamura L, Benk C, Trummer G, et al. Acquired von Willebrand syndrome in patients with extracorporeal life support (ECLS). *Intensive Care Med*. (2012) 38:62–8. doi: 10.1007/s00134-011-2370-6
15. Esper SA, Welsby IJ, Subramaniam K, John Wallisch W, Levy JH, Waters JH, et al. Adult extracorporeal membrane oxygenation: an international survey of transfusion and anticoagulation techniques. *Vox Sang*. (2017) 112:443–52. doi: 10.1111/vox.12514
16. Lukito P, Wong A, Jing J, Arthur JF, Marasco SF, Murphy DA, et al. Mechanical circulatory support is associated with loss of platelet receptors glycoprotein Ibalpha and glycoprotein VI. *J Thromb Haemost*. (2016) 14:2253–60. doi: 10.1111/jth.13497
17. Chung JH, Yeo HJ, Kim D, Lee SM, Han J, Kim M, et al. Changes in the levels of beta-thromboglobulin and inflammatory mediators during extracorporeal membrane oxygenation support. *Int J Artif Organs*. (2017) 40:575–80. doi: 10.5301/ijao.5000617
18. Laine A, Niemi T, Suojäranta-Ylinen R, Raivio P, Soininen L, Lemstrom K, et al. Decreased maximum clot firmness in rotational thromboelastometry (ROTEM(R)) is associated with bleeding during extracorporeal mechanical circulatory support. *Perfusion* (2016) 28:0267659116647473. doi: 10.1177/0267659116647473
19. Tauber H, Streif W, Fritz J, Ott H, Weigel G, Loacker L, et al. Predicting transfusion requirements during extracorporeal membrane oxygenation. *J Cardiothorac Vasc Anesth*. (2016) 30:692–701. doi: 10.1053/j.jvca.2016.01.009
20. Mutlak H, Reyher C, Meybohm P, Papadopoulos N, Hanke AA, Zacharowski K, et al. Multiple electrode aggregometry for the assessment of acquired platelet dysfunctions during extracorporeal circulation. *Thorac Cardiovasc Surg*. (2015) 63:21–7. doi: 10.1055/s-0034-1383817
21. Nair P, Hoechter DJ, Buscher H, Venkatesh K, Whittam S, Joseph J, et al. Prospective observational study of hemostatic alterations during adult extracorporeal membrane oxygenation (ECMO) using point-of-care thromboelastometry and platelet aggregometry. *J Cardiothorac Vasc Anesth*. (2015) 29:288–96. doi: 10.1053/j.jvca.2014.06.006
22. Kalbhenn J, Schlagenhauf A, Rosenfelder S, Schmutz A, Zieger B. Acquired von Willebrand syndrome and impaired platelet function during venovenous extracorporeal membrane oxygenation: Rapid onset and fast recovery. *J Heart Lung Transplant*. (2018) 37:985–91. doi: 10.1016/j.healun.2018.03.013
23. Moher D, Liberati A, Tetzlaff J, Altman DG. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLoS Med*. (2009) 6:e1000097. doi: 10.1371/journal.pmed.1000097
24. Ferraris VA, Brown JR, Despotis GJ, Hammon JW, Reece TB, Saha SP, et al. 2011 Update to the society of thoracic surgeons and the society of cardiovascular anesthesiologists blood conservation clinical practice guidelines. *Ann Thorac Surg*. (2011) 91:944–82. doi: 10.1016/j.athoracsurg.2010.11.078
25. National Heart Lung and Blood Institute. *Study Quality Assessment Tools*. (2018). Available online at: <https://www.nhlbi.nih.gov/health-topics/study-quality-assessment-tools> (Accessed January 2, 2018).
26. Massberg S, Gawaz M, Gruner S, Schulte V, Konrad I, Zohnhofer D, et al. A crucial role of glycoprotein VI for platelet recruitment to the injured arterial wall in vivo. *J Exp Med*. (2003) 197:41–9. doi: 10.1084/jem.20020945
27. Bergmeier W, Piffath CL, Goerge T, Cifuni SM, Ruggeri ZM, Ware J, et al. The role of platelet adhesion receptor GPIIb/IIIa far exceeds that of its main ligand, von Willebrand factor, in arterial thrombosis. *Proc Natl Acad Sci USA*. (2006) 103:16900–5. doi: 10.1073/pnas.0608207103
28. Al-Tamimi M, Tan CW, Qiao J, Pennings GJ, Javadzadegan A, Yong AS, et al. Pathologic shear triggers shedding of vascular receptors: a novel mechanism for down-regulation of platelet glycoprotein VI in stenosed coronary vessels. *Blood* (2012) 119:4311–20. doi: 10.1182/blood-2011-10-386607
29. Andrews RK, Karunakaran D, Gardiner EE, Berndt MC. Platelet receptor proteolysis: a mechanism for downregulating platelet reactivity. *Arterioscler Thromb Vasc Biol*. (2007) 27:1511–20. doi: 10.1161/atvbaha.107.141390
30. Gardiner EE, Al-Tamimi M, Andrews RK, Berndt MC. Platelet receptor shedding. *Methods Mol Biol*. (2012) 788:321–39. doi: 10.1007/978-1-61779-307-3_22
31. Stephens G, Yan Y, Jandrot-Perrus M, Villevall JL, Clemetson KJ, Phillips DR. Platelet activation induces metalloproteinase-dependent GP VI cleavage to down-regulate platelet reactivity to collagen. *Blood* (2005) 105:186–91. doi: 10.1182/blood-2004-07-2842
32. de Boer AC, Genton E, Turpie AG. Chemistry, measurement, and clinical significance of platelet specific proteins. *Crit Rev Clin Lab Sci*. (1982) 18:183–211. doi: 10.3109/10408368209083495
33. Kaplan KL, Broekman MJ, Chernoff A, Lesznik GR, Drillings M. Platelet alpha-granule proteins: studies on release and subcellular localization. *Blood* (1979) 53:604–18.
34. Kaplan KL, Owen J. Plasma levels of beta-thromboglobulin and platelet factor 4 as indices of platelet activation in vivo. *Blood* (1981) 57:199–202.
35. Sagedal S, Sandvik L, Klingenberg O, Sandset PM. beta-Thromboglobulin may not reflect platelet activation during haemodialysis with the HeprAN membrane. *Scand J Clin Lab Invest*. (2017) 77:679–84. doi: 10.1080/00365513.2017.1397288
36. Skipper MT, Rubak P, Larsen OH, Hvas A-M. Thrombocytopenia model with minimal manipulation of blood cells allowing whole blood assessment of platelet function. *Platelets* (2016) 27:295–300. doi: 10.3109/09537104.2015.1095873
37. Femia EA, Scavone M, Lecchi A, Cattaneo M. Effect of platelet count on platelet aggregation measured with impedance aggregometry (Multiplate analyzer) and with light transmission aggregometry. *J Thromb Haemost*. (2013) 11:2193–6. doi: 10.1111/jth.12432
38. Würtz M, Hvas A-M, Kristensen SD, Grove EL. Platelet aggregation is dependent on platelet count in patients with coronary artery disease. *Thromb Res*. (2012) 129:56–61. doi: 10.1016/j.thromres.2011.08.019
39. Stissing T, Dridi NP, Ostrowski SR, Bochen L, Johansson PI. The influence of low platelet count on whole blood aggregometry assessed by Multiplate. *Clin Appl Thromb Hemost*. (2011) 17:E211–7. doi: 10.1177/1076029610397183
40. Hanke AA, Roberg K, Monaca E, Sellmann T, Weber CF, Rahe-Meyer N, et al. Impact of platelet count on results obtained from multiple electrode platelet aggregometry (Multiplate). *Eur J Med Res*. (2010) 15:214–9.
41. Rubak P, Villadsen K, Hvas A-M. Reference intervals for platelet aggregation assessed by multiple electrode platelet aggregometry. *Thromb Res*. (2012) 130:420–3. doi: 10.1016/j.thromres.2012.06.017
42. Goerge T, Ho-Tin-Noe B, Carbo C, Benarafa C, Remold-O'Donnell E, Zhao BQ, et al. Inflammation induces hemorrhage in thrombocytopenia. *Blood* (2008) 111:4958–64. doi: 10.1182/blood-2007-11-123620
43. Skipper MT, Rubak P, Stentoft J, Hvas AM, Larsen OH. Evaluation of platelet function in thrombocytopenia. *Platelets* (2017) 2017:1–7. doi: 10.1080/09537104.2017.1296566

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Balle, Jeppesen, Christensen and Hvas. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Inherited Thrombocytopenia: Update on Genes and Genetic Variants Which may be Associated With Bleeding

Ibrahim Almazni, Rachel Stapley and Neil V. Morgan*

Institute of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham, Birmingham, United Kingdom

OPEN ACCESS

Edited by:

Vera Ignjatovic,
Murdoch Childrens Research
Institute, Australia

Reviewed by:

Patricia B. Maguire,
University College Dublin, Ireland
Cécile Oury,
University of Liège, Belgium

*Correspondence:

Neil V. Morgan
n.v.morgan@bham.ac.uk

Specialty section:

This article was submitted to
Atherosclerosis and Vascular
Medicine,
a section of the journal
Frontiers in Cardiovascular Medicine

Received: 24 April 2019

Accepted: 31 May 2019

Published: 19 June 2019

Citation:

Almazni I, Stapley R and Morgan NV
(2019) Inherited Thrombocytopenia:
Update on Genes and Genetic
Variants Which may be Associated
With Bleeding.
Front. Cardiovasc. Med. 6:80.
doi: 10.3389/fcvm.2019.00080

Inherited thrombocytopenia (IT) is comprised of a group of hereditary disorders characterized by a reduced platelet count as the main feature, and often with abnormal platelet function, which can subsequently lead to impaired haemostasis. Inherited thrombocytopenia results from genetic mutations in genes implicated in megakaryocyte differentiation and/or platelet formation and clearance. The identification of the underlying causative gene of IT is challenging given the high degree of heterogeneity, but important due to the presence of various clinical presentations and prognosis, where some defects can lead to hematological malignancies. Traditional platelet function tests, clinical manifestations, and hematological parameters allow for an initial diagnosis. However, employing Next-Generation Sequencing (NGS), such as Whole Genome and Whole Exome Sequencing (WES) can be an efficient method for discovering causal genetic variants in both known and novel genes not previously implicated in IT. To date, 40 genes and their mutations have been implicated to cause many different forms of inherited thrombocytopenia. Nevertheless, despite this advancement in the diagnosis of IT, the molecular mechanism underlying IT in some patients remains unexplained. In this review, we will discuss the genetics of thrombocytopenia summarizing the recent advancement in investigation and diagnosis of IT using phenotypic approaches, high-throughput sequencing, targeted gene panels, and bioinformatics tools.

Keywords: inherited thrombocytopenia, platelets, megakaryocytes, genes, mutations, bleeding

INTRODUCTION

Platelets are small anucleate cells produced by megakaryocytes in the bone marrow (BM) where they circulate in the blood to protect the integrity of blood vessels. They play an important role in normal haemostasis to prevent excessive bleeding at the site of blood vessel injury (1, 2). Inherited Thrombocytopenias (ITs) are a heterogeneous group of disorders characterized by low platelet counts, often manifesting as bleeding diathesis which subsequently result in impaired haemostasis (3). In 1948, the disease inheritance pattern of one IT was initially discovered in a disorder called Bernard-Soulier syndrome (BSS) (4). Since then, the advancement in clinical and scientific research has led to an increased understanding of the molecular defects in patients with ITs. These defects are variable in severity, ranging from severe bleeding, which can be recognized within a few weeks after birth, to mild bleeding that may remain undiagnosed until incidental recognition during

routine blood testing in adulthood (5). They manifest with different symptoms including epistaxis, easy bruising, petechiae, prolonged bleeding from cuts, gum bleeding, excessive bleeding after surgery, hematuria, and menorrhagia in women (6, 7). As bleeding is considered the main clinical complication for patients with IT, some patients with common ITs have the propensity to develop other disorders such as hematological malignancies and kidney failure (4, 8). Although there are other causes of thrombocytopenia, such as infections and immune disorders, IT is primarily caused by mutations in genes involved in megakaryocyte differentiation, maturation and platelet release (9). Since the last decade, next generation sequencing technologies, namely Whole Exome Sequencing (WES) and Whole Genome Sequencing (WGS) coupled with conventional Sanger sequencing and *in-silico* bioinformatic tools have been used in parallel to uncover novel genes with a pivotal role in megakaryocyte biology and platelet biogenesis (10, 11). To date, 40 genes have been reported to cause different forms of IT, which reflects the immense difficulty in identifying a single causative gene, particularly when accompanied by other hematological disorders [Table 1; Figure 1; (27, 28, 54)]. These genetic forms have various clinical manifestations, phenotypic presentations and sometimes associated with secondary qualitative defects in platelet function (7). Diverse platelet phenotypes mean there are several approaches in which they can be characterized. One such way is to classify genes based on their influence on megakaryocyte differentiation, platelet production, and removal (54), and will be discussed below. However, despite these advancements, nearly 50% of patients with IT of unknown genetic etiology still remain undiagnosed (6, 10).

IT GENES ASSOCIATED WITH MEGAKARYOCYTE DIFFERENTIATION AND MATURATION

The process of megakaryopoiesis and thrombopoiesis involves a complicated biological series of events. Megakaryocytes, like all blood cells, are derived from the hematopoietic stem cells (HSC) in the bone marrow during the lineage commitment stages. The hematopoietic stem cell differentiation process involves committed precursors that include the common myeloid progenitor (CMP) and the megakaryocyte-erythroid progenitor (MEP). The erythrocyte cells and megakaryocyte cells result from the MEP. Megakaryocyte precursors encompass a maturation process that results in mature polyploid megakaryocytes, and then leads to the formation of pro-platelets (54, 55). The process of megakaryopoiesis and thrombopoiesis involves multiple genes and transcription factors (detailed below) which play important roles in megakaryocyte differentiation, platelet formation, and release. IT can result from defects in these genes which present with variable phenotypic display and clinical presentation. As a result of the numerous clinical demonstrations of ITs, they can be characterized based on genes and their role during megakaryocyte differentiation, platelet production, and release (9, 56).

Some ITs result from defective changes from haemopoietic progenitor cells to MKs, leading to reduction or absence in the number of bone marrow MKs. Thrombopoietin (TPO), an acidic glycoprotein, is the main regulator of the megakaryopoiesis and thrombopoiesis mechanism in humans, acting through its receptor c-Mpl. It is required for megakaryocytes to fulfill their developmental proliferation and for the subsequent maturation of platelets (57). Affected individuals from a large Micronesian family displayed idiopathic anemia and mild thrombocytopenia as a result of mutations in *TPO* and *MPL* genes (21, 58). The main defective mechanism in several forms of IT is a change in MK maturation which therefore leads to the production of immature and dysfunctional MKs. However, the differentiation and maturation of MKs is regulated by several transcription factors such as *GATA1*. *GATA1* is highly expressed in the erythroid and megakaryocytic lineage, and plays a vital role in the maturation and development of erythroid cells and megakaryocytes (59). X-linked thrombocytopenia with thalassemia and X-linked thrombocytopenia with dyserythropoietic anemia are both caused by mutations in *GATA1*, resulting in impaired MK and erythroid cell maturation. As a consequence, *GATA1*-mutated patients are characterized with large platelets and reduced α -granule contents. They also display a variable degree of anemia and abnormal morphology of red blood cells (60). Additional transcription factors known to be involved in the maturation of megakaryocytes are *RUNX1*, *ETV6*, *ANKRD26*, *FLI-1*, and the transcriptional repressor *GFI1B* acting by binding to promoter regions in MK expressed genes. Thus, multiple mechanisms in MK and platelet maturation are affected as result of alterations in these genes (61, 62). A previous study identified a point mutation in the third helix of *HOXA11* homeodomain causing an inherited syndrome of congenital amegakaryocytic thrombocytopenia and radio-ulnar synostosis (19). Thrombocytopenia absent radii (TAR) syndrome results from a combination of a microdeletion on Chromosome 1 including the *RBM8A* gene alongside a low frequency non-coding single nucleotide polymorphism (SNP) within the regulatory region of *RBM8A* (23). As a consequence, hematopoietic progenitors from patients with TAR syndrome fail to differentiate into MKs *in vitro* (63). Gray Platelet syndrome is characterized by a deficiency in α granule content which also results in a platelet function defect. It is associated with enlarged platelets and mild thrombocytopenia with moderate to severe bleeding as a result of biallelic mutations in *NBEAL2*, the gene encoding the neurobeachin-like-2 protein (22). Variants in the 5'UTR of *ANKRD26* cause familial thrombocytopenia type-2 (THC2) with propensity to leukemia, which result in loss of *RUNX1* and *FLI1* binding and prevents gene silencing (12). Moreover, heterozygous variants specifically located in the promoter region between c.-134G and c.-113 region highly affect gene expression. Patients with THC2 are characterized by small MKs with hypolobulated nuclei as a result of dysmegakaryopoiesis (64). A mutation in the *FYB1* gene has recently been identified to cause IT and although the exact mechanism of the mutation is still ambiguous, it has been suggested that thrombocytopenia arises from a reduction of mature MKs in the bone marrow and synthesis of small platelets (16).

TABLE 1 | The direct genetic causes of inherited thrombocytopenia and their associated syndromes.

Area of mutational effect	Gene	Syndrome	Other syndromic and features	References
Megakaryopoiesis	<i>ANKRD26</i>	<i>ANKRD26</i> -related thrombocytopenia	Predisposition to leukemia. Reduction of platelet α -granules. Normal <i>in vitro</i> platelet aggregation and mean platelet volume. Some patients have high level of hemoglobin and leukocyte.	(12, 13)
	<i>ETV6</i>	<i>ETV6</i> -related thrombocytopenia	Leukemia predisposition. High erythrocyte mean corpuscular volume (MCV). Some patients have elevated red cell MCV.	(14)
	<i>FLI1</i>	Paris-Trousseau thrombocytopenia/Jacobsen syndrome	Abnormal development of heart and face. Intellectual disabilities. Large α -granules. Abnormal MKs morphology. Normal RBCs and WBCs counts. Moderate thrombocytopenia.	(15)
	<i>FYB</i>	<i>FYB</i> -related thrombocytopenia	Small platelets. Reduction of mature MKs in BM. Significant bleeding tendency. Normal WBCs count. Low mean platelet volume MPV. Mild iron deficiency anemia.	(16)
	<i>GATA1</i>	GATA1-related disease: X-linked thrombocytopenia (XLT) and X-linked thrombocytopenia with thalassemia (XLTT)	Dyserythropoietic anemia. Macrothrombocytopenia. Beta-thalassemia. Congenital erythropoietic porphyria. Erythrocyte abnormalities. Splenomegaly.	(17)
	<i>GFI1B</i>	Macrothrombocytopenia and platelet function defects	Macrothrombocytopenia. Red cell anisopoikilocytosis. Platelet dysfunction. Reduction of platelet α -granules.	(18)
	<i>HOXA11</i>	Amegakaryocytic thrombocytopenia with radio-ulnar synostosis	Bilateral radioulnar synostosis. Severe bone marrow failure. Cardiac and renal malformations. B-cell deficiency. Hearing loss. Clinodactyly. Some patients show skeletal anomalies. Some patients have developed pancytopenia.	(19)
	<i>MECOM</i>	Congenital amegakaryocytic thrombocytopenia and radioulnar synostosis		(20)
	<i>MPL</i>	Congenital amegakaryocytic thrombocytopenia (CAMT)	Absence or reduced of MKs in BM. No physical anomalies. Development to BM aplasia in infancy.	(21)
	<i>NBEAL2</i>	Gray platelet syndrome	Impaired platelet function. Severe reduction of platelet α -granules contents. Large platelets. Development of myelofibrosis and splenomegaly in some patients. Abnormalities in megakaryocyte development.	(22)
	<i>RBM8A</i>	Thrombocytopenia-absent radius syndrome	Bilateral radial aplasia. Elevated hemoglobin level in patients with 5' UTR SNP. Normal WBCs count and some patients have leucocytosis and eosinophilia. Anemia. Skeletal, urogenital, kidney, and heart defects. Reduced MKs in BM.	(23)
	<i>RUNX1</i>	Familial platelet disorder with propensity to acute myelogenous leukemia (FPD/AML)	Platelet defects. Variable platelet counts. Reduction in dense granule secretion observed in secondary qualitative abnormality. Myelodysplasia. Reduced response to several platelet agonists.	(8)
	<i>SLFN14</i>	<i>SLFN14</i> -related thrombocytopenia	Giant platelets. Decreased ATP secretion. Reduced number of dense granules.	(24)
	<i>SRC</i>	<i>SRC</i> -related thrombocytopenia	Myelofibrosis, bleeding, and bone pathologies. Hypercellular bone marrow with trilineage dysplasia. Platelets are dysmorphic and variable in size. Paucity of α -granules. Splenomegaly, congenital facial dysmorphism. Abundant vacuoles.	(25)
	<i>THPO</i>	Inherited thrombocytopenia from monoallelic <i>THPO</i> mutation	Bone marrow aplasia. Normal or enlarged platelet morphology.	(26)
	<i>PTPRJ</i>	Inherited thrombocytopenia	Syndromic thrombocytopenia characterized by spontaneous bleeding, small-sized platelets. Impaired platelet function.	(27)
	<i>GALE</i>	Inherited thrombocytopenia	Dysplastic megakaryocytes. Some patients have mild anemia and febrile neutropenia. Big and pale platelets. Galactosemia, hypotonia, seizures, jaundice, galactosuria, and hepatomegaly.	(28)
Platelet production/clearance	<i>ACTN1</i>	<i>ACTN1</i> -related thrombocytopenia	Congenital macrothrombocytopenia. Anisocytosis. Absent or mild bleeding diathesis.	(29)
	<i>CYCS</i>	<i>CYCS</i> -related thrombocytopenia	Normal platelet size and volume.	(30)
	<i>GNE</i>	<i>GNE</i> myopathy with congenital thrombocytopenia	Rimmed vacuoles. Hematological complications are rare. Proteinuria and hematuria in some patients. Membranoproliferative glomerulonephritis. Platelets size are normal to large.	(31–33)

(Continued)

TABLE 1 | Continued

Area of mutational effect	Gene	Syndrome	Other syndromic and features	References
	<i>GP1BA</i>	Bernard-Soulier Syndrome (BSS) + platelet type von-Willebrand disease (PTvWD)	Macrothrombocytopaenia. Severe bleeding tendency with platelet function defect. Platelet anisocytosis.	(34–36)
	<i>GPIBB</i>			
	<i>GP9</i>			
	<i>ITGA2B</i>	Glanzmann thrombasthenia	Impaired platelet function.	(37, 38)
	<i>ITGB3</i>			
	<i>MYH9</i>	<i>MYH9</i> -related disease (MYH9-RD)	Congenital macrothrombocytopaenia. Mild bleeding tendency. Development of kidney dysfunction, deafness, cataracts, and Döhle-like bodies. Elevated liver enzymes.	(39)
	<i>PRKACG</i>	<i>PRKACG</i> -related thrombocytopenia	Giant platelet. Impaired platelet function.	(40)
	<i>TRPM7</i>	<i>TRPM7</i> -related thrombocytopenia	Macrothrombocytopaenia. Atrial fibrillation.	(41)
	<i>TPM4</i>	Tropomyosin 4-related thrombocytopenia	Macrothrombocytopaenia. All other blood cell counts are normal. Mild effect on platelet function.	(42)
	<i>TUBB1</i>	<i>TUBB1</i> -related thrombocytopenia	Congenital macrothrombocytopaenia.	(43)
	<i>WAS</i>	Wiskott-Aldrich syndrome, X-linked thrombocytopenia (XLT)	Mild or severe immunodeficiency, hematopoietic malignancies, and eczema. Thrombocytopenia with small platelets. Autoimmune haemolytic anemia.	(44)
	<i>FLNA</i>	Filaminopathies A	X-linked dominant form of periventricular nodular heterotopia (FLNA-PVNH) and the otopalatodigital syndrome spectrum of disorders. Hemorrhage and coagulopathy. Abnormal platelet morphology.	(45)
	<i>DIAPH1</i>	Macrothrombocytopenia (MTP) and hearing loss	–	(46)
Other/unknown	<i>ABCG5</i>	Macrothrombocytopenia associated with sitosterolemia	Xanthomas and pre-mature coronary atherosclerosis due to hypercholesterolemia. Hematologic abnormalities.	(47)
	<i>ABCG8</i>			
	<i>ADAMTS13</i>	Thrombotic thrombocytopenic purpura	Upshaw_Schulman syndrome. Anemia.	(48)
	<i>STIM1</i>	Stormorken syndrome and york platelet syndrome	Tubular myopathy and congenital miosis. Severe immune dysfunction.	(49, 50)
	<i>vWF</i>	Von Willebrand disease type IIB		(51)
	<i>ORAI1</i>	Stormorken syndrome	CRAC channelopathy. Severe combined immunodeficiency, autoimmunity, muscular hypotonia, and ectodermal dysplasia.	(52)
	<i>MASTL</i>	Autosomal dominant thrombocytopenia	–	(53)

DEFECTS IN PROPLATELET FORMATION AND PLATELET RELEASE

After megakaryopoiesis, proplatelets form extensions which lead to “budding” at the tips and platelet release into the circulation. Mature MKs undergo essential processing by extending long branches called proplatelets via the bone marrow sinusoids, and subsequently release platelets into the blood circulation. These processes are underpinned by cytoskeletal changes and cellular signaling where most causative mutations of IT disrupt the pathway reducing the circulating platelet count (65, 66). Mature polyploid MKs cytoplasm extend long beaded cytoplasmic protrusions, as a result of microtubule sliding. The dimerisation of β 1-tubulin with α -tubulin polymerizes into long microtubule bundles inside the MK cortex. A mixed polarity of microtubule bundles runs throughout the extension of proplatelets which are thought to provide fundamental force for microtubule sliding and proplatelet elongation (65). *TUBB1* encodes for

β 1-tubulin and mutations within *TUBB1* are associated with an autosomal dominant form of IT known as a congenital macrothrombocytopenia (43). WASp is a multidomain protein belonging to a family of actin nucleation-promoting factors (NPFs) which are specifically expressed in hematopoietic cells. WASp plays an important role in actin polymerization by transmission of surface signals via the actin-related protein (Arp)2/3 complex (44, 67). Mutations have been identified in the *WAS* gene which cause a rare X-linked disorder called Wiskott-Aldrich syndrome (WAS). Patients are characterized by microthrombocytopenia and immunodeficiency with predisposition to malignancies (68). The transmission of extracellular signals into the cytoskeleton is mediated via membrane bound receptors which have been associated with mutations in IT. One of the main membrane receptors in platelets/MKs is the GP1b-IX-V complex, which binds specifically to Von-Willebrand factor (VWF). This receptor is comprised of four subunits including GP1b α , GP1b β , GPIX, and GPV. Binding of VWF with GP1b α

leads to activation and signal transmission to form the extending proplatelet. Mutations in the encoding genes *GP1BA*, *GP1BB*, and *GP9* cause monoallelic and biallelic forms of BSS (69, 70). Other receptors include the receptor for fibrinogen, integrin GPIIb-IIIa, which is encoded by the genes *ITGA2B* and *ITGB3*. Affected mutations in *ITGA2B* and *ITGB3* have been identified to cause Glanzmann thrombasthenia (GT) (71), but patients have a normal platelet count.

INITIAL DIAGNOSIS OF HEREDITARY THROMBOCYTOPENIA

Identification of the genetic cause in patients with IT is challenging and many patients may be misdiagnosed with acquired thrombocytopenia such as immune thrombocytopenic purpura (ITP). IT can be recognized in patients when a low platelet count has been identified after birth, the presence of familial medical history with similar clinical presentations, evaluation of peripheral blood films, and physical examination. Moreover, the presence of a severe bleeding tendency (in combination with a low platelet count), a lifelong history of diathesis, and evidence of other clinical complications that are typically associated with thrombocytopenia in syndromic forms, and all help to diagnose IT (4, 72). Platelets are involved in other biological roles beyond hemostasis, such as immunity and inflammation (73–75) therefore, mutations in platelet specific genes may cause functional disruption in hemostasis, other biological pathways or both. Furthermore, some proteins are expressed in megakaryocytes and platelets and can be found in other cell types. *GATA1* is a prominent example which involves megakaryopoiesis and erythropoiesis (76). *MYH9* also has an important role in the platelet cytoskeleton and has been found expressed in kidney and inner ear cilia (77). Based on this, inherited bleeding disorders can be classified into three categories including disorders that (i) affect only platelets, (ii) disorders that are associated with syndromic or non-syndromic phenotypes, and (iii) disorders with increased risk of haematological malignancies. This classification can be used for both diagnostic and prognostic purposes (4, 11).

SYNDROMIC DISORDERS ASSOCIATED WITH IT

The number of IT forms identified has increased over the last few years since the implementation of NGS. Consequently, it has been shown that the bleeding is not the only clinical phenotype with IT, but patients with some IT forms have propensity to develop more syndromic disorders as result of molecular defects in genes responsible for thrombocytopenia. For instance, hematological malignancies, bone marrow aplasia, skeletal malformation, liver and kidney malfunction, and deafness (Figure 2). The development of these diseases can be more severe for patients than the bleeding itself (4) however, it is still important to recognize if these manifestations are present in the relatives. Some syndromic phenotypes associated with ITs are variable between family members or can arise later in

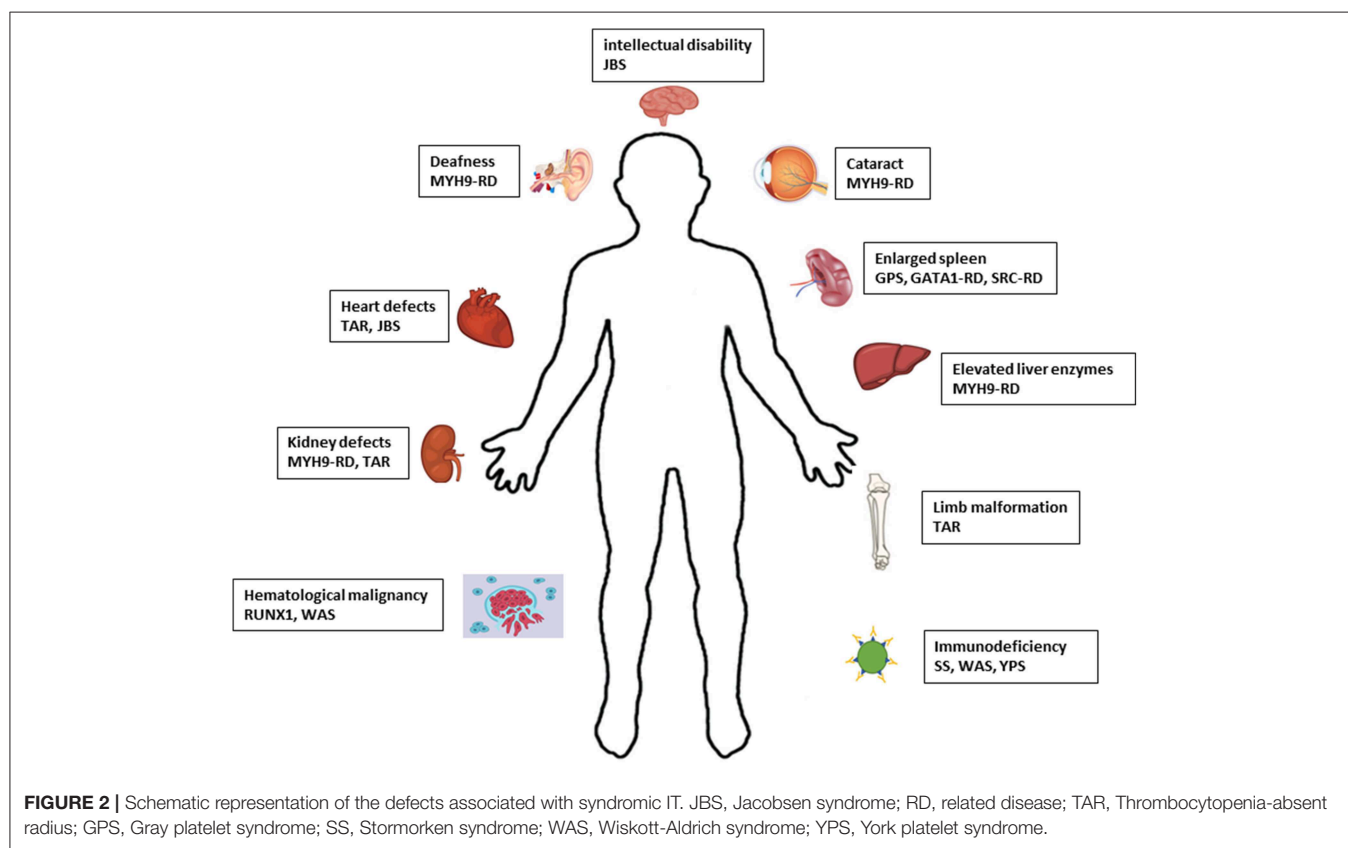
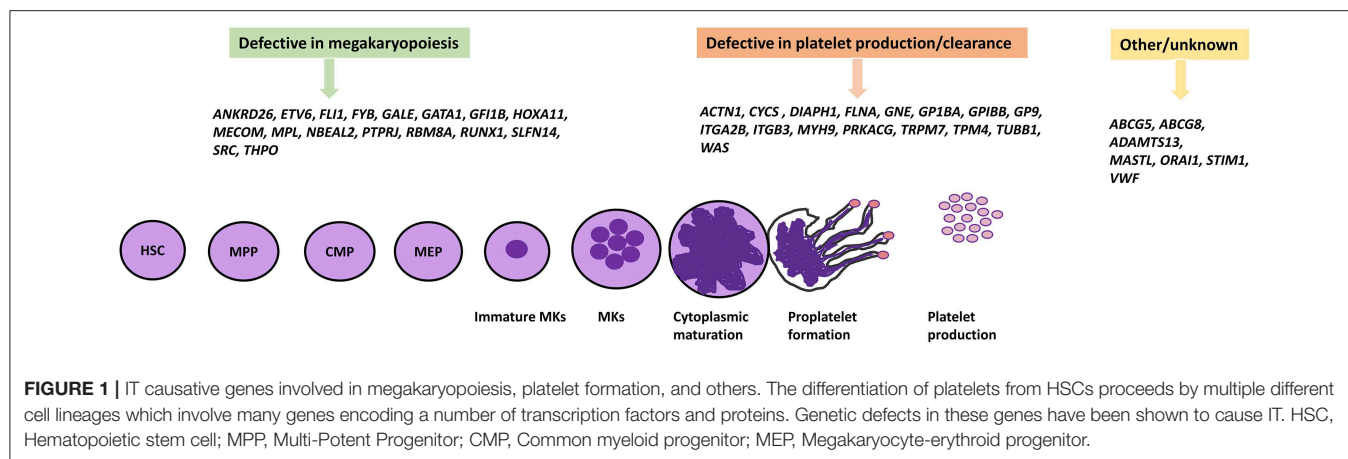
life. For example, development of deafness, kidney malfunction and/or cataract in patients with *MYH9*-RD occur only in adult individuals and it has been reported that patients of the same *MYH9*-RD pedigrees have variable clinical manifestations (78).

THE GENETIC DIAGNOSIS OF IT

Some patients with a low platelet count may be falsely diagnosed and receive unnecessary treatments such as immunosuppression and splenectomies and therefore it is paramount that strong evidence must prove that the condition is truly genetic. Genetic diagnosis is a vital approach in providing patients with clinical benefits and prevent unnecessary treatments. Patients with genetic mutations in *RUNX1* have a predisposition to develop hematological malignancies where the genetic information can be used to monitor the patients' hematological parameters very closely. This emphasizes the importance and need for definitive genetic diagnostic tools to provide quick and cost-effective diagnosis for screening patients with IT (6, 8). The molecular basis of ITs has been elucidated since the adoption of Sanger sequencing and linkage analysis in the 1990s. Recently, Sanger sequencing is considered a low throughput and time-consuming approach which can be used initially as a standard tool to investigate patients based on precise clinical findings and phenotype (4). A targeted thrombocytopenia gene specific panel is a useful approach which can be used as initial screening prior to WES. This targeted panel encompasses all known genes associated with IT and their related genes. The aim of using an IT gene specific panel is to filter out patients based on variants in known IT-causal genes and subsequently allowing for WES for patients with unknown genetic etiology (4, 6). The ThromboGenomics project provided a multi-gene high-throughput sequencing platform (HTS) for the diagnosis of heritable bleeding disorders (79). The HTS platform covers approximately 96 genes associated with inherited bleeding, thrombotic, coagulation, and platelet disorders. The panel showed high sensitivity in detecting causative variants in patients who had not been previously investigated at the molecular level. It has a high sensitivity to detect variants in the exonic region as well as many of exonic-intronic boundaries and untranslated regions (UTRs) (6, 79).

NEXT GENERATION SEQUENCING

Targeted NGS platforms can be efficiently applied to determine the causative genes of IT. As the molecular basis of ITs remain unknown in many patients, WGS or WES may be required which improves the knowledge of ITs at the molecular level. Several national and international consortia have adopted these approaches to identify disease-causing genes associated with IT. The genes *SLFN14*, *FYB*, *STIM1*, *GFI1b*, and *ETV6* are some examples of causative genes detected by these approaches. The results obtained by HTS improves the understanding of the functional role in some causative genes, whose function in platelet production was previously unknown. These techniques will bring substantial benefits to improve our



understanding of the molecular mechanisms in megakaryocyte and platelet biogenesis (14, 16, 24, 49, 80, 81). However, distinguishing pathogenic variants from non-pathogenic variants often requires complex functional and cell line studies to prove causality (7).

BIOINFORMATIC TOOLS

Bioinformatic tools can be conducted to determine candidate variants from WES or WGS data. A wide range of variants,

~25,000–40,000 variants can be identified per single patient in WES. These variants can be filtered for novelty by direct comparison using a database from the 100,000 Genomes Project, Exon Variant Server (EVS), dbSNP versions, Exome Aggregation Consortium (ExAC) (gnomAD), and in-house databases of whole exomes and/or whole genomes. A database of known platelet-related genes and genes involved in platelet formation, function, lifespan, or death can be compared with the patient's genes in order to narrow the candidate genetic variants down. Variants with MAF (minor allele frequency) ≥ 0.01 are generally excluded given the rarity of most of

these genetic defects in IT. Variants not known to change the amino acid or those that do not have a potential effect on protein, such as synonymous variants and intron variants can also be excluded. Splice site variants occurring >5 base pair away from the exome can be also excluded, although this can potentially result in splicing or regulatory mutations being missed. Comparisons with other affected and unaffected family members on the database can be used to select candidate variants. Also, pathogenicity prediction can be assessed by using different tools such PolyPhen2, Provean, SIFT, Mutation Taster, mRNA expression levels which predict the potential effect of amino acid changes on protein structure and function and also measure the conservation of amino acids among different species. Sapiencia is a recently developed clinical diagnostic platform established by Congenica to help clinicians, clinical scientists, and researchers with genetic diagnosis and identification of disease-causing genes, by interrogating the human genome with multiple bioinformatic tools. It can help streamline the process of diagnosis, ensuring patients are receiving accurate information and treatments for their individual platelet or megakaryocyte defect.

CONCLUSIONS

The advances in NGS techniques improves our knowledge about the molecular mechanisms of IT. The major risk factor for patients with ITs is the development of additional syndromic disorders rather than bleeding itself. Due to the polygenic nature of ITs and disorders involved in hematopoiesis, identifying a

singular causative gene for platelet and megakaryocyte function is particularly difficult. A combination of whole blood counting and platelet functional assays will highlight the platelet phenotype, however only familial studies and genetic sequencing will help to identify any genetic defect. With the introduction of HTS and various genome browser software such as Sapiencia, the diagnosis process has been modernized to highlight candidate variants in known platelet affected genes and reveal variants in novel genes in which hemostatic input remains to be explored. Approaches such as these may be implemented within clinical settings in the future, however, bioinformatic pipelines are yet to be standardized across all facilities. Aside from bioinformatic training and the initial financial burden of installation of the software, there are clear reasons that updating current genetic analysis in hematological disorders benefits healthcare in the wider community. This will ensure IT families obtain a clear diagnosis and receive correct treatment based on their genetically influenced megakaryocyte or platelet defect.

AUTHOR CONTRIBUTIONS

IA wrote the manuscript. IA, RS, and NM critically reviewed and edited the review.

FUNDING

The work in the author's laboratories is supported by the British Heart Foundation (PG/16/103/32650; FS/18/11/33443) and the Saudi Arabia Cultural Bureau in London.

REFERENCES

1. D'Andrea G, Chetta M, Margaglione M. Inherited platelet disorders: thrombocytopenias and thrombocytopathies. *Blood Transfus.* (2009) 7:278. doi: 10.2450/2009.0078-08
2. Davi G, Patrono C. Platelet activation and atherothrombosis. *N Engl J Med.* (2007) 357:2482–94. doi: 10.1056/NEJMra071014
3. Balduini A, Raslova H, Di Buduo CA, Donada A, Ballmaier M, Germeshausen M, et al. Clinic, pathogenic mechanisms and drug testing of two inherited thrombocytopenias, ANKRD26- and MYH9-related diseases. *Eur J Med Genet.* (2018) 61:715–22. doi: 10.1016/j.ejmg.2018.01.014
4. Noris P, Pecci A. Hereditary thrombocytopenias: a growing list of disorders. *Hematology.* (2017) 2017:385–99. doi: 10.1182/asheducation-2017.1.385
5. Drachman JG. Review in translational hematology inherited thrombocytopenia : when a low platelet count does not mean ITP. *Blood.* (2004) 103:390–8. doi: 10.1182/blood-2003-05-1742
6. Johnson B, Doak R, Allsup D, Astwood E, Evans G, Grimley C, et al. A comprehensive targeted next-generation sequencing panel for genetic diagnosis of patients with suspected inherited thrombocytopenia. *Res Pract Thromb Haemost.* (2018) 2:640–52. doi: 10.1002/rth2.12151
7. Johnson B, Lowe GC, Futterer J, Lordkipanidzé M, MacDonald D, Simpson MA, et al. Whole exome sequencing identifies genetic variants in inherited thrombocytopenia with secondary qualitative function defects. *Haematologica.* (2016) 101:1170–9. doi: 10.3324/haematol.2016.146316
8. Morgan NV, Daly ME. Gene of the issue: RUNX1 mutations and inherited bleeding. *Platelets.* (2017) 28:208–10. doi: 10.1080/09537104.2017.1280151
9. Balduini CL, Melazzini F, Pecci A. Inherited thrombocytopenias—recent advances in clinical and molecular aspects. *Platelets.* (2017) 28:3–13. doi: 10.3109/09537104.2016.1171835
10. Savoia A. Molecular basis of inherited thrombocytopenias: an update. *Curr Opin Hematol.* (2016) 23:486–92. doi: 10.1097/MOH.0000000000000269
11. Greinacher A, Eekels JJM. Diagnosis of hereditary platelet disorders in the era of next-generation sequencing: “primum non nocere.” *J Thromb Haemost.* (2019) 17:551–4. doi: 10.1111/jth.14377
12. Bluteau D, Balduini A, Balayn N, Currao M, Nurden P, Deswarte C, et al. Thrombocytopenia-associated mutations in the ANKRD26 regulatory region induce MAPK hyperactivation. *J Clin Invest.* (2014) 124:580–91. doi: 10.1172/JCI71861
13. Pippucci T, Savoia A, Perrotta S, Ria Pujol-Moix N, Noris P, Castegnaro G, et al. Mutations in the 5'UTR of ANKRD26, the ankirin repeat domain 26 gene, cause an autosomal-dominant form of inherited thrombocytopenia, THC2. *Am J Hum Genet.* (2011) 88:115–20. doi: 10.1016/j.ajhg.2010.12.006
14. Noetzi L, Lo RW, Lee-Sherick AB, Callaghan M, Noris P, Savoia A, et al. Germline mutations in ETV6 are associated with thrombocytopenia, red cell macrocytosis and predisposition to lymphoblastic leukemia. *Nat Genet.* (2015) 47:535–8. doi: 10.1038/ng.3253
15. Stevenson WS, Rabbolini DJ, Beutler L, Chen Q, Gabrielli S, Mackay JP, et al. Paris-Trousseau thrombocytopenia is phenocopied by the autosomal recessive inheritance of a DNA-binding domain mutation in FLI1. *Blood.* (2015) 126:2027–30. doi: 10.1182/blood-2015-06-650887
16. Koren A, Khayat M, Hauschner H, Pretorius E, Rosenberg N, Zalman L, et al. Deleterious mutation in the FYB gene is associated with congenital autosomal recessive small-platelet thrombocytopenia. *J Thromb Haemost.* (2015) 13:1285–92. doi: 10.1111/jth.12966

17. Freson K, Wijgaerts A, Van Geet C. GATA1 gene variants associated with thrombocytopenia and anemia. *Platelets*. (2017) 28:731–4. doi: 10.1080/09537104.2017.1361525
18. Stevenson WS, Morel-Kopp MC, Chen Q, Liang HP, Bromhead CJ, Wright S, et al. GFI1B mutation causes a bleeding disorder with abnormal platelet function. *J Thromb Haemost*. (2013) 11:2039–47. doi: 10.1111/jth.12368
19. Horvat-Switzer RD, Thompson AA. HOXA11 mutation in amegakaryocytic thrombocytopenia with radio-ulnar synostosis syndrome inhibits megakaryocytic differentiation *in vitro*. *Blood Cells Mol Dis*. (2006) 37:55–63. doi: 10.1016/j.bcmd.2006.04.001
20. Germeshausen M, Ancliff P, Estrada J, Metzler M, Ponstingl E, Schwabe D, et al. MECOM-associated syndrome: a heterogeneous inherited bone marrow failure syndrome with amegakaryocytic thrombocytopenia. *Blood Adv*. (2018) 2:586–96. doi: 10.1182/bloodadvances.2018016501
21. Ihara K, Ishii E, Eguchi M, Takada H, Suminoe A, Good RA, et al. Identification of mutations in the c-mpl gene in congenital amegakaryocytic thrombocytopenia. *Med Sci*. (1999) 96:3132–6. doi: 10.1073/pnas.96.6.3132
22. Pluthero FG, Di Paola J, Carcao MD, Kahr WHA. NBEAL2 mutations and bleeding in patients with gray platelet syndrome. *Platelets*. (2018) 29:632–5. doi: 10.1080/09537104.2018.1478405
23. Manukjan G, Bösing H, Schmugge M, Strauß G, Schulze H. Impact of genetic variants on haematopoiesis in patients with thrombocytopenia absent radii (TAR) syndrome. *Br J Haematol*. (2017) 179:606–17. doi: 10.1111/bjh.14913
24. Fletcher SJ, Johnson B, Lowe GC, Bem D, Drake S, Lordkipanidzé M, et al. SLFN14 mutations underlie thrombocytopenia with excessive bleeding and platelet secretion defects. *J Clin Invest*. (2015) 125:3600–5. doi: 10.1172/JCI80347
25. Turro E, Greene D, Wijgaerts A, Thys C, Lentaigne C, Bariana TK, et al. A dominant gain-of-function mutation in universal tyrosine kinase SRC causes thrombocytopenia, myelofibrosis, bleeding, and bone pathologies. *Sci Transl Med*. (2016) 8:328ra30. doi: 10.1126/scitranslmed.aad7666
26. Noris P, Marconi C, De Rocco D, Melazzini F, Pippucci T, Loffredo G, et al. A new form of inherited thrombocytopenia due to monoallelic loss of function mutation in the thrombopoietin gene. *Br J Haematol*. (2017) 181:698–701. doi: 10.1111/bjh.14694
27. Wen R, Wang D. PTPRJ: a novel inherited thrombocytopenia gene. *Blood*. (2019) 133:1272–4. doi: 10.1182/blood-2019-01-895102
28. Seo A, Gulsuner S, Pierce S, Ben-Harosh M, Shalev H, Walsh T, et al. Inherited thrombocytopenia associated with mutation of UDP-galactose-4-epimerase (GALE). *Hum Mol Genet*. (2019) 28:133–42. doi: 10.1093/hmg/ddy334
29. Kunishima S, Okuno Y, Yoshida K, Shiraishi Y, Sanada M, Muramatsu H, et al. ACTN1 mutations cause congenital macrothrombocytopenia. *Am J Hum Genet*. (2013) 92:431–8. doi: 10.1016/j.ajhg.2013.01.015
30. Ong L, Morison IM, Ledgerwood EC. Megakaryocytes from CYCS mutation-associated thrombocytopenia release platelets by both proplatelet-dependent and -independent processes. *Br J Haematol*. (2017) 176:268–79. doi: 10.1111/bjh.14421
31. Futterer J, Dalby A, Lowe GC, Johnson B, Simpson MA, Motwani J, et al. Mutation in GNE is associated with severe congenital thrombocytopenia. *Blood*. (2018) 132:1855–8. doi: 10.1182/blood-2018-04-847798
32. Revel-Vilk S, Shai E, Turro E, Jahshan N, Hi-Am E, Spectre G, et al. GNE variants causing autosomal recessive macrothrombocytopenia without associated muscle wasting. *Blood*. (2018) 132:1851–4. doi: 10.1182/blood-2018-04-845545
33. Izumi R, Niihori T, Suzuki N, Sasahara Y, Rikiishi T, Nishiyama A, et al. GNE myopathy associated with congenital thrombocytopenia: a report of two siblings. *Neuromuscul Disord*. (2014) 24:1068–72. doi: 10.1016/j.nmd.2014.07.008
34. Sivapalaratnam S, Westbury SK, Stephens JC, Greene D, Downes K, Kelly AM, et al. Rare variants in GPIIB are responsible for autosomal dominant macrothrombocytopenia. *Blood*. (2017) 129:520–4. doi: 10.1182/blood-2016-08-732248
35. Balduini A, Malara A, Balduini CL, Noris P. Megakaryocytes derived from patients with the classical form of Bernard-Soulier syndrome show no ability to extend proplatelets *in vitro*. *Platelets*. (2011) 22:308–11. doi: 10.3109/09537104.2010.547960
36. Wright SD, Michaelides K, Johnson DJD, West NC, Tuddenham EGD. Double heterozygosity for mutations in the platelet glycoprotein IX gene in three siblings with Bernard-Soulier syndrome. *Blood*. (1993) 81:2339–47.
37. Burk CD, Newman PJ, Lyman S, Gill J, Collier BS, Poncz M. A deletion in the gene for glycoprotein IIB associated with Glanzmann's thrombasthenia. *J Clin Invest*. (1991) 87:270–6. doi: 10.1172/JCI114982
38. Nurden AT, Pillois X, Wilcox DA. Glanzmann Thrombasthenia: state of the art and future directions. *Semin Thromb Hemost*. (2013) 39:642. doi: 10.1055/s-0033-1353393
39. Balduini CL, Pecci A, Savoia A. Recent advances in the understanding and management of MYH9-related inherited thrombocytopenias. *Br J Haematol*. (2011) 154:161–74. doi: 10.1111/j.1365-2141.2011.08716.x
40. Manchev VT, Hilpert M, Berrou E, Elaib Z, Aouba A, Boukour S, et al. A new form of macrothrombocytopenia induced by a germ-line mutation in the PRKACG gene. *Blood*. (2014) 124:2554–63. doi: 10.1182/blood-2014-01-551820
41. Stritt S, Nurden P, Favier R, Favier M, Ferioli S, Gotru SK, et al. Defects in TRPM7 channel function deregulate thrombopoiesis through altered cellular Mg2+ homeostasis and cytoskeletal architecture. *Nat Commun*. (2016) 7:11097. doi: 10.1038/ncomms11097
42. Pleines I, Woods J, Chappaz S, Kew V, Foad N, Ballester-Beltrán J, et al. Mutations in tropomyosin 4 underlie a rare form of human macrothrombocytopenia. *J Clin Invest*. (2017) 127:814–29. doi: 10.1172/JCI86154
43. Kunishima S, Kobayashi R, Itoh TJ, Hamaguchi M, Saito H. Mutation of the β 1-tubulin gene associated with congenital macrothrombocytopenia affecting microtubule assembly. *Blood*. (2009) 113:458–61. doi: 10.1182/blood-2008-06-162610
44. Massaad MJ, Ramesh N, Geha RS. Wiskott-Aldrich syndrome: a comprehensive review. *Ann N Y Acad Sci*. (2013) 1285:26–43. doi: 10.1111/nyas.12049
45. Nurden P, Debili N, Couprie I, Bryckaert M, Youlyouze-Marfak I, Solé G, et al. Thrombocytopenia resulting from mutations in filamin A can be expressed as an isolated syndrome. *Blood*. (2011) 118:5928–37. doi: 10.1182/blood-2011-07-365601
46. Stritt S, Nurden P, Turro E, Greene D, Jansen SB, Westbury SK, et al. A gain-of-function variant in DIAPH1 causes dominant macrothrombocytopenia and hearing loss. *Blood*. (2016) 127:2903–14. doi: 10.1182/blood-2015-10-675629
47. Bastida JM, Benito R, Janusz K, Díez-Campelo M, Hernández-Sánchez JM, Marcellini S, et al. Two novel variants of the ABCG5 gene cause xanthelasmas and macrothrombocytopenia: a brief review of hematologic abnormalities of sitosterolemia. *J Thromb Haemost*. (2017) 15:1859–66. doi: 10.1111/jth.13777
48. Levy GG, Nichols WC, Lian EC, Foroud T, Mcclintick JN, McGee BM, et al. Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. *Nature*. (2001) 413:488–94. doi: 10.1038/35097008
49. Nesin V, Wiley G, Kousi M, Ong EC, Lehmann T, Nicholl DJ, et al. Activating mutations in STIM1 and ORAI1 cause overlapping syndromes of tubular myopathy and congenital miosis. *Proc Natl Acad Sci USA*. (2014) 111:4197–202. doi: 10.1073/pnas.1312520111
50. Markello T, Chen D, Kwan JY, Horkayne-Szakaly I, Morrison A, Simakova O, et al. York platelet syndrome is a CRAC channelopathy due to gain-of-function mutations in STIM1 HHS public access. *Mol Genet Metab*. (2015) 114:474–82. doi: 10.1016/j.ymgme.2014.12.307
51. Cooney KA, Nichols WC, Bruck ME, Bahou WF, Shapiro AD, Bowie EJW, et al. The molecular defect in type IIB von Willebrand disease. *J Clin Invest*. (1991) 87:1227–33. doi: 10.1172/JCI115123
52. Lacruz RS, Feske S. Diseases caused by mutations in ORAI1 and STIM1. *Ann N Y Acad Sci*. (2015) 1356:45–79. doi: 10.1111/nyas.12938
53. Hurtado B, Trakala M, Ximénez-Embún P, El Bakkali A, Partida D, Sanz-Castillo B, et al. Thrombocytopenia-associated mutations in Ser/Thr kinase MASTL deregulate actin cytoskeletal dynamics in platelets. *J Clin Invest*. (2018) 128:5351–67. doi: 10.1172/JCI121876
54. Johnson B, Fletcher SJ, Morgan NV. Inherited thrombocytopenia: novel insights into megakaryocyte maturation, proplatelet formation and platelet lifespan. *Platelets*. (2016) 27:519–25. doi: 10.3109/09537104.2016.1148806
55. Schulze H, Shivdasani RA. Mechanisms of thrombopoiesis. *J Thromb Haemost*. (2005) 3:1717–24. doi: 10.1111/j.1538-7836.2005.01426.x

56. Ogawa M. Differentiation and proliferation of hematopoietic stem cells. *Blood*. (1993) 81:2844–53.
57. Drachman JG, Griffin JD, Kaushansky K. The c-Mpl ligand (thrombopoietin) stimulates tyrosine phosphorylation of Jak2, Shc, and c-Mpl. *J Biol Chem*. (1995) 270:4979–82. doi: 10.1074/jbc.270.10.4979
58. Dasouki MJ, Rafi SK, Olm-Shipman AJ, Wilson NR, Abhyankar S, Ganter B, et al. Exome sequencing reveals a thrombopoietin ligand mutation in a Micronesian family with autosomal recessive aplastic anemia. *Blood*. (2013) 122:3440–9. doi: 10.1182/blood-2012-12-473538
59. Geddis AE. Megakaryopoiesis. *Semin Hematol*. (2010) 47:212–9. doi: 10.1053/j.seminhematol.2010.03.001
60. Millikan PD, Balamohan SM, Raskind WH, Kacena MA. Inherited thrombocytopenia due to GATA-1 mutations. *Semin Thromb Hemost*. (2011) 37:682–9. doi: 10.1055/s-0031-1291378
61. Tijssen MR, Ghevaert C. Transcription factors in late megakaryopoiesis and related platelet disorders. *J Thromb Haemost*. (2013) 11:593–604. doi: 10.1111/jth.12131
62. Foudi A, Kramer DJ, Qin J, Ye D, Behlich A-S, Mordecai S, et al. Distinct, strict requirements for Gfi-1b in adult bone marrow red cell and platelet generation. *J Exp Med*. (2014) 211:909–27. doi: 10.1084/jem.2013.1065
63. Letestu R, Vitrat N, Massé A, Le Couedic J-P, Lazar V, Rameau P, et al. Existence of a differentiation blockage at the stage of a megakaryocyte precursor in the thrombocytopenia and absent radii (TAR) syndrome. *Blood*. (2000) 95:1633–41.
64. Loffredo G, Balduini CL, Notarangelo LD, Casale M, Perrotta S, Pujol-Moix N, et al. Mutations in ANKRD26 are responsible for a frequent form of inherited thrombocytopenia: analysis of 78 patients from 21 families. *Blood*. (2011) 117:6673–80. doi: 10.1182/blood-2011-02-336537
65. Bender M, Thon JN, Ehrlicher AJ, Wu S, Mazutis L, Deschmann E, et al. Microtubule sliding drives proplatelet elongation and is dependent on cytoplasmic dynein. *Blood*. (2015) 125:860–8. doi: 10.1182/blood-2014-09-600858
66. Machlus KR, Italiano JE. The incredible journey: from megakaryocyte development to platelet formation. *J Cell Biol*. (2013) 201:785–96. doi: 10.1083/jcb.201304054
67. Snapper SB, Rosen FS. The Wiskott-Aldrich Syndrome Protein (WASP): roles in signaling and cytoskeletal organization. *Annu. Rev. Immunol*. (1999) 17:905–29. doi: 10.1146/annurev.immunol.17.1.905
68. Kirchhausen T, Rosen FS. Disease mechanism: unravelling Wiskott-Aldrich syndrome. *Curr Biol*. (1996) 6:676–8. doi: 10.1016/S0960-9822(09)00447-3
69. Savoia A, Kunishima S, De Rocco D, Zieger B, Rand ML, Pujol-Moix N, et al. Spectrum of the mutations in bernard-soulier syndrome. *Hum Mutat*. (2014) 35:1033–45. doi: 10.1002/humu.22607
70. Balduini A, Malara A, Pecci A, Badalucco S, Bozzi V, Pallotta I, et al. Proplatelet formation in heterozygous Bernard-Soulier syndrome type Bolzano. *J Thromb Haemost*. (2009) 7:478–84. doi: 10.1111/j.1538-7836.2008.03255.x
71. Kunishima S, Kashiwagi H, Otsu M, Takayama N, Eto K, Onodera M, et al. Heterozygous ITGA2B R995W mutation inducing constitutive activation of the IIb3 receptor affects proplatelet formation and causes congenital macrothrombocytopenia. *Blood*. (2011) 117:5479–84. doi: 10.1182/blood-2010-12-323691
72. Pecci A. Diagnosis and treatment of inherited thrombocytopenias. *Clin Genet*. (2016) 89:141–53. doi: 10.1111/cge.12603
73. Broadley SP, Plaumann A, Coletti R, Lehmann C, Wanisch A, Seidlmeier A, et al. Dual-track clearance of circulating bacteria balances rapid restoration of blood sterility with induction of adaptive immunity. *Cell Host Microbe*. (2016) 20:36–48. doi: 10.1016/j.chom.2016.05.023
74. Kim S-J, Davis R, Jenne C. Platelets as modulators of inflammation. *Semin Thromb Hemost*. (2018) 44:91–101. doi: 10.1055/s-0037-1607432
75. Swinkles M, Rijkers M, Voorberg C, Vidarsson G, Leebeek FWG, Jansen AJG. Emerging concepts in immune thrombocytopenia. *Front Immunol*. (2018) 9:880. doi: 10.3389/fimmu.2018.00880
76. Leonard M, Brice M, Engel JD, Papayannopoulou T. Dynamics of GATA transcription factor expression during erythroid differentiation. *Blood*. (1993) 82:1071–9.
77. Marigo V, Nigro A, Pecci A, Montanaro D, Stazio M Di, Balduini CL, et al. Correlation between the clinical phenotype of MYH9-related disease and tissue distribution of class II nonmuscle myosin heavy chains. *Genomics*. (2004) 83:1125–33. doi: 10.1016/j.ygeno.2003.12.012
78. Pecci A, Klersy C, Gresele P, Lee KJD, De Rocco D, Bozzi V, et al. MYH9-related disease: a novel prognostic model to predict the clinical evolution of the disease based on genotype-phenotype correlations Europe PMC funders group. *Hum Mutat*. (2014) 35:236–47. doi: 10.1002/humu.22476
79. Simeoni I, Stephens JC, Hu F, Deevi SV, Megy K, Bariana TK, et al. A high-throughput sequencing test for diagnosing inherited bleeding, thrombotic, and platelet disorders. *Blood*. (2016) 127:2791–803. doi: 10.1182/blood-2015-12-688267
80. Kahr WH, Hinckley J, Li L, Schwertz H, Christensen H, Rowley JW, et al. Mutations in NBEAL2, encoding a BEACH protein, cause gray platelet syndrome. *Nat Genet*. (2011) 43:738–40. doi: 10.1038/ng.884
81. Monteferrario D, Bolar NA, Marneth AE, Hebeda KM, Bergevoet SM, Veenstra H, et al. A dominant-negative GFIIIB mutation in the gray platelet syndrome. *N Engl J Med*. (2014) 370:245–53. doi: 10.1056/NEJMoa1308130

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Almazni, Stapley and Morgan. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Platelet-Neutrophil Interplay: Insights Into Neutrophil Extracellular Trap (NET)-Driven Coagulation in Infection

Amanda Z. Zucoloto and Craig N. Jenne*

Department of Microbiology, Immunology and Infectious Diseases, Snyder Institute for Chronic Diseases, The University of Calgary, Calgary, AB, Canada

OPEN ACCESS

Edited by:

Marie Lordkipanidzé,
Université de Montréal, Canada

Reviewed by:

Nigel S. Key,
University of North Carolina at Chapel
Hill, United States
Daniel Duerschmied,
Department of Cardiology, University
of Freiburg, Germany

*Correspondence:

Craig N. Jenne
cnjenne@ucalgary.ca

Specialty section:

This article was submitted to
Atherosclerosis and Vascular
Medicine,
a section of the journal
Frontiers in Cardiovascular Medicine

Received: 26 March 2019

Accepted: 06 June 2019

Published: 20 June 2019

Citation:

Zucoloto AZ and Jenne CN (2019)
Platelet-Neutrophil Interplay: Insights
Into Neutrophil Extracellular Trap
(NET)-Driven Coagulation in Infection.
Front. Cardiovasc. Med. 6:85.
doi: 10.3389/fcvm.2019.00085

Well established for their central role in hemostasis, platelets have increasingly been appreciated as immune cells in recent years. This emerging role should not come as a surprise as the central immune cells of invertebrates, hemocytes, are able to phagocytose, secrete soluble mediators and promote coagulation of hemolymph, blurring the line between immunity and hemostasis. The undeniable evolutionary link between coagulation and immunity becomes even clearer as the role of platelets in inflammation is better understood. Platelets exert a range of immune-related functions, many of which involve an intimate interplay with leukocytes. Platelets promote leukocyte recruitment via endothelial activation and can serve as “landing pads” for leukocytes, facilitating cellular adhesion in vascular beds devoid of classic adhesion molecules. Moreover, platelets enhance leukocyte function both through direct interactions and through release of soluble mediators. Among neutrophil-platelets interactions, the modulation of neutrophil extracellular traps (NETs) is of great interest. Platelets have been shown to induce NET formation; and, in turn, NET components further regulate platelet and neutrophil function. While NETs have been shown to ensnare and kill pathogens, they also initiate coagulation via thrombin activation. In fact, increased NET formation has been associated with hypercoagulability in septic patients as well as in chronic vascular disorders. This review will delve into current knowledge of platelet-neutrophil interactions, with a focus on NET-driven coagulation, in the context of infectious diseases. A better understanding of these mechanisms will shed a light on the therapeutic potential of uncoupling immunity and coagulation through targeting of NETs.

Keywords: platelets, neutrophils, neutrophil extracellular traps, NETs, coagulation, inflammation

INTRODUCTION

Poised at the interface of immunity and coagulation, platelets express a plethora of surface molecules and receptors and carry granules packed with hundreds of biologically active products. Platelets arise from megakaryocytes, which in turn differentiate from pluripotent hematopoietic cells restricted to the bone-proximal osteoblastic niche in the bone marrow (1, 2). Proplatelets are released from this specialized site into the circulation, continue to mature, ultimately releasing mature platelets (3).

Platelets are undoubtedly critical for hemostasis, in a large part by supporting blood coagulation. Upon activation, platelets expose negatively-charged phospholipids on the outer leaflet of their

plasma membrane, providing an ideal surface for the assembly of coagulation factors complexes, such as the VIIIa-IXa complex and the Xa-Va complex. Moreover, factor XI and thrombin are brought into close proximity through interactions with the glycoprotein (GP) Ib/V/IX complex on the surface of platelets, facilitating factor XI cleavage by thrombin. This not only sustains the coagulation cascade, but also overcomes coagulation arrest in the presence of TFPI (inhibitor of the extrinsic pathway) (4).

Platelets are equipped with numerous immune receptors. For instance, signaling through TLRs 2/6 and 1/2 triggers platelet activation, marked by increased expression of surface CD62P (P-selectin), degranulation and aggregation (5–7). TLR4 engagement was shown to induce platelet aggregation and interaction with leukocytes as well as affect CD62P expression in a ligand-dependent manner (8–10). Moreover, human platelets have been shown to secrete antimicrobial peptides targeting both bacteria and fungi in response to thrombin, a key enzyme in the coagulation cascade (11). Platelets have also been increasingly recognized for their role in cellular recruitment. In that sense, platelets have been shown to serve as a “landing pad” in endothelial beds devoid of adhesion molecules, such as the brain (12, 13). In a model of hepatitis, CD8+ T cells were also shown to preferentially dock onto platelets adherent within liver sinusoids rather than adhering to endothelial cells themselves, suggesting a role for platelets in adaptive immunity (14).

The active role of platelets in both coagulation and immunity hints at an evolutionary link to the central immune cells of invertebrates, hemocytes. Hemocytes not only provide host defense through secretion of microbicidal peptides and phagocytosis, but also through coagulation of the hemolymph. In these invertebrate organisms, clot formation is a potent host defense mechanism as it isolates and contains the infectious agent (15–17). This ability to sequester and contain pathogens undeniably resembles the role of neutrophil extracellular traps (NETs), an immune effector mechanism of higher vertebrates. The complex interplay between platelets and neutrophils, which is most likely a long-evolving relationship, will be discussed in this review, with a focus on NET-driven coagulation.

PLATELET DEPENDENT NEUTROPHIL RECRUITMENT AND ACTIVATION

The interactions between platelets and neutrophils are orchestrated by both their surface and secreted molecules, with the former allowing for physical interactions between these cells. Activated platelets express CD62P, which binds to P-selectin glycoprotein ligand-1 (PSGL-1) on the surface of neutrophils (**Figure 1A**) (21, 22). Alternatively, both GPIb or the integrin α IIb β 3 on platelets interact with the integrin α M β 2 on leukocytes either directly, or through fibrinogen as a bridging molecule (23–25). Secreted molecules, such as cathepsin G produced by activated neutrophils, can disrupt these interactions through cleavage of GPIb and PSGL-1 (26). Moreover, there is increasing evidence of platelet-derived products modulating neutrophil recruitment, activation and function. For instance, CD40L secreted by platelets has been shown to upregulate

integrin expression on neutrophils (27). Serotonin and CXCL4 have also been implicated in platelet-dependent neutrophil recruitment in models of abdominal inflammation and acute pancreatitis (28, 29).

Platelets mediate leukocyte recruitment via two main mechanisms: (a) by serving as a docking site for immune cells along the endothelium surrounding the inflammatory focus and (b) through secretion of chemoattractants. The extent to which platelets promote cellular recruitment appears to be tissue- and model-dependent. For instance, neutrophil infiltration into the peritoneal cavity, skin and brain in response to LPS was shown to be platelet-dependent whereas in the lung, a compensatory mechanism characterized by the upregulation of CXCL1 and CCL5 was able to overcome platelet depletion (30). In a model of *Pseudomonas aeruginosa* pulmonary infection, however, platelet depletion was shown to reduce neutrophil infiltration in the lung (31). Similarly, platelet-driven neutrophil recruitment to the colon and kidney has been demonstrated in models of dextran sodium sulfate (DSS)-induced acute colitis and cecal ligation and puncture (CLP) (32, 33). In these studies, platelet depletion was shown to improve clinical and histopathological scores, whereas in the aforementioned model of *P. aeruginosa* pulmonary infection, platelet depletion led to increased bacterial dissemination and mortality (31–33). These differences further emphasize the complexity of the interplay between platelets and other immune cells, such as neutrophils.

Indeed, platelets are not limited to providing a port-of-entry to neutrophils into sites of tissue insult. Platelets have been shown to directly stimulate the production of NETs through the process of NETosis (18, 34–37). In turn, NETs amplify platelet activation, aggregation and thrombin activation, and all three act in synergy to promote intravascular coagulation in sepsis (19, 38, 39). Evidence supporting the deleterious effects of NET-induced coagulation in infectious diseases will be discussed in the next section.

PLATELET-DRIVEN NETosis

Unsurprisingly, platelet and neutrophil interactions are greatly increased during inflammatory responses. These interactions are, for the most part, initiated by soluble mediators, which directly activate these cells (**Table 1**). Co-incubation of healthy platelets and neutrophils with plasma from septic patients has been shown to promote platelet adhesion to neutrophils in a TLR4-dependent manner, a result similar to what is observed following co-incubation of platelets, neutrophils and LPS (34, 36). Moreover, LPS-induced intravascular NETosis and trapping of *Escherichia coli* in NETs has been shown to be augmented in the presence of platelets (34). Platelet-driven NETosis has been observed in the presence of all classic platelet agonists (i.e., thrombin, ADP, collagen, arachidonic acid) as well as several TLR ligands; however, in these models, NET formation does not occur in the absence of platelet activation (35–37). Hence, it is consensus that, for platelet-induced NETosis *in vitro*, platelets must be first activated. CD62P is also required for platelet-induced NETosis as CD62P^{−/−} platelets have been shown to

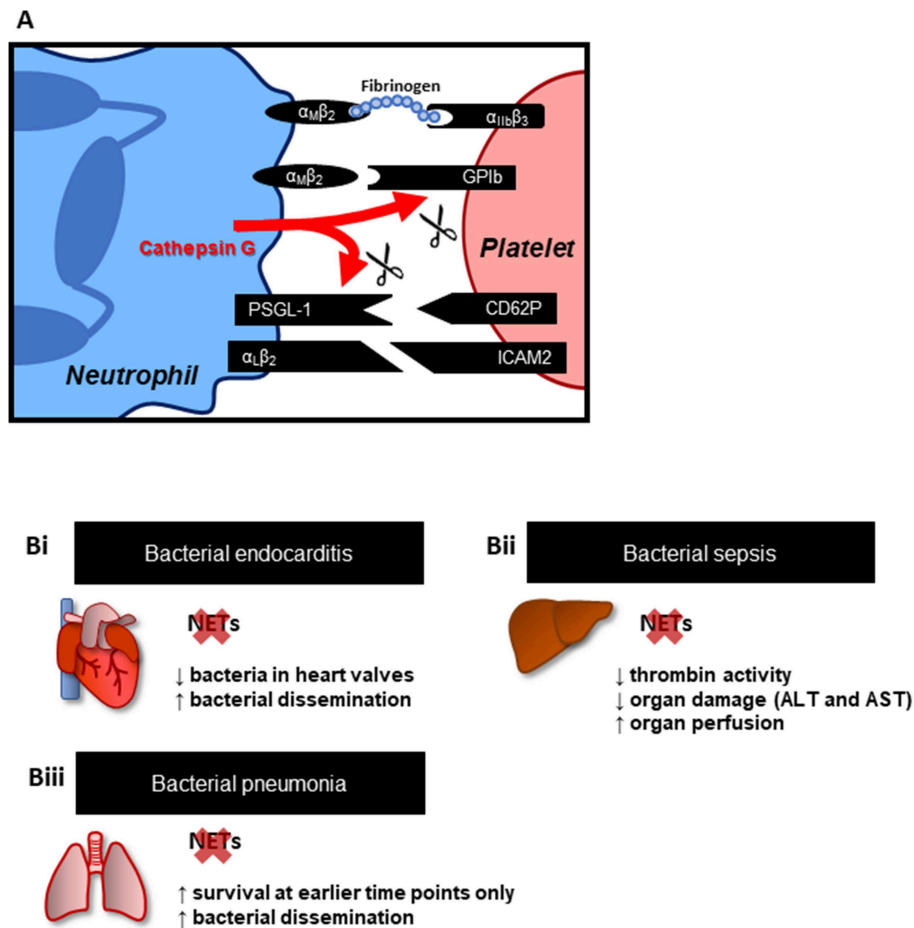


FIGURE 1 | Platelets-neutrophil interactions and NETs. **(A)** Key adhesion molecules involved in platelet-neutrophil interactions. These interactions not only provide mechanisms of cell attachment but may also trigger intracellular signaling, promoting cell activation, resulting in the upregulation of additional adhesion and effector molecules. Effector molecules from both cells, such as cathepsin G from neutrophils, in turn modulate neutrophil-platelet physical interactions through cleavage of PSGL-1 and GPIb. **(B)** Effect of NET inhibition (PAD4^{-/-} mice) or disruption (DNase treatment) in animal models of **(Bi)** endocarditis (18), **(Bii)** bacterial sepsis (19), and **(Biii)** bacterial pneumonia (20). Overall, targeting NETs is associated with reduced inflammation and organ damage; however, this effect has been shown to favor bacterial dissemination.

fail to promote the release of NETs whereas overexpression of platelet CD62P enhanced phorbol 12-myristate 13-acetate (PMA)- and ionomycin-induced NETosis (35). The role of other surface molecules involved in platelet-neutrophil interactions, GPIb/IIa, CD11b (integrin α -chain L), and CD18 (integrin β -chain 2), remains debatable as some studies have shown these molecules to be dispensable (37) while other models have indicated a clear need for integrin-mediated platelet adhesion in the induction of NETosis (69). Although there is strong evidence that platelet-neutrophil adhesion plays a central role in platelet-induced NET formation, physical interactions between platelets and neutrophils may not be absolutely required as activated platelets are known to shed CD62P. Indeed, neutrophils, in the presence of *Streptococcus mutans* and soluble CD62P (sCD62P) have been shown to produce NETs, thus indicating that direct interactions are dispensable in some situations, at least *in vitro* (18).

In addition, to CD62P/PSGL-1 signaling, other mediators have been shown to be involved in triggering NET release. For instance, antibody-mediated blockade of platelet-derived high-mobility box group 1 protein (HMBG1) has been shown to inhibit NET formation *in vitro* (37). In fact, NET release was inhibited in the presence of anti-HMBG1 to a greater extent than in the presence of anti-CD62P. Moreover, HMGB1 was shown to be required for activation of autophagy pathways, which are required for NETosis in a RAGE-dependent manner, a key receptor for HMGB1 (37). A role for thromboxane A2 (TXA2) has also been demonstrated in platelet-driven NET release. In a study by Caudrillier et al. (66), activated, but not resting, platelets were shown to induce NET formation *in vitro*. This process was shown to be dependent on TXA2 receptor-mediated signaling, which activates the MAPK pathway (66). Moreover, platelet-derived β -defensin 1 was shown to induce NET formation in a ROS-dependent manner (70). Human platelets store β -defensin

TABLE 1 | Platelet molecules that modulate neutrophil activation.

Factors stored in granules	Adhesive glycoproteins: P-selectin [*] (27, 40), Fibrinogen (41), vWF [*] (36, 42, 43), Fibronectin (44), Thrombospondin (44) Coagulation factors: Protein S (45), Factor XI (46) Mitogenic factors: PDGF (47), TGF- β (48), EGF (49) Angiogenic / Vasoactive factors: VEGF (50), PF4 inhibitor (51), Serotonin (52) Chemokines: CXCL7 (53), CXCL4 [*] (PF4) (54, 55), CXCL1 (GRO α) (56, 57), CXCL5 [*] (58), CCL5 [*] (RANTES) (59, 60), CCL3 (MIP1 α) (61)
Unknown location	CCL7(MCP3) (56), IL1 β (62), HMGB1 [*] (63), Defensins [*] (11)
Plasma Membrane	Thromboxane A2 [*] (36), PAF (64), CD40L (9), TREM-1 ligand (65), α IIb β 3 Integrin [*] (66, 67), GPIIb [*] (36), ICAM2 (68), P-selectin [*] (35)

vWF, von Willebrand factor; PDGF, platelet derived growth factor; TGF- β , transforming growth factor β ; VEGF, vascular endothelial growth factor; PF4, Platelet Factor 4; IL1 β , interleukin 1 β ; HMGB1, high mobility group box protein 1; PAF, platelet activating factor; TREM-1, triggering receptor expressed on myeloid cells 1; GPIIb, glycoprotein IIb; ICAM2, intercellular adhesion molecule 2. Factors known to be associated with NET production by neutrophils are denoted with an^{*}.

1, an antimicrobial peptide primarily expressed by epithelial cells, in extragranular cytoplasmic compartments. Thus, β -defensin is not released during classic platelet degranulation stimulated by agonists such as thrombin or platelet activating factor (PAF). Instead, β -defensin 1 was shown to be released when platelets were stimulated in the presence of *Staphylococcus aureus*-derived α -toxin but not LPS (15). This α -toxin-platelet- β -defensin 1 axis could represent a novel mechanism by which platelets directly induce NETs in response to Gram-positive infections. The generation of NETs, however, was only demonstrated in the presence of purified β -defensin 1 (70). Co-incubation of α -toxin, platelets and neutrophils could provide more conclusive evidence of this putative interplay.

EFFECTS OF NETS ON PLATELETS AND COAGULATION

The downstream effects of platelet-induced NET release have also been studied. Activated, but not resting, platelets and neutrophils were shown to result in NET release and increased monolayer permeability in LPS-stimulated endothelial cells *in vitro* (66). Importantly, NETs also affect platelet function and direct evidence demonstrates that the platelet-NET axis is by no means a one-way road. In a report by Elaskalani et al. (38), cell-free NETs (collected from PMA-stimulated neutrophils) were incubated with human platelets where the presence of NETs alone (with no other platelet agonists added) was shown to promote platelet aggregation, secretion of ATP and ADP, and increased expression of CD62L and phosphatidylserine (PS) on the surface of the platelets (38). An increase in protein phosphorylation at tyrosine residues pointed to NET-mediated activation of intracellular signaling in platelets. Accordingly, Fuchs et al. (39) have demonstrated a role for DNA as well as histones H3 and H4 in NET-induced platelet aggregation *in vitro* (39). These results

strongly hint at a positive feedback loop between platelets and NETs. NET-dependent platelet aggregation, however, was shown to be unaffected by DNase or heparin, suggesting mechanisms independent of the DNA scaffold and thrombin. Inhibition of cathepsin G, a NET component, reduced surface expression of CD62P and PS exposure in platelets whereas blockade of GPIIb/IIIa significantly inhibited platelet aggregation without affecting CD62P expression (38). These observations suggest multiple pathways are likely involved in NET-induced platelet activation and aggregation.

Furthermore, NETs have been implicated in directly inducing thrombin generation through both platelet-dependent and independent mechanisms. NETs released from PMA-stimulated neutrophils have been shown to increase thrombin generation in platelet-poor plasma. This activation required coagulation factors XII and XI, pointing to the involvement of the intrinsic coagulation pathway (71). Importantly, this mechanism was DNA-dependent, as thrombin generation was abrogated when DNase was added to the system (71, 72), although the exact role of DNA scaffold in this context is unknown. Given that coagulation factors optimally assemble on negatively-charged surfaces (such as the PS-rich plasma membrane of activated platelets and microparticles), and that DNA is a negatively-charged molecule, perhaps the DNA backbone of NETs serves as a somewhat ideal surface for the formation of coagulation factor complexes. Importantly, some studies have demonstrated a failure of purified NETs to induce thrombin generation. Whereas, the purified DNA backbone from NETs was able to induce thrombin generation in platelet-poor plasma, intact NETs, failed to generate active thrombin pointing out clear differences between *in vivo* and *in vitro* assays and suggesting qualitative differences in NETs themselves exist (73). In the presence of platelets, NETs were also shown to contribute to thrombin generation. The effect was dependent on platelet expression of TLR-2 and TLR-4, suggesting that NET components may act as receptor agonists, inducing platelet activation. Interestingly, DNase potentiated platelet- and NET-driven thrombin generation, likely because dismantling of NETs led to enhanced release of NET components, making them more readily available to act as platelet agonists (71).

In addition to NET production, PMA-stimulated neutrophils have also been shown to release microparticles, which attach themselves to NETs via PS residues. Blocking this interaction was implicated in reduced NET-dependent thrombin generation pointing to a role for neutrophil-derived microparticles in addition to DNA and other NET components (72). While these reports were largely done *in vitro*, the direct effect of NETs on coagulation *in vivo* has been also been investigated (19, 72). Using a model of sepsis following CLP, increased cell-free DNA in plasma was shown to be largely derived from neutrophils. Plasma levels of DNA-histone complexes were significantly inhibited in neutrophil-depleted animals, strongly suggesting that neutrophils undergo NETosis in the vasculature during sepsis. Thrombin-antithrombin (TAT) complex levels, a marker of thrombin generation, were increased 24 h after CLP. Moreover, thrombin generation *ex vivo* was decreased in these animals due to consumption of coagulation factors, a clinical manifestation of disseminated intravascular coagulation (DIC)

in septic patients. In animals treated with DNase prior to CLP, thrombin generation *ex vivo* was restored, placing NETs as critical factor for the depletion of coagulation factors during sepsis (72). Additionally, blockade of NETs has been shown to reduce thrombin activity in the liver and lung microvasculature and inhibition of thrombin prevents NET-induced liver damage in an *in vivo* model of *E. coli*-induced sepsis (19), providing a direct link between NETs and coagulation. Interestingly, NETs have been shown to contribute to the occlusion of vessels in the lung microvasculature independently of thrombin generation in animals deficient for DNase1 and 3 during sepsis (74). These data indicate that NETs engage multiple mechanisms leading to microvascular obstruction, impairing organ perfusion and driving tissue damage.

Taken together, these studies have contributed greatly to mapping of the molecular mechanisms underlying platelet-NET interplay. While these mechanisms remain incompletely known, crucial molecules, such as CD62P and HMGB1 in platelets, PSGL1 in neutrophils and cathepsin G, histones and DNA in NETs, have been identified as potential targets for uncoupling immunity and coagulation. Targeting of these interactions, however, may have profound impact on host defense, underscoring the need for studying platelet-neutrophil crosstalk in *in vivo* models of infection and inflammatory disease.

TARGETING PLATELET-NET INTERACTIONS IN INFECTION-INDUCED VASCULAR DYSFUNCTION

In support of experimental evidence of NET-induced coagulation, a correlation between NETs and thrombosis has been previously demonstrated in clinical studies (75–77). Various components of NETs (cell-free DNA, citrullinated H3 and nucleosome) were shown to be significantly increased following acute ischemic stroke. Levels of citrullinated H3 were also associated with mortality at the 1-year follow up assessment of the study, which included over 200 patients with acute ischemic stroke (75). Of note, H3 citrullination weakens histone binding to negatively charged DNA, which in turn favors chromatin decondensation, a critical step for NETosis (78). Furthermore, a descriptive study of the composition of thrombi retrieved from ischemic stroke patients has revealed the presence of activated neutrophils (CD66b+ and neutrophil elastase+ granulocytes) and as well as NETs (extracellular DNA with citrullinated H3) (76). In the context of infectious diseases, a study by Yang et al. (77) has compared the ability of neutrophils from septic patients to undergo NETosis and its implications in thrombin and fibrin generation (77). Neutrophils from septic patients were able to promote thrombin and fibrin generation in the presence of control plasma in a DNA-dependent manner. Thus, NET release alone was associated with risk of thromboembolism. However, it is still unclear if NETs are causative of thrombosis or if NETosis is a consequence of thrombus formation.

Clinical evidence has supported the role of NETs in driving coagulation and ultimately, vascular dysfunction in both primarily cardiovascular disorders (i.e., ischemic stroke) as well as conditions initiated by infections, such as sepsis. These reports, however, did not delve into the implications of disrupting NET-induced coagulation for disease outcome. Perhaps the main concern when considering this strategy is the impairment of the innate immune response. To address this issue, animal models of systemic or highly invasive infections have been very insightful (**Figure 1B**). Infective endocarditis is a condition involving thrombi formation following an immune response to bacterial colonization in the heart. Vegetations, a pathological feature of endocarditis, are composed of bacteria embedded in a mass of platelets, fibrin, and immune cells, such as neutrophils. In a rat model of *Streptococcus mutans*-induced endocarditis, NETs were identified in vegetations on damaged heart valves (18). Accordingly, pre-treatment with DNase I, which dismantles the NET web-like structure, led to a decrease in vegetation weight and bacterial load. However, the effect was also accompanied by increased bacterial dissemination. These results suggest a protective role of thrombus and NET formation on heart valves, at least in restricting pathogen invasion and dissemination throughout the body. Indeed, *in vitro*, in the presence of neutrophils, platelets were shown to form aggregates around bacteria. The effect was abrogated in the presence of DNase I, supporting the involvement of NETs in pathogen trapping (18).

Furthermore, in models of bacterial sepsis, NET formation, platelet aggregation and thrombin activity were associated with impaired perfusion of the liver microvasculature and organ damage. PAD4^{-/-} mice (unable to release NETs from neutrophils) or pre-treatment with DNase I markedly reduced thrombin activity, suggesting that NETs directly contributed to thrombin activation, likely through platelet-dependent mechanisms. Moreover, organ perfusion and function were improved in the absence of NETs, suggesting that direct targeting of NETs may be beneficial in the context of disseminated infections characterized by overt inflammation, such as sepsis (19). Importantly, this beneficial effect of NET prevention/dismantlement is very much context dependent. In a study by Lefrançois et al. (20), NETs, as expected, were associated with increased inflammation, lung damage and early mortality in a model of bacteria-induced lung injury (20). However, NET-deficient mice or removal of NETs with DNase I, was associated with increased bacterial loads, demonstrating a clear role for NETs in restricting pathogen dissemination. Critically, the survival rate of animals in this model was only improved if NETs were targeted at earlier time points post-infection. At later time points (>40 h from the i.t., administration of *S. aureus*), blockade of NETs was no longer protective. These results suggest that although NETs play a role in immunopathology early in infection, their role in trapping and sequestering microorganisms is also critical to limit dissemination of infection and as such, complete abrogation of NET production may be just as deleterious to the host as NET-induced pathology.

SUMMARY

Platelet-neutrophil interactions are undoubtedly a two-way relationship due, in large part, to the role of NETs in modulating both platelet and neutrophil function. Moreover, clinical and experimental studies have supported that NET-mediated coagulation and immunity are critical to disease outcome. Perhaps not surprisingly, NETs seem to play a dual role in models of infectious diseases: they orchestrate both immunopathology and infection clearance. Interestingly, the direct participation of NETs in amplifying coagulation through platelet activation, thrombin generation and microparticle release, seems to ultimately underlie NET-induced immunopathology. Importantly, while blocking or dismantling NETs ameliorates

coagulation dysfunction, it may also impair pathogen clearance. Although separating immunity and coagulopathy is the ultimate goal, the question still remains... is this a matter of simply fine tuning of platelet-neutrophil interactions, or, is targeting the procoagulant components of NETs the key to this therapeutic avenue? In the end, a more thorough understanding of the molecular mechanisms underlying NET-driven coagulation will be needed if we are to uncouple immunity and coagulation in the setting of infectious disease.

AUTHOR CONTRIBUTIONS

AZ and CJ contributed to manuscript generation and revision and read and approved the submitted version.

REFERENCES

1. Avecilla ST, Hattori K, Heissig B, Tejada R, Liao F, Shido K, et al. Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. *Nat Med.* (2004) 10:64–71. doi: 10.1038/nm973
2. Larson MK, Watson SP. A product of their environment: do megakaryocytes rely on extracellular cues for proplatelet formation? *Platelets.* (2006) 17:435–40. doi: 10.1080/09537100600772637
3. Italiano JE Jr, Lecine P, Shivdasani RA, Hartwig JH. Blood platelets are assembled principally at the ends of proplatelet processes produced by differentiated megakaryocytes. *J Cell Biol.* (1999) 147:1299–312. doi: 10.1083/jcb.147.6.1299
4. Heemskerk JW, Bevers EM, Lindhout T. Platelet activation and blood coagulation. *Thromb Haemost.* (2002) 88:186–93. doi: 10.1055/s-0037-1613209
5. Damien P, Cognasse F, Payrastre B, Spinelli SL, Blumberg N, Arthaud CA, et al. NF-kappaB Links TLR2 and PAR1 to soluble immunomodulator factor secretion in human platelets. *Front Immunol.* (2017) 8:85. doi: 10.3389/fimmu.2017.00085
6. Assinger A, Kral JB, Yaiw KC, Schrottmaier WC, Kurzejamska E, Wang Y, et al. Human cytomegalovirus-platelet interaction triggers toll-like receptor 2-dependent proinflammatory and proangiogenic responses. *Arterioscler Thromb Vasc Biol.* (2014) 34:801–9. doi: 10.1161/ATVBAHA.114.303287
7. Keane C, Tilley D, Cunningham A, Smolenski A, Kadioglu A, Cox D, et al. Invasive *Streptococcus pneumoniae* trigger platelet activation via toll-like receptor 2. *J Thromb Haemost.* (2010) 8:2757–65. doi: 10.1111/j.1538-7836.2010.04093.x
8. Rigg RA, Healy LD, Nowak MS, Mallet J, Thierheimer ML, Pang J, et al. Heat shock protein 70 regulates platelet integrin activation, granule secretion and aggregation. *Am J Physiol Cell Physiol.* (2016) 310:C568–75. doi: 10.1152/ajpcell.00362.2015
9. Carestia A, Rivadeneyra L, Romaniuk MA, Fondevila C, Negrotto S, Schattner M. Functional responses and molecular mechanisms involved in histone-mediated platelet activation. *Thromb Haemost.* (2013) 110:1035–45. doi: 10.1160/TH13-02-0174
10. Andonegui G, Kerfoot SM, McNagny K, Ebbert KV, Patel KD, Kubes P. Platelets express functional toll-like receptor-4. *Blood.* (2005) 106:2417–23. doi: 10.1182/blood-2005-03-0916
11. Tang YQ, Yeaman MR, Selsted ME. Antimicrobial peptides from human platelets. *Infect Immun.* (2002) 70:6524–33. doi: 10.1128/IAI.70.12.6524-6533.2002
12. Langer HF, Choi EY, Zhou H, Schleicher R, Chung KJ, Tang Z, et al. Platelets contribute to the pathogenesis of experimental autoimmune encephalomyelitis. *Circ Res.* (2012) 110:1202–10. doi: 10.1161/CIRCRESAHA.111.256370
13. Carvalho-Tavares J, Hickey MJ, Hutchison J, Michaud J, Sutcliffe IT, Kubes P. A role for platelets and endothelial selectins in tumor necrosis factor-alpha-induced leukocyte recruitment in the brain microvasculature. *Circ Res.* (2000) 87:1141–8. doi: 10.1161/01.RES.87.12.1141
14. Iannaccone M. Platelet-mediated modulation of adaptive immunity. *Semin Immunol.* (2016) 28:555–60. doi: 10.1016/j.smim.2016.10.008
15. Salzet M. Vertebrate innate immunity resembles a mosaic of invertebrate immune responses. *Trends Immunol.* (2001) 22:285–8. doi: 10.1016/S1471-4906(01)01895-6
16. Iwanaga S, Lee BL. Recent advances in the innate immunity of invertebrate animals. *J Biochem Mol Biol.* (2005) 38:128–50. doi: 10.5483/BMBRep.2005.38.2.128
17. Jenne CN, Kubes P. Platelets in inflammation and infection. *Platelets.* (2015) 26:286–92. doi: 10.3109/09537104.2015.1010441
18. Jung CJ, Yeh CY, Hsu RB, Lee CM, Shun CT, Chia JS. Endocarditis pathogen promotes vegetation formation by inducing intravascular neutrophil extracellular traps through activated platelets. *Circulation.* (2015) 131:571–81. doi: 10.1161/CIRCULATIONAHA.114.011432
19. McDonald B, Davis RP, Kim SJ, Tse M, Esmon CT, Kolaczowska E, et al. Platelets and neutrophil extracellular traps collaborate to promote intravascular coagulation during sepsis in mice. *Blood.* (2017) 129:1357–67. doi: 10.1182/blood-2016-09-741298
20. Lefrancais E, Mallavia B, Zhuo H, Calfee CS, Looney MR. Maladaptive role of neutrophil extracellular traps in pathogen-induced lung injury. *JCI Insight.* (2018) 3:98178. doi: 10.1172/jci.insight.98178
21. Yang J, Furie BC, Furie B. The biology of P-selectin glycoprotein ligand-1: its role as a selectin counterreceptor in leukocyte-endothelial and leukocyte-platelet interaction. *Thromb Haemost.* (1999) 81:1–7. doi: 10.1055/s-0037-1614407
22. Evangelista V, Manarini S, Sideri R, Rotondo S, Martelli N, Piccoli A, et al. Platelet/polymorphonuclear leukocyte interaction: P-selectin triggers protein-tyrosine phosphorylation-dependent CD11b/CD18 adhesion: role of PSGL-1 as a signaling molecule. *Blood.* (1999) 93:876–85.
23. Diacovo TG, deFougerolles AR, Bainton DF, Springer TA. A functional integrin ligand on the surface of platelets: intercellular adhesion molecule-2. *J Clin Invest.* (1994) 94:1243–51. doi: 10.1172/JCI117442
24. Kazzaz NM, Sule G, Knight JS. Intercellular interactions as regulators of NETosis. *Front Immunol.* (2016) 7:453. doi: 10.3389/fimmu.2016.00453
25. Simon DI, Chen Z, Xu H, Li CQ, Dong J, McIntire LV, et al. Platelet glycoprotein Ibalpha is a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18). *J Exp Med.* (2000) 192:193–204. doi: 10.1084/jem.192.2.193
26. Gardiner EE, De LM, McNally T, Michelson AD, Andrews RK, Berndt MC. Regulation of P-selectin binding to the neutrophil P-selectin counter-receptor P-selectin glycoprotein ligand-1 by neutrophil elastase and cathepsin G. *Blood.* (2001) 98:1440–7. doi: 10.1182/blood.V98.5.1440

27. Rahman M, Zhang S, Chew M, Ersson A, Jeppsson B, Thorlacius H. Platelet-derived CD40L (CD154) mediates neutrophil upregulation of Mac-1 and recruitment in septic lung injury. *Ann Surg.* (2009) 250:783–90. doi: 10.1097/SLA.0b013e3181bd95b7
28. Duerschmied D, Suidan GL, Demers M, Herr N, Carbo C, Brill A, et al. Platelet serotonin promotes the recruitment of neutrophils to sites of acute inflammation in mice. *Blood.* (2013) 121:1008–15. doi: 10.1182/blood-2012-06-437392
29. Wetterholm E, Linders J, Merza M, Regner S, Thorlacius H. Platelet-derived CXCL4 regulates neutrophil infiltration and tissue damage in severe acute pancreatitis. *Transl Res.* (2016) 176:105–18. doi: 10.1016/j.trsl.2016.04.006
30. Giles JA, Greenhalgh AD, Denes A, Nieswandt B, Coutts G, McColl BW, et al. Neutrophil infiltration to the brain is platelet-dependent, and is reversed by blockade of platelet GPIIb/IIIa. *Immunology.* (2018) 154:322–8. doi: 10.1111/imm.12892
31. Amison RT, O'Shaughnessy BG, Arnold S, Cleary SJ, Nandi M, Pitchford SC, et al. Platelet depletion impairs host defense to pulmonary infection with *Pseudomonas aeruginosa* in mice. *Am J Respir Cell Mol Biol.* (2018) 58:331–40. doi: 10.1165/rcmb.2017-0083OC
32. Li XH, Qian YB, Meng XX, Wang RL. Effect of platelet-derived P-selectin on neutrophil recruitment in a mouse model of sepsis-induced acute kidney injury. *Chin Med J.* (2017) 130:1694–9. doi: 10.4103/0366-6999.209889
33. Yu C, Zhang S, Wang Y, Zhang S, Luo L, Thorlacius H. Platelet-derived CCL5 regulates CXC chemokine formation and neutrophil recruitment in acute experimental colitis. *J Cell Physiol.* (2016) 231:370–6. doi: 10.1002/jcp.25081
34. Clark SR, Ma AC, Tavenor SA, McDonald B, Goodarzi Z, Kelly MM, et al. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat Med.* (2007) 13:463–9. doi: 10.1038/nm1565
35. Etulain J, Martinod K, Wong SL, Cifuni SM, Schattner M, Wagner DD. P-selectin promotes neutrophil extracellular trap formation in mice. *Blood.* (2015) 126:242–6. doi: 10.1182/blood-2015-01-624023
36. Carestia A, Kaufman T, Rivadeneyra L, Landoni VI, Pozner RG, Negrotto S, et al. Mediators and molecular pathways involved in the regulation of neutrophil extracellular trap formation mediated by activated platelets. *J Leukoc Biol.* (2016) 99:153–62. doi: 10.1189/jlb.3A0415-161R
37. Maugeri N, Campana L, Gavina M, Covino C, De MM, Panciroli C, et al. Activated platelets present high mobility group box 1 to neutrophils, inducing autophagy and promoting the extrusion of neutrophil extracellular traps. *J Thromb Haemost.* (2014) 12:2074–88. doi: 10.1111/jth.12710
38. Elaskalani O, Bdo Razak NB, Metharom P. Neutrophil extracellular traps induce aggregation of washed human platelets independently of extracellular DNA and histones. *Cell Commun Signal.* (2018) 16:24. doi: 10.1186/s12964-018-0235-0
39. Fuchs TA, Brill A, Duerschmied D, Schatzberg D, Monestier M, Myers DD Jr, et al. Extracellular DNA traps promote thrombosis. *Proc Natl Acad Sci USA.* (2010) 107:15880–5. doi: 10.1073/pnas.1005743107
40. Larsen E, Celi A, Gilbert GE, Furie BC, Erban JK, Bonfanti R, et al. PADGEM protein: a receptor that mediates the interaction of activated platelets with neutrophils and monocytes. *Cell.* (1989) 59:305–12. doi: 10.1016/0092-8674(89)90292-4
41. Wencel-Drake JD, Painter RG, Zimmerman TS, Ginsberg MH. Ultrastructural localization of human platelet thrombospondin, fibrinogen, fibronectin, and von Willebrand factor in frozen thin section. *Blood.* (1985) 65:929–38.
42. Lenting PJ, Christophe OD, Denis CV. von Willebrand factor biosynthesis, secretion, and clearance: connecting the far ends. *Blood.* (2015) 125:2019–28. doi: 10.1182/blood-2014-06-528406
43. Kaweck C, Lenting PJ, Denis CV. von Willebrand factor and inflammation. *J Thromb Haemost.* (2017) 15:1285–94. doi: 10.1111/jth.13696
44. Suzuki H, Katagiri Y, Tsukita S, Tanoue K, Yamazaki H. Localization of adhesive proteins in two newly subdivided zones in electron-lucent matrix of human platelet alpha-granules. *Histochemistry.* (1990) 94:337–44. doi: 10.1007/BF00266440
45. Schwarz HP, Heeb MJ, Wencel-Drake JD, Griffin JH. Identification and quantitation of protein S in human platelets. *Blood.* (1985) 66:1452–5.
46. Zucker M, Hauschner H, Seligsohn U, Rosenberg N. Platelet factor XI: intracellular localization and mRNA splicing following platelet activation. *Blood Cells Mol Dis.* (2018) 69:30–7. doi: 10.1016/j.bcmd.2017.04.006
47. Tzeng DY, Deuel TF, Huang JS, Senior RM, Boxer LA, Baehner RL. Platelet-derived growth factor promotes polymorphonuclear leukocyte activation. *Blood.* (1984) 64:1123–8.
48. Assoian RK, Sporn MB. Type beta transforming growth factor in human platelets: release during platelet degranulation and action on vascular smooth muscle cells. *J Cell Biol.* (1986) 102:1217–23. doi: 10.1083/jcb.102.4.1217
49. Kiuru J, Viinikka L, Myllyla G, Pesonen K, Perheentupa J. Cytoskeleton-dependent release of human platelet epidermal growth factor. *Life Sci.* (1991) 49:1997–2003. doi: 10.1016/0024-3205(91)90642-O
50. Gunsilius E, Petzer A, Stockhammer G, Nussbaumer W, Schumacher P, Clausen J, et al. Thrombocytes are the major source for soluble vascular endothelial growth factor in peripheral blood. *Oncology.* (2000) 58:169–74. doi: 10.1159/000012095
51. Struyf S, Burdick MD, Proost P, Van Damme J, Strieter RM. Platelets release CXCL4L1, a nonallelic variant of the chemokine platelet factor-4/CXCL4 and potent inhibitor of angiogenesis. *Circ Res.* (2004) 95:855–7. doi: 10.1161/01.RES.0000146674.38319.07
52. Mauler M, Herr N, Schoenichen C, Witsch T, Marchini T, Hardtner C, et al. Platelet serotonin aggravates myocardial ischemia/reperfusion injury via neutrophil degranulation. *Circulation.* (2019) 139:918–31. doi: 10.1161/CIRCULATIONAHA.118.033942
53. Brown AJ, Sepuru KM, Sawant KV, Rajarathnam K. Platelet-derived chemokine CXCL7 dimer preferentially exists in the glycosaminoglycan-bound form: implications for neutrophil-platelet crosstalk. *Front Immunol.* (2017) 8:1248. doi: 10.3389/fimmu.2017.01248
54. Giddings JC, Brookes LR, Piovela F, Bloom AL. Immunohistological comparison of platelet factor 4 (PF4), fibronectin (Fn) and factor VIII related antigen (VIII:Ag) in human platelet granules. *Br J Haematol.* (1982) 52:79–88. doi: 10.1111/j.1365-2141.1982.tb03863.x
55. Perdomo J, Leung HHL, Ahmadi Z, Yan F, Chong JJH, Passam FH, et al. Neutrophil activation and NETosis are the major drivers of thrombosis in heparin-induced thrombocytopenia. *Nat Commun.* (2019) 10:1322. doi: 10.1038/s41467-019-09160-7
56. Boehlen F, Clemetson KJ. Platelet chemokines and their receptors: what is their relevance to platelet storage and transfusion practice? *Transfus Med.* (2001) 11:403–17. doi: 10.1046/j.1365-3148.2001.00340.x
57. Jin L, Batra S, Jeyaseelan S. Diminished neutrophil extracellular trap (NET) formation is a novel innate immune deficiency induced by acute ethanol exposure in polymicrobial sepsis, which can be rescued by CXCL1. *PLoS Pathog.* (2017) 13:e1006637. doi: 10.1371/journal.ppat.1006637
58. Power CA, Furness RB, Brawand C, Wells TN. Cloning of a full-length cDNA encoding the neutrophil-activating peptide ENA-78 from human platelets. *Gene.* (1994) 151:333–4. doi: 10.1016/0378-1119(94)90682-3
59. Appay V, Rowland-Jones SL. RANTES: a versatile and controversial chemokine. *Trends Immunol.* (2001) 22:83–7. doi: 10.1016/S1471-4906(00)01812-3
60. Rossaint J, Herter JM, Van Aken H, Napirei M, Doring Y, Weber C, et al. Synchronized integrin engagement and chemokine activation is crucial in neutrophil extracellular trap-mediated sterile inflammation. *Blood.* (2014) 123:2573–84. doi: 10.1182/blood-2013-07-516484
61. Klinger MH, Wilhelm D, Bubel S, Sticherling M, Schroder JM, Kuhnel W. Immunocytochemical localization of the chemokines RANTES and MIP-1 alpha within human platelets and their release during storage. *Int Arch Allergy Immunol.* (1995) 107:541–6. doi: 10.1159/000237097
62. Brown GT, Narayanan P, Li W, Silverstein RL, McIntyre TM. Lipopolysaccharide stimulates platelets through an IL-1beta autocrine loop. *J Immunol.* (2013) 191:5196–203. doi: 10.4049/jimmunol.1300354
63. Dyer MR, Chen Q, Haldeman S, Yazdani H, Hoffman R, Loughran P, et al. Deep vein thrombosis in mice is regulated by platelet HMGB1 through release of neutrophil-extracellular traps and DNA. *Sci Rep.* (2018) 8:2068. doi: 10.1038/s41598-018-20479-x
64. Gill P, Jindal NL, Jagdis A, Vadas P. Platelets in the immune response: revisiting platelet-activating factor in anaphylaxis. *J Allergy Clin Immunol.* (2015) 135:1424–32. doi: 10.1016/j.jaci.2015.04.019
65. Jolly L, Lemarie J, Carrasco K, Popovic B, Derive M, Boufenzar A, et al. Triggering receptor expressed on myeloid cells-1: a

- new player in platelet aggregation. *Thromb Haemost.* (2017) 117:1772–81. doi: 10.1160/TH17-03-0156
66. Cadrillier A, Kessenbrock K, Gilliss BM, Nguyen JX, Marques MB, Monestier M, et al. Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury. *J Clin Invest.* (2012) 122:2661–71. doi: 10.1172/JCI61303
 67. Jenne CN, Wong CH, Zemp FJ, McDonald B, Rahman MM, Forsyth PA, et al. Neutrophils recruited to sites of infection protect from virus challenge by releasing neutrophil extracellular traps. *Cell Host Microbe.* (2013) 13:169–80. doi: 10.1016/j.chom.2013.01.005
 68. Kuijper PH, Gallardo Torres HI, Lammers JW, Sixma JJ, Koenderman L, Zwaginga JJ. Platelet associated fibrinogen and ICAM-2 induce firm adhesion of neutrophils under flow conditions. *Thromb Haemost.* (1998) 80:443–8. doi: 10.1055/s-0037-1615227
 69. McDonald B, Urrutia R, Yipp BG, Jenne CN, Kubes P. Intravascular neutrophil extracellular traps capture bacteria from the bloodstream during sepsis. *Cell Host Microbe.* (2012) 12:324–33. doi: 10.1016/j.chom.2012.06.011
 70. Kraemer BF, Campbell RA, Schwertz H, Cody MJ, Franks Z, Tolley ND, et al. Novel anti-bacterial activities of beta-defensin 1 in human platelets: suppression of pathogen growth and signaling of neutrophil extracellular trap formation. *PLoS Pathog.* (2011) 7:e1002355. doi: 10.1371/journal.ppat.1002355
 71. Gould TJ, Vu TT, Swystun LL, Dwivedi DJ, Mai SH, Weitz JI, et al. Neutrophil extracellular traps promote thrombin generation through platelet-dependent and platelet-independent mechanisms. *Arterioscler Thromb Vasc Biol.* (2014) 34:1977–84. doi: 10.1161/ATVBAHA.114.304114
 72. Wang Y, Luo L, Braun OO, Westman J, Madhi R, Herwald H, et al. Neutrophil extracellular trap-microparticle complexes enhance thrombin generation via the intrinsic pathway of coagulation in mice. *Sci Rep.* (2018) 8:4020. doi: 10.1038/s41598-018-22156-5
 73. Noubouossie DE, Whelihan ME, Yu YB, Sparkenbaugh E, Pawlinski R, Monroe DM, et al. *In vitro* activation of coagulation by human neutrophil DNA and histone proteins but not neutrophil extracellular traps. *Blood.* (2017) 129:1021–9. doi: 10.1182/blood-2016-06-722298
 74. Jimenez-Alcazar M, Rangaswamy C, Panda R, Bitterling J, Simsek YJ, Long AT, et al. Host DNases prevent vascular occlusion by neutrophil extracellular traps. *Science.* (2017) 358:1202–6. doi: 10.1126/science.aam8897
 75. Valles J, Lago A, Santos MT, Latorre AM, Tembl JI, Salom JB, et al. Neutrophil extracellular traps are increased in patients with acute ischemic stroke: prognostic significance. *Thromb Haemost.* (2017) 117:1919–29. doi: 10.1160/TH17-02-0130
 76. Laridan E, Denorme F, Desender L, Francois O, Andersson T, Deckmyn H, et al. Neutrophil extracellular traps in ischemic stroke thrombi. *Ann Neurol.* (2017) 82:223–32. doi: 10.1002/ana.24993
 77. Yang S, Qi H, Kan K, Chen J, Xie H, Guo X, et al. Neutrophil extracellular traps promote hypercoagulability in patients with sepsis. *Shock.* (2017) 47:132–9. doi: 10.1097/SHK.0000000000000741
 78. Leshner M, Wang S, Lewis C, Zheng H, Chen XA, Santy L, et al. PAD4 mediated histone hypercitrullination induces heterochromatin decondensation and chromatin unfolding to form neutrophil extracellular trap-like structures. *Front Immunol.* (2012) 3:307. doi: 10.3389/fimmu.2012.00307

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Zucoloto and Jenne. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Circadian Rhythm of Cardiovascular Disease: The Potential of Chronotherapy With Aspirin

Marleen Buurma^{1*}, Jeske J. K. van Diemen², Abel Thijs², Mattijs E. Numans¹ and Tobias N. Bonten¹

¹ Department of Public Health and Primary Care, Leiden University Medical Center, Leiden, Netherlands, ² Department of Internal Medicine, Amsterdam University Medical Center, Amsterdam, Netherlands

OPEN ACCESS

Edited by:

Marie Lordkipanidzé,
Université de Montréal, Canada

Reviewed by:

Paola Patrignani,
Università degli Studi G. d'Annunzio
Chieti e Pescara, Italy
Erik Lerkevang Grove,
Aarhus University Hospital, Denmark

*Correspondence:

Marleen Buurma
m.buurma.HAGK@lumc.nl

Specialty section:

This article was submitted to
Atherosclerosis and Vascular
Medicine,
a section of the journal
Frontiers in Cardiovascular Medicine

Received: 09 April 2019

Accepted: 04 June 2019

Published: 20 June 2019

Citation:

Buurma M, van Diemen JJK, Thijs A,
Numans ME and Bonten TN (2019)
Circadian Rhythm of Cardiovascular
Disease: The Potential of
Chronotherapy With Aspirin.
Front. Cardiovasc. Med. 6:84.
doi: 10.3389/fcvm.2019.00084

Almost all the systems in our body adhere to a daily 24 h rhythm. The cardiovascular system is also affected by this 24 h rhythm. In the morning there is a change in various cardiovascular processes, including platelet aggregability. These changes may play a role in the relative excess of early morning cardiovascular events. The number of recurrent cardiovascular diseases (CVD) could, in theory, be reduced by responding to this 24 h rhythm with timed medication intake (chronotherapy), which also applies to aspirin. Multiple studies on chronotherapy with low-dose aspirin are promising, showing a decrease in early morning platelet activity with evening intake compared with morning intake. However, in order to further demonstrate its clinical impact, randomized trials with cardiovascular events as a primary outcome are needed. This review discusses the available evidence of the effects of circadian rhythm on CVD and the potential positive effect of chronotherapy with aspirin.

Keywords: aspirin, cardiovascular disease, platelet aggregation, circadian rhythm, chronotherapy

INTRODUCTION

In the past centuries, more and more has been discovered about the different mechanisms in our body as well as how to apply this in our medical treatment regimes. This article will discuss one of those discoveries, namely the circadian rhythm and its influence on the development of acute events of cardiovascular disease (CVD). Platelet aggregation is one of the mechanisms responsible for the development of adverse cardiovascular events. In current medicine, antiplatelet medication is the cornerstone of prevention of recurrent cardiovascular events. Recent studies assessed whether optimal timing of intake of medication, such as aspirin, can give a further reduction of CVD.

THE DISCOVERY OF ASPIRIN AND CIRCADIAN RHYTHMS

Around 2,400 years ago Hippocrates was one of the first who described the analgesic effects of willow bark (1). Aspirin, a medicine derived from the willow bark, is still one of the most widespread used medications in the world (2). It is sometimes called the wonder drug, because of its different properties like antipyretic, analgesic, antiplatelet, anti-inflammatory, and even anti-cancer effects (1, 3).

These observations were already made well before the use of evidence-based medicine. In 1563, Reverend Edward Stone carried out a carefully planned clinical study on ~50 patients suffering from inflammatory disorders and pains with encouraging results (1, 4). This research provided the first scientific basis for the common use of willow bark.

The next step in the scientific discovery was the isolation of the active ingredient of the willow bark (1). In 1897, acetylsalicylic acid was created by Felix Hoffman, by modifying salicylic acid by acetylation, and named it Aspirin. This acetylated salicylic acid also provided for the properties of Aspirin to prevent (recurring) CVD events (4).

In 1950, before the exact working mechanism of aspirin was known, the family doctor Lawrence Craven wrote about his experience with aspirin in a number of American journals. In one of his articles, he mentioned that in patients using a higher dosage of aspirin impregnated chewing gum than that he prescribed after undergoing a tonsillectomy, more complications such as bleeding occurred. Following this, he reasoned that aspirin might have a thrombolytic effect (1).

Twenty years later (1971), Vane and co-workers discovered that aspirin inhibits the synthesis of prostaglandin. This mechanism explains the antipyretic, anti-inflammatory, and antiplatelet effects of aspirin (5). For this discovery, Vane received the Nobel prize for Medicine in 1982. Even today, scientists are discovering new properties of aspirin and are reassessing its value in the light of new knowledge of mechanisms in pathophysiology. This includes recent knowledge about circadian rhythms in our body, which seems a new opportunity to optimize today's medical treatment for CVD.

For a long time, it was thought that almost everything in our body occurred randomly. In 1984, a gene was identified in fruit flies, that ensures the 24 h rhythm (circadian rhythm) in physiological processes, like hormone concentrations (6). The circadian rhythm in physiology and human behavior (e.g., sleep, activity, and eating), are essential for all organisms enabling them to anticipate and adapt to the natural environment (7, 8).

This circadian rhythm is controlled by the central or master clock and peripheral clocks. The central clock situated in the hypothalamus in the central nervous system, called the suprachiasmatic nucleus (SCN), coordinates the expression of the clock genes throughout the body (9). This central clock is mainly driven by the alternation of light and dark (10, 11). The peripheral clocks can be found in almost any tissue of the body, including the cardiovascular system (11, 12). An example of one of those genes is the CLOCK gene. This gene affects, among other things, platelet aggregation, and the expression of plasminogen activator inhibitor-1 (PAI-1). A mutation in the CLOCK gene can result in several changes. For example, the daily variation in platelet aggregation disappeared in CLOCK mutant mice. Besides, mice with CLOCK mutation had reduced and non-rhythmic secretion of PAI-1 by the endothelial cells (7).

The internal clock has a lot of influence on the functioning of our body. Chronic disruption of this clock, for example by shift work, is thought to be related to metabolic syndrome, cancer, diabetes, and cardiovascular disease (7, 9, 13).

CARDIOVASCULAR DISEASE AND CIRCADIAN RHYTHM

The human cardiovascular system has different activity patterns with cycles of 24 h, including heart rate, blood pressure,

TABLE 1 | Important changes in cardiovascular processes during the morning.

Cardiovascular processes	Activation during morning hours	Negative effect on cardiovascular disease
Sympathetic tone	Increased	Increase in blood pressure, heart rate, and vasoconstriction (15).
Parasympathetic function	Decreased	Increase in heart rate and vasoconstriction (15).
Blood pressure	Increased	There is an associations between an increase in blood pressure and ischemic heart disease and stroke (16).
Heart rate	Increased	High resting heart rate is associated with adverse cardiovascular events (17).
Vascular endothelial function	Decreased	Endothelial dysfunction is associated with atherosclerotic plaque formation (18).
Platelet aggregation	Increased	A higher platelet aggregation increases the change of the formation of blood clots and thereby, adverse cardiovascular events (19).
Thrombolytic activity	Decreased	Lower thrombolytic activity increases the change of the formation of blood clots, and thereby adverse cardiovascular events (20).

circulating catecholamines, blood coagulation markers, vascular endothelial function, and autonomic nervous system (8, 14). In the morning there is a change in a number of cardiovascular processes (Table 1). Importantly, these rhythm modifications in the morning correspond to the occurrence of CVD. An apparent increase in the number of adverse cardiovascular events is observed during morning hours (06.00–12.00 h), such as stroke, myocardial infarction, ventricular arrhythmias, and sudden cardiac arrest (8, 21, 22). Patients who had a heart attack during morning hours will mostly have a larger infarct size and worse prognosis, compared with patients having a myocardial infarction during the rest of the day (23).

AUTONOMIC NERVOUS SYSTEM AND CIRCADIAN RHYTHM

The autonomic nervous system plays a vital role in physiological and pathological responses of the cardiovascular system. The sympathetic system induces an increase in heart rate, myocardial contractility, and peripheral resistance. The parasympathetic system, on the other hand, has the opposite effect, where it primarily causes a reduction in heart rate and, to a lesser extent, a decrease in cardiac contractility (15). The parasympathetic and sympathetic system both follow a circadian rhythm. Whereas, the parasympathetic activity is more pronounced during the night, the sympathetic system has a peak in activity during the morning. Moreover, this early morning peak coincides with a high sensitivity of vascular receptors (14, 21). This morning peak of the sympathetic nervous system and the increased activity of the renin-angiotensin-aldosterone axis along with the decreased parasympathetic system contribute to the rise in blood pressure

and heart rate in the morning (21). These changes in the morning probably add to the increased incidence of CVD during the same time period (8).

BLOOD PRESSURE AND CIRCADIAN RHYTHM

Blood pressure follows a 24 h rhythm. In the morning there is a rise, which reaches a plateau around 11 am. Then it gradually decreases, and around midnight it reaches its lowest value (24). Blood pressure usually is about 10 to 20% lower while sleeping than during daytime (25). The change in blood pressure is related to the change in activity of the sympathetic nervous system running parallel to the normal sleep-wake cycle (24). Night-time blood pressure is lower due to the reduction of sympathetic tone and the parallel increase in vagal activity during the sleep period. External factors influence blood pressure as well, such as physical activity, emotions, intake of food and drinks, and the variation in light and dark (20).

The time frame of the rise in blood pressure in the morning coincides with the increase in the number of cardiovascular events in the morning (8). Due to the rise in blood pressure in the morning, vulnerable plaques in arteries are more prone to rupture, which can lead to acute adverse cardiovascular events (26). Not only the morning blood pressure rise increases the risk of developing CVD. Research has shown that having continues high blood pressure during nighttime (non-dipping) increases this risk as well. As a matter of fact, it is the lack of a nightly drop in blood pressure which is a risk of the development of CVD (8, 24). The MAPEC study showed that, with the use of chronotherapy, nighttime blood pressure would be reduced, thereby diminishing the risk of CVD (27).

VASCULAR ENDOTHELIUM AND CIRCADIAN RHYTHM

The vascular endothelium has anti-atherosclerotic functions, plays a role in the aggregation of platelets and regulates the patency of vessels via excretion of nitrogen oxide (8, 28). All of these factors influence the cardiovascular system, and therefore, dysfunction may lead to adverse cardiovascular events (8). Dysfunction of the endothelium results in an imbalance in the production and consumption of nitrogen oxide (18). The endothelium-dependent vasodilator function is normally reduced during morning hours, because of the lower production of nitrogen oxide (20). This vasodilating function of the endothelium protects against diseases such as hypertension, stroke, and myocardial infarction. The deterioration of this function might contribute to the morning peak of CVD (8, 28, 29).

BLOOD CLOTTING AND CIRCADIAN RHYTHM

The ability of blood to form clots can be life-saving during a bleeding event. On the other side, the formation of clots in blood

vessels can also lead to ischemic stroke, myocardial infarction, and sudden cardiac arrest. As a counterpart to the development of blood clot, there is thrombolysis, which helps to break down clots and keep blood vessels open. The balance between clotting and bleeding tendency changes during the day. Coagulation is increased during morning hours, partly because platelet aggregation and platelet surface activation markers experience a peak in the morning between 06.00 and 12.00 h (2, 30, 31). In this same period, there is reduced thrombolysis which can partly be explained by a reduced concentration of plasmin-plasmin inhibitor complex in blood during morning hours (20). Secondly by an increased level of plasminogen activator inhibitor-1 (PAI-1). PAI-1 is one of the most important inhibitors of plasma fibrinolytic activity. So, increased PAI-1 concentrations enhance the chance of the development of blood clots (32, 33). These phenomena lead to an increased risk of developing blood clots in the morning hours, possibly contributing to the peak of CVD in this period (2, 8, 33).

The development of CVD is influenced by factors such as continuous interaction between the various circadian phases in the body, the behavior of the individual such as physical exercise and personal risk factors. The circadian rhythms of different processes in the body, such as vagal withdrawal, sympathetic activation, and increased blood clotting, have a homeostatic advantage in most circumstances, anticipating the expected behavioral changes in the morning, and other periods of the day. But in a person susceptible to CVD, the circadian change in the morning can exceed the theoretical risk threshold, which leads to the development of adverse cardiovascular events. The question is whether the circadian rhythm is actually the cause of CVD or whether it reveals the underlying vulnerability (8, 33).

Based on currently available evidence we believe that the circadian rhythms and their influence on CVD, as described above, should be taken into account in the treatment of CVD. Also, the pharmacokinetics of the drug is maybe influenced by this rhythm (34). By reckoning the importance of the circadian rhythm in total during treatment, this might lead to increased effectiveness and fewer side effects. Furthermore, the effectiveness of medicines can also be influenced by comedication. A recent study shows that coadministration of low-dose aspirin and atorvastatin, and possibly other statins, reduces cardiovascular events (35). Still, an important role may be reserved for using the circadian rhythm for drug treatment, such as with acetylsalicylic acid.

ASPIRIN AND TIME OF INTAKE

Platelets are key players in the development of arterial thrombosis. Consequently, aspirin is a cornerstone of secondary prevention because it inhibits platelets. The majority of patients take maintenance doses of 75–325 mg once a day in the morning, after awakening (36). However, research shows that, despite the improvement of lifestyle and drug prevention, 10–33% of these patients have a recurrence of an adverse cardiovascular event within 5 years after their first event (37). The reasons for this recurrence is not completely clear, but probably multifactorial. In theory, it could be partially explained due to the pharmacokinetic

properties of aspirin and the circadian physiology of platelets. Aspirin is absorbed reasonably quickly with a T_{max} of 0.5–2 h for non-enteric-coated aspirin and 3–4 h for enteric-coated aspirin. But also rapidly excreted with a half-life of acetylsalicylic acid of ~20 min (38). The current once-daily regimen is associated with a slow increase in platelet activity within the 24 h dosing interval (39–41). A likely explanation for this phenomenon could be the production of new platelets (42). These new platelets are released at a rate of 10–15% per day (43, 44). Because of the pharmacokinetic properties of aspirin, the cyclooxygenase-1 (COX-1) of the newly released platelets is not inhibited, and so they are able to form blood clots. Previous studies have shown that 95% of all platelets' COX-1 must be inhibited to achieve effective reduction of platelet aggregation (45, 46). This percentage is supported by a recent study in which insufficient inhibition of platelet aggregation was seen 24 h after morning aspirin intake in 25% of patients with CVD (40). The production and delivery of these new platelets follow a circadian rhythm with peak release in the late night and early morning (47). This, too, coincides with the occurrence of adverse cardiovascular events (8, 21, 22). Therefore, it can be essential to ensure adequate platelet inhibition during the morning hours. By taking the aspirin on awakening, 10% uninhibited platelets are present during the morning hours. By responding to the circadian rhythm and taking aspirin at bedtime, the proportion of unrestrained platelets during the morning hours would theoretically be reduced to 5% (2). This results in increased inhibition of the platelet reactivity during morning hours, and possibly a reduction in adverse cardiovascular events.

Several studies try to accomplish a further decrease in the number of recurrences of adverse cardiovascular events by further reducing the number of uninhibited platelets. These studies are assessing the effect on platelet aggregation by increasing dosage, increasing intake frequency, and the use of chronotherapy.

Increasing Aspirin Dosage

When comparing standard morning low-dose aspirin with a higher dosage on platelet aggregation, the effectiveness of the increase differ. In most studies, the inhibition of the sTx_{B2} shows sufficient inhibition at a dosage between 80 and 325 mg aspirin per day (48–52). However, three trials reported higher sTx_{B2} inhibition after dosages above 325 mg of aspirin in comparison with a standard once daily low-dose regimen (52–54). There was a small to no increase in platelet inhibition measured by the Light Transmission Aggregometry if the dosages were above 325 mg (52, 54–57). The results of platelet aggregation measured with the Platelet Function Analyzer and VerifyNow were in line with the sTx_{B2} results: two trials demonstrated sufficient levels of platelet inhibition with once-daily dosages of 75–325 mg of aspirin (50, 56, 58).

Increased Intake Frequency of Aspirin

Regarding the increase of the intake frequency, a higher sTx_{B2} inhibition was reported in three studies, when intake was increased to more than one intake per day in comparison with the standard once daily low-dose regimen (50, 59, 60). Capodanno

et al. reported a superior inhibition when intake was increased to more than once-daily of aspirin in comparison with the standard once-daily dosing, measured by the Light Transmission Aggregometry and VerifyNow (50). On the other hand, five trials did not demonstrate superiority in the level of inhibition by increasing the intake frequency of aspirin (49, 59, 61–63). In addition, when measuring the platelets aggregation by Platelet Function Analyzer, a difference was seen between patients with stable CVD and healthy volunteers (59, 64). In patients with stable CVD, a significant decrease in platelet aggregation was seen when comparing intake of once daily with twice a day (64). This difference was not demonstrated in healthy volunteers (59).

Chronotherapy of Aspirin

Two randomized cross-over trials on chronotherapy concerning platelet function inhibition suggested a superior sTx_{B2} inhibition after once-daily evening intake of low-dose aspirin in comparison with morning intake (2, 59). van Diemen et al. reported an increased platelet inhibition, measured with Light Transmission Aggregometry, after low-dose aspirin intake in the evening in comparison with the standard low-dose aspirin once daily in the morning (65). When measuring the platelets aggregation by Platelet Function Analyzer, a different effect on platelet aggregation was found between patients with stable CVD and healthy volunteers. In stable cardiovascular patients, a statistically significantly higher level of platelet inhibition was demonstrated after evening intake, whereas a trial conducted in healthy subjects did not show a difference (59, 65).

One parallel RCT and three randomized cross-over trials reported more platelet inhibition, measured by the VerifyNow, after evening intake of low-dose aspirin compared with the standard once daily morning dosing (2, 59, 66, 67).

The Different Intake Regimens

When analyzing these different studies on platelet aggregation, one could conclude that an aspirin dosage between 75 and 325 mg once daily provides sufficient platelet inhibition in early morning hours (48–52, 56, 58, 59, 61–63). Especially when we consider that changes in the dosage, frequency, or intake time, in addition to their positive effect on platelet aggregation, may also give a higher chance of side effects. When increasing the dosage above 325 mg once daily minor improvement of platelet inhibition is obtained (52–56). Besides, the risk of side effects such as gastrointestinal bleeding increases with increasing dosage (68). Increasing the intake frequency seems also beneficial on the level of platelet inhibition (50, 59, 60, 64). Again, the total daily dose should not exceed 325 mg, due to the increased chance of complications. Beside, adherence deteriorated with an increasing number of intake moments (69). Importantly, the application of chronotherapy showed, in most of the studies, a higher level of platelet inhibition when comparing the evening intake with the standard once-daily morning intake (2, 59, 65–67). The chance of side effects with the use of low-dose aspirin is similar between the different intake regimens (70). But there have been few long-term studies on the effects of increased intake frequency and chronotherapy so far. However, the evidence for chronotherapy seems hitherto to be the most consistent compared with the

other interventions (increasing dosages and intake frequency). Chronotherapy probably has a lower expectancy of side effects on the long-term, because there is no increase in dosage.

CONCLUSION

It is clear that the cardiovascular system of our body follows a circadian rhythm. Several studies have shown that adverse cardiac events also follow a circadian rhythm, with a vulnerable period in the early-morning hours. The described circadian processes of the cardiovascular system, each with its own rhythm, presumably all contribute to the development of adverse cardiac events. It is essential to investigate how the medical profession can respond to this circadian rhythm in the treatment of CVD leading to increased effectiveness of treatments and/or fewer side effects. The available evidence already shows positive effects of chronotherapy, for instance by reducing nighttime blood pressure. Also, recent studies on chronotherapy suggest

that low-dose aspirin taken at bedtime compared with intake on awakening can improve platelet inhibition during morning hours. There is a need for randomized controlled trials with cardiovascular events and side-effects as endpoints, to further supports the promising chronotherapy hypothesis.

AUTHOR CONTRIBUTIONS

MB, TB, JD, AT, and MN made substantial contributions to conception and design and gave final approval of the version to be submitted and any revised version. MB, JD, and TB participated in drafting the article. TB, AT, and MN participated in revising it critically for important intellectual content.

FUNDING

This work was supported by ZonMw (grant number 848016006).

REFERENCES

- Mueller RL, Scheidt S. History of drugs for thrombotic disease. Discovery, development, and directions for the future. *Circulation*. (1994) 89:432–49. doi: 10.1161/01.CIR.89.1.432
- Bonten TN, Saris A, van Oostrom MJ, Snoep JD, Rosendaal FR, Zwaginga J, et al. Effect of aspirin intake at bedtime versus on awakening on circadian rhythm of platelet reactivity. A randomised cross-over trial. *Thromb Haemost*. (2014) 112:1209–18. doi: 10.1160/th14-05-0453
- Rothwell PM, Wilson M, Price JF, Belch JF, Meade TW, Mehta Z. Effect of daily aspirin on risk of cancer metastasis: a study of incident cancers during randomised controlled trials. *Lancet*. (2012) 379:1591–601. doi: 10.1016/S0140-6736(12)60209-8
- Miner J, Hoffhines A. The discovery of aspirin's antithrombotic effects. *Tex Heart Inst J*. (2007) 34:179–86.
- Vane JR. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature*. (1971) 231:232–5. doi: 10.1038/newbio231232a0
- Burki T. Nobel Prize awarded for discoveries in circadian rhythm. *Lancet*. (2017) 390:e25. doi: 10.1016/S0140-6736(17)32661-2
- Pritchett D, Reddy AB. Circadian clocks in the hematologic system. *J Biol Rhythms*. (2015) 30:374–88. doi: 10.1177/0748730415592729
- Thosar SS, Butler MP, Shea SA. Role of the circadian system in cardiovascular disease. *J Clin Invest*. (2018) 128:2157–67. doi: 10.1172/JCI80590
- Kuehn BM. The heart's circadian rhythms point to potential treatment strategies. *Circulation*. (2016) 134:1907–8. doi: 10.1161/CIRCULATIONAHA.116.026039
- Takeda N, Maemura K. The role of clock genes and circadian rhythm in the development of cardiovascular diseases. *Cell Mol Life Sci*. (2015) 72:3225–34. doi: 10.1007/s00018-015-1923-1
- Crnko S, Cour M, Van Laake LW, Lecour S. Vasculature on the clock: circadian rhythm and vascular dysfunction. *Vasc Pharmacol*. (2018) 108:1–7. doi: 10.1016/j.vph.2018.05.003
- Boivin DB, Boudreau P. Impacts of shift work on sleep and circadian rhythms. *Pathol-Biol*. (2014) 62:292–301. doi: 10.1016/j.patbio.2014.08.001
- Wehrens SM, Hampton SM, Skene DJ. Heart rate variability and endothelial function after sleep deprivation and recovery sleep among male shift and non-shift workers. *Scand J Work Environ. Health*. (2012) 38:171–81. doi: 10.5271/sjweh.3197
- Cooke-Ariel H. Circadian variations in cardiovascular function and their relation to the occurrence and timing of cardiac events. *Am J Health-Syst Pharm*. (1998) 55(Suppl. 3):S5–11. doi: 10.1093/ajhp/55.suppl_3.S5
- La Rovere MT, Christensen JH. The autonomic nervous system and cardiovascular disease: role of n-3 PUFAs. *Vasc Pharmacol*. (2015) 71:1–10. doi: 10.1016/j.vph.2015.02.005
- Rapsomaniki E, Timmis A, George J, Pujades-Rodriguez M, Shah AD, Denaxas S, et al. Blood pressure and incidence of twelve cardiovascular diseases: lifetime risks, healthy life-years lost, and age-specific associations in 1.25 million people. *Lancet*. (2014) 383:1899–911. doi: 10.1016/S0140-6736(14)60685-1
- Zhang D, Wang W, Li F. Association between resting heart rate and coronary artery disease, stroke, sudden death and noncardiovascular diseases: a meta-analysis. *Canad Med Assoc J*. (2016) 188:e384–92. doi: 10.1503/cmaj.160050
- Widmer RJ, Lerman A. Endothelial dysfunction and cardiovascular disease. *Glob Cardiol Sci Pract*. (2014) 2014:291–308. doi: 10.5339/gcsp.2014.43
- Willoughby S, Holmes A, Loscalzo J. Platelets and cardiovascular disease. *Euro J Cardiovasc Nurs*. (2002) 1:273–88. doi: 10.1016/S1474-51510200038-5
- Takeda N, Maemura K. Circadian clock and vascular disease. *Hypertens Res*. (2010) 33:645–51. doi: 10.1038/hr.2010.68
- Manfredini R, Boari B, Smolensky MH, Salmi R, la Cecilia O, Maria Malagoni A, et al. Circadian variation in stroke onset: identical temporal pattern in ischemic and hemorrhagic events. *Chronobiol Int*. (2005) 22:417–53. doi: 10.1081/CBI-200062927
- Butt MU, Zakaria M, Hussain HM. Circadian pattern of onset of ischaemic and haemorrhagic strokes, and their relation to sleep/wake cycle. *J PMA*. (2009) 59:129–32.
- Suarez-Barrientos A, Lopez-Romero P, Vivas D, Castro-Ferreira F, Nunez-Gil I, Franco E, et al. Circadian variations of infarct size in acute myocardial infarction. *Heart*. (2011) 97:970–6. doi: 10.1136/hrt.2010.212621
- Neutel JM. The importance of 24-h blood pressure control. *Blood Pressure Monitor*. (2001) 6:9–16. doi: 10.1097/00126097-200102000-00002
- Cuspidi C, Sala C, Tadic M, Gherbesi E, De Giorgi A, Grassi G, et al. Clinical and prognostic significance of a reverse dipping pattern on ambulatory monitoring: an updated review. *J Clin Hypertens*. (2017) 19:713–21. doi: 10.1111/jch.13023
- Atkinson G, Jones H, Ainslie PN. Circadian variation in the circulatory responses to exercise: relevance to the morning peaks in strokes and cardiac events. *Euro J Appl Physiol*. (2010) 108:15–29. doi: 10.1007/s00421-009-1243-y
- Hermida RC, Ayala DE, Mojon A, Fernandez JR. Influence of circadian time of hypertension treatment on cardiovascular risk: results of the MAPEC study. *Chronobiol Int*. (2010) 27:1629–51. doi: 10.3109/07420528.2010.510230
- Verma S, Anderson TJ. Fundamentals of endothelial function for the clinical cardiologist. *Circulation*. (2002) 105:546–9. doi: 10.1161/hc0502.104540
- Hirsch L, Shechter A, Feinberg MS, Koren-Morag N, Shechter M. The impact of early compared to late morning hours on brachial endothelial

- function and long-term cardiovascular events in healthy subjects with no apparent coronary heart disease. *Int J Cardiol.* (2011) 151:342–7. doi: 10.1016/j.ijcard.2010.08.069
30. McLoughlin SC, Haines P, FitzGerald GA. Clocks and cardiovascular function. *Methods Enzymol.* (2015) 552:211–28. doi: 10.1016/bs.mie.2014.11.029
 31. Sibbing D, Gross L, Aradi D. Prevention of cardiovascular events with antiplatelet treatment: does time of intake matter for aspirin and ADP receptor blockers? *Thromb Haemost.* (2016) 115:3–6. doi: 10.1160/TH15-05-0430
 32. Cesari M, Pahor M, Incalzi RA. Plasminogen activator inhibitor-1 (PAI-1): a key factor linking fibrinolysis and age-related subclinical and clinical conditions. *Cardiovasc Ther.* (2010) 28:e72–91. doi: 10.1111/j.1755-5922.2010.00171.x
 33. Scheer FA, Shea SA. Human circadian system causes a morning peak in prothrombotic plasminogen activator inhibitor-1 (PAI-1) independent of the sleep/wake cycle. *Blood.* (2014) 123:590–3. doi: 10.1182/blood-2013-07-517060
 34. Baraldo M. The influence of circadian rhythms on the kinetics of drugs in humans. *Expert Opin Drug Metabo Toxicol.* (2008) 4:175–92. doi: 10.1517/17425255.4.2.175
 35. Tacconelli S, Dovizio M, Di Francesco L, Meneguzzi A, D'Agostino I, Evangelista V, et al. Reduced variability to aspirin antiplatelet effect by the coadministration of statins in high-risk patients for cardiovascular disease. *Clin Pharmacol Ther.* (2018) 104:111–9. doi: 10.1002/cpt.1075
 36. Windecker S, Kolh P, Alfonso F, Collet JP, Cremer J, Falk V, et al. 2014 ESC/EACTS guidelines on myocardial revascularization: the task force on myocardial revascularization of the European Society of Cardiology (ESC) and the European Association for Cardio-Thoracic Surgery (EACTS) developed with the special contribution of the European Association of Percutaneous Cardiovascular Interventions (EAPCI). *Euro Heart J.* (2014) 35:2541–619. doi: 10.1093/eurheartj/ehu278
 37. Roger VL, Go AS, Lloyd-Jones DM, Adams RJ, Berry JD, Brown TM, et al. Heart disease and stroke statistics—2011 update: a report from the American Heart Association. *Circulation.* (2011) 123:e18–209. doi: 10.1161/CIR.0b013e31820c7a50
 38. Needs CJ, Brooks PM. Clinical pharmacokinetics of the salicylates. *Clin Pharmacokinet.* (1985) 10:164–77. doi: 10.2165/00003088-198510020-00004
 39. Wurtz M, Hvas AM, Jensen LO, Kalltoft AK, Tilsted HH, Kristensen SD, et al. 24-hour antiplatelet effect of aspirin in patients with previous definite stent thrombosis. *Int. J. Cardiol.* (2014) 175:274–9. doi: 10.1016/j.ijcard.2014.05.013
 40. Henry P, Vermillet A, Boval B, Guyetand C, Petroni T, Dillinger JG, et al. 24-hour time-dependent aspirin efficacy in patients with stable coronary artery disease. *Thromb Haemost.* (2011) 105:336–44. doi: 10.1160/TH10-02-0082
 41. Vernstrom L, Funck KL, Grove EL, Laugesen E, Baier JM, Hvas AM, et al. Antiplatelet effect of aspirin during 24 h in patients with type 2 diabetes without cardiovascular disease. *Thromb Res.* (2018) 161:1–6. doi: 10.1016/j.thromres.2017.11.013
 42. Grove EL, Storey RF, Kristensen SD. Can we improve the efficacy of low-dose aspirin? *Thromb Haemost.* (2014) 112:1077–8. doi: 10.1160/th14-08-0702
 43. Warner TD, Nylander S, Whatling C. Anti-platelet therapy: cyclooxygenase inhibition and the use of aspirin with particular regard to dual anti-platelet therapy. *Br J Clin Pharmacol.* (2011) 72:619–33. doi: 10.1111/j.1365-2125.2011.03943.x
 44. Lordkipanidze M. Platelet turnover in atherothrombotic disease. *Curr Pharm Des.* (2012) 18:5328–43. doi: 10.2174/138161212803251952
 45. Chen WH, Cheng X, Lee PY, Ng W, Kwok JY, Tse HF, et al. Aspirin resistance and adverse clinical events in patients with coronary artery disease. *Am J Med.* (2007) 120:631–5. doi: 10.1016/j.amjmed.2006.10.021
 46. McAdam B, Keimowitz RM, Maher M, Fitzgerald DJ. Transdermal modification of platelet function: an aspirin patch system results in marked suppression of platelet cyclooxygenase. *J Pharmacol Exp Ther.* (1996) 277:559–64.
 47. Scheer FA, Michelson AD, Frelinger AL III, Evoniuk H, Kelly EE, McCarthy M, et al. The human endogenous circadian system causes greatest platelet activation during the biological morning independent of behaviors. *PLoS ONE.* (2011) 6:e24549. doi: 10.1371/journal.pone.0024549
 48. Gow JA, Ebbeling L, Gerrard JM. The effect of regular and enteric-coated aspirin on bleeding time, thromboxane, and prostacyclin. *Prost Leukotr Essen Fatty Acids.* (1993) 49:515–20. doi: 10.1016/0952-3278(93)90040-4
 49. Lorenz RL, Boehlig B, Uedelhoven WM, Weber PC. Superior antiplatelet action of alternate day pulsed dosing versus split dose administration of aspirin. *Am J Cardiol.* (1989) 64:1185–8. doi: 10.1016/0002-9149(89)90875-8
 50. Capodanno D, Patel A, Dharmashankar K, Ferreira JL, Ueno M, Kodali M, et al. Pharmacodynamic effects of different aspirin dosing regimens in type 2 diabetes mellitus patients with coronary artery disease. *J Am Coll Cardiol.* (2011) 57:E1910. doi: 10.1016/S0735-1097(11)61910-4
 51. Sinzinger H, Kaliman J, Fitscha P, O'Grady J. Diminished platelet residence time on active human atherosclerotic lesions *in-vivo*—evidence for an optimal dose of aspirin? *Prost Leukotr Essen Fatty Acids.* (1988) 34:89–93. doi: 10.1016/0952-3278(88)90068-3
 52. DiMinno G, Silver MJ, Cerbone AM, Murphy S. Trial of repeated low-dose aspirin in diabetic angiopathy. *Blood.* (1986) 68:886–91.
 53. Hart RG, Leonard AD, Talbert RL, Pearce LA, Cornell E, Bovill E, et al. Aspirin dosage and thromboxane synthesis in patients with vascular disease. *Pharmacotherapy.* (2003) 23:579–84. doi: 10.1592/phco.23.5.579.32206
 54. Tohgi H, Konno S, Tamura K, Kimura B, Kawano K. Effects of low-to-high doses of aspirin on platelet aggregability and metabolites of thromboxane A2 and prostacyclin. *Stroke.* (1992) 23:1400–3. doi: 10.1161/01.STR.23.10.1400
 55. Saleem SM, Shah S, Shah BA, Dhobi GN, Feroz S. Role of platelet aggregation and effect of aspirin in ischemic strokes—a randomized study. *JK Pract.* (2000) 7:39–42. doi: 10.1161/01.STR.0000181081.09262.e1
 56. DiChiara J, Bliden KP, Tantry US, Hamed MS, Antonino MJ, Suarez TA, et al. The effect of aspirin dosing on platelet function in diabetic and nondiabetic patients: an analysis from the aspirin-induced platelet effect (ASPECT) study. *Diabetes.* (2007) 56:3014–9. doi: 10.2337/db07-0707
 57. Chamorro A, Escolar G, Revilla M, Obach V, Vila N, Reverter JC, et al. *Ex vivo* response to aspirin differs in stroke patients with single or recurrent events: a pilot study. *J Neurol Sci.* (1999) 171:110–4. doi: 10.1016/S0022-510X(99)00260-9
 58. Perneby C, Wallen NH, Rooney C, Fitzgerald D, Hjelm Dahl P. Dose- and time-dependent antiplatelet effects of aspirin. *Thromb Haemost.* (2006) 95:652–8. doi: 10.1160/TH05-10-0653
 59. Racca C, van Diemen JK, Fuijkschot WW, Spit K, Bonten TN, Numans ME, et al. Aspirin intake in the morning is associated with suboptimal platelet inhibition, as measured by serum Thromboxane B2, during infarct-prone early-morning hours. *Platelets.* (2018) 1–7. doi: 10.1080/09537104.2018.1528347
 60. Santos MT, Valles J, Aznar J, Lago A, Sanchez E, Cosin J, et al. Aspirin therapy for inhibition of platelet reactivity in the presence of erythrocytes in patients with vascular disease. *J Lab Clin Med.* (2006) 147:220–7. doi: 10.1016/j.lab.2005.12.005
 61. Spectre G, Arnetz L, Ostenson CG, Brismar K, Li N, Hjelm Dahl P. Twice daily dosing of aspirin improves platelet inhibition in whole blood in patients with type 2 diabetes mellitus and micro- or macrovascular complications. *Thromb Haemost.* (2011) 106:491–9. doi: 10.1160/TH11-04-0216
 62. McLeod LJ, Roberts MS, Cossum PA, Vial JH. The effects of different doses of some acetylsalicylic acid formulations on platelet function and bleeding times in healthy subjects. *Scand J Haematol.* (1986) 36:379–84. doi: 10.1111/j.1600-0609.1986.tb01753.x
 63. Furuno T, Yamasaki F, Yokoyama T, Sato K, Sato T, Doi Y, et al. Effects of various doses of aspirin on platelet activity and endothelial function. *Heart Vessels.* (2011) 26:267–73. doi: 10.1007/s00380-010-0054-8
 64. Addad F, Chakroun T, Elalamy I, Abderazek F, Chouchene S, Dridi Z, et al. Antiplatelet effect of once- or twice-daily aspirin dosage in stable coronary artery disease patients with diabetes. *Inter J Hematol.* (2010) 92:296–301. doi: 10.1007/s12185-010-0652-3
 65. van Diemen JJ, Fuijkschot WW, Wessels TJ, Veen G, Smulders YM, Thijs A. Evening intake of aspirin is associated with a more stable 24-h platelet inhibition compared to morning intake: a study in chronic aspirin users. *Platelets.* (2016) 27:351–6. doi: 10.3109/09537104.2015.1107536
 66. Bonten TN, Snoep JD, Assendelft WJ, Zwaginga JJ, Eikenboom J, Huisman MV, et al. Time-dependent effects of aspirin on blood pressure and morning platelet reactivity: a randomized cross-over trial. *Hypertension.* (2015) 65:743–50. doi: 10.1161/HYPERTENSIONAHA.114.04980

67. Krasinska B, Paluszkiwicz L, Miciak-Lawicka E, Krasinski M, Rzymiski P, Tykarski A, et al. The effect of acetylsalicylic acid dosed at bedtime on the anti-aggregation effect in patients with coronary heart disease and arterial hypertension: a randomized, controlled trial. *Cardiol J.* (2018). doi: 10.5603/CJ.a2018.0142. [Epub ahead of print].
68. Garcia Rodriguez LA, Martin-Perez M, Hennekens CH, Rothwell PM, Lanan A. Bleeding risk with long-term low-dose aspirin: a systematic review of observational studies. *PLoS ONE.* (2016) 11:e0160046. doi: 10.1371/journal.pone.0160046
69. Claxton AJ, Cramer J, Pierce C. A systematic review of the associations between dose regimens and medication compliance. *Clin Ther.* (2001) 23:1296–310. doi: 10.1016/S0149-2918(01)80109-0
70. Bem D, Lordkipanidze M, Hodgkinson J, Stevens S, Bayliss S, Moore D, et al. The effects of different aspirin dosing frequencies and the timing

of aspirin intake in primary and secondary prevention of cardiovascular disease: a systematic review. *Clin Pharmacol Ther.* (2016) 100:500–12. doi: 10.1002/cpt.438

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Buurma, van Diemen, Thijs, Numans and Bonten. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Comparative Analysis of Microfluidics Thrombus Formation in Multiple Genetically Modified Mice: Link to Thrombosis and Hemostasis

Magdolna Nagy¹, Johanna P. van Geffen¹, David Stegner², David J. Adams³, Attila Braun², Susanne M. de Witt¹, Margitta Elvers⁴, Mitchell J. Geer⁵, Marijke J. E. Kuijpers¹, Karl Kunzelmann⁶, Jun Mori⁵, Cécile Oury⁷, Joachim Pircher⁸, Irina Pleines², Alastair W. Poole⁹, Yotis A. Senis⁵, Remco Verdood¹, Christian Weber^{1,10}, Bernhard Nieswandt², Johan W. M. Heemskerk^{1*} and Constance C. F. M. J. Baaten^{1,11*}

OPEN ACCESS

Edited by:

Paul Jurasz,
University of Alberta, Canada

Reviewed by:

Plinio Cirillo,
University of Naples Federico II, Italy
Daniel Duerschmied,
University of Freiburg, Germany

*Correspondence:

Johan W. M. Heemskerk
jwm.heemskerk@
maastrichtuniversity.nl
Constance C. F. M. J. Baaten
cbaaten@ukaachen.de

Specialty section:

This article was submitted to
Atherosclerosis and Vascular
Medicine,
a section of the journal
Frontiers in Cardiovascular Medicine

Received: 06 May 2019

Accepted: 03 July 2019

Published: 30 July 2019

Citation:

Nagy M, van Geffen JP, Stegner D, Adams DJ, Braun A, de Witt SM, Elvers M, Geer MJ, Kuijpers MJE, Kunzelmann K, Mori J, Oury C, Pircher J, Pleines I, Poole AW, Senis YA, Verdood R, Weber C, Nieswandt B, Heemskerk JWM and Baaten CCFMJ (2019) Comparative Analysis of Microfluidics Thrombus Formation in Multiple Genetically Modified Mice: Link to Thrombosis and Hemostasis. *Front. Cardiovasc. Med.* 6:99. doi: 10.3389/fcvm.2019.00099

¹ Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, Netherlands, ² Rudolf Virchow Center, Institute of Experimental Biomedicine, University Hospital Würzburg, University of Würzburg, Würzburg, Germany, ³ Wellcome Trust Sanger Institute, Cambridge, United Kingdom, ⁴ Department of Vascular Surgery, Experimental Vascular Medicine, Heinrich Heine University, Düsseldorf, Germany, ⁵ Institute of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham, Birmingham, United Kingdom, ⁶ Institute of Physiology, University of Regensburg, Regensburg, Germany, ⁷ GIGA-Cardiovascular Sciences, University of Liège, Liège, Belgium, ⁸ Medizinische Klinik und Poliklinik I, Klinikum der Universität München, Ludwig-Maximilians-University, and DZHK (German Center for Cardiovascular Research), Partner Site Munich Heart Alliance, Munich, Germany, ⁹ Department of Physiology and Pharmacology, School of Medical Sciences, University of Bristol, Bristol, United Kingdom, ¹⁰ Institute for Cardiovascular Prevention (IPEK), Ludwig-Maximilians-Universität München, Munich, Germany, ¹¹ Institute for Molecular Cardiovascular Research (IMCAR), University Hospital Aachen, Aachen, Germany

Genetically modified mice are indispensable for establishing the roles of platelets in arterial thrombosis and hemostasis. Microfluidics assays using anticoagulated whole blood are commonly used as integrative proxy tests for platelet function in mice. In the present study, we quantified the changes in collagen-dependent thrombus formation for 38 different strains of (genetically) modified mice, all measured with the same microfluidics chamber. The mice included were deficient in platelet receptors, protein kinases or phosphatases, small GTPases or other signaling or scaffold proteins. By standardized re-analysis of high-resolution microscopic images, detailed information was obtained on altered platelet adhesion, aggregation and/or activation. For a subset of 11 mouse strains, these platelet functions were further evaluated in rhodocytin- and laminin-dependent thrombus formation, thus allowing a comparison of glycoprotein VI (GPVI), C-type lectin-like receptor 2 (CLEC2) and integrin $\alpha_6\beta_1$ pathways. High homogeneity was found between wild-type mice datasets concerning adhesion and aggregation parameters. Quantitative comparison for the 38 modified mouse strains resulted in a matrix visualizing the impact of the respective (genetic) deficiency on thrombus formation with detailed insight into the type and extent of altered thrombus signatures. Network analysis revealed strong clusters of genes involved in GPVI signaling and Ca^{2+} homeostasis. The majority of mice demonstrating an antithrombotic phenotype *in vivo* displayed with a larger or smaller reduction in multi-parameter analysis of collagen-dependent thrombus formation *in vitro*. Remarkably, in only approximately half of the mouse strains that displayed reduced arterial thrombosis *in vivo*, this was

accompanied by impaired hemostasis. This was also reflected by comparing *in vitro* thrombus formation (by microfluidics) with alterations in *in vivo* bleeding time. In conclusion, the presently developed multi-parameter analysis of thrombus formation using microfluidics can be used to: (i) determine the severity of platelet abnormalities; (ii) distinguish between altered platelet adhesion, aggregation and activation; and (iii) elucidate both collagen and non-collagen dependent alterations of thrombus formation. This approach may thereby aid in the better understanding and better assessment of genetic variation that affect *in vivo* arterial thrombosis and hemostasis.

Keywords: arterial thrombus formation, bleeding, collagen, glycoprotein VI, platelets, microfluidics

INTRODUCTION

Current concepts of platelet activation pathways in thrombosis and hemostasis rely to a large extent on the summation of single observations. Frequently, the role of a particular protein or signaling pathway is deduced from the consequences of a genetic knockout in mouse on platelet responses, such as in comparison to changes in experimental arterial thrombosis and tail bleeding. A large set of such studies has resulted in the concept of collagen-induced arterial thrombus formation (1–4). Herein, it is stipulated that the exposure of subendothelial collagen to flowing blood is a key trigger to start shear-dependent thrombus formation. Collagen causes platelet adhesion and furthermore binds von Willebrand factor (VWF), which can decelerate flowing platelets at high shear rate. Firm VWF/collagen-mediated adhesion and subsequent platelet activation requires synergy between the VWF receptor, glycoprotein (GP)Ib-V-IX, and the collagen receptors, GPVI and integrin $\alpha_2\beta_1$ (5–8).

Collagen is known to induce a number of stimulating pathways via GPVI, in particular: (i) activation of Src-family and Syk tyrosine kinases, resulting in phospholipase C (PLC) γ_2 activation and intracellular Ca^{2+} mobilization (2, 9); (ii) additional Ca^{2+} influx via ORAI1 channels which couple to the calcium sensor STIM1 in the reticular membrane (10); (iii) activation of several isoforms of protein kinase C (PKC) (11), phosphoinositide 3-kinases (PI3K) (12), and small GTPases, the latter including CDC42, RAC1 and RHOA (13). Additional, modifying signaling pathways include: (iv) activation of phospholipase D, augmenting flow-dependent platelet activation (14); and (v) activation of multiple (tyrosine) phosphatases, a part of which act downstream of immunoreceptor tyrosine-based inhibition motif (ITIM)-containing receptors. Such phosphatases can have a direct or indirect platelet-stimulating (e.g., CD148, DUSP) or a platelet-inhibiting effect (PECAM1, G6b-B) in response to collagen (15). Together, these signaling routes cooperate to control the activation state of the platelet fibrinogen receptor, integrin $\alpha_{IIb}\beta_3$, the secretion of granular contents, and the release of thromboxane- A_2 . Locally secreted or generated soluble agonists, acting via G-protein coupled receptors (GPCR) ensure the capture and incorporation of passing platelets into an aggregate or thrombus (2–4). In a subset of platelets in the thrombus, procoagulant activity is generated by Ca^{2+} -dependent activation of the anoctamin-6 (TMEM16F) channel (16),

regulating phosphatidylserine (PS) exposure and platelet ballooning (17–19).

Although all these signaling components are known to play a certain role in collagen-dependent thrombus formation, there is still limited insight into the relative contribution of individual proteins. In addition, it is unclear to which extent C-type lectin-like receptor-2 (CLEC2), another receptor that signals via tyrosine kinases (20, 21), is capable to regulate the process of thrombus formation. The same holds for integrin $\alpha_6\beta_1$, an adhesive receptor, which mediates flow-dependent adhesion of platelets to the matrix protein laminin (22).

In a recent synthesis approach, a quantitative evaluation was made of the contribution of 431 mouse genes to experimental arterial thrombosis and hemostasis *in vivo*, thereby revealing several genes with a role in thrombosis without affecting bleeding (23). For the total cohort of studies and mouse genes, it appeared that microfluidics assays where thrombus formation is measured *in vitro*—by whole blood perfusion over a collagen surface—predict the consequences of a gene knockout on thrombosis models *in vivo* (23). However, the standard microfluidic tests only report on changes in platelet adhesion (surface area coverage, SAC%), which is a limitation given that the recorded microscopic images also contain information on platelet aggregate formation (24, 25). In comparison, for human blood samples from a large cohort of healthy subjects, it could be shown that a multi-parameter image analysis can provide detailed information on the sub-processes of platelet adhesion, aggregation and activation at the same time (26).

To better understand the alterations in thrombus phenotypes using microfluidics, we applied a similar multi-parameter approach to quantitatively compare the effects of deficiency of 37 signaling proteins on collagen-induced pathways. We therefore re-analyzed earlier recorded microscopic images, in all cases from thrombi generated using the same microfluidic flow chamber setup.

MATERIALS AND METHODS

Mice

Mice were included from 38 strains, in each case with a monogenetic or antibody-induced deficiency, as well as 22 sets of corresponding wild-type or control mice, as described in the original publications (see **Table 1**). As a selection criterion for

inclusion, microscopic images needed to be available from $n \geq 3$ animals per modified group and $n \geq 4$ for wild-types. Scaled effects on *in vivo* arterial thrombosis and tail bleeding are for the majority of strains described previously (23).

In all cases, mouse blood was collected into anticoagulant medium, consisting of PPACK (40 μ M), unfractionated heparin (5 U/ml) and low molecular weight heparin (fragmin, 50 U/ml). Samples were immediately processed. For original data, experiments were approved by the district government of Lower Franconia (Bezirksregierung Unterfranken) and by the Animal Experimental Committee in Hinxton/Cambridge. For previously published data, experiments were approved by the local Animal Experimental Committees as indicated in the original publications (see references in Table 1).

Whole Blood Thrombus Formation Under Flow

For all data sets, thrombus formation in whole blood under flow was assessed with the Maastricht flow chamber (depth 50 μ m, width 3 mm, length 30 mm) (24). In short, PPACK/heparin anticoagulated blood (400–500 μ l) was perfused for 3.5–4.0 min at room temperature at a wall shear rate of 1000 s^{-1} (where indicated 1700 s^{-1}) over (i) Horm-type collagen (100 μ g/ml, Nycomed Pharma, Munich, Germany), allowing platelet interaction via GPIIb, GPVI and integrin $\alpha_2\beta_1$.

Where specified, two additional surfaces were used, similarly as described before (24): (ii) VWF-binding peptide (VWF-BP, 12.5 μ g/ml, obtained from Prof. Dr. R. Farndale, Cambridge University, UK) + laminin (50 μ g/ml, from human plasma, Sigma-Aldrich, St. Louis MO, USA; binding integrin $\alpha_6\beta_1$) (22), and (iii) VWF-BP + laminin + rhodocytin (250 μ g/ml, activating CLEC2) (54). Rhodocytin purified from *Calloselasma rhodostoma* (55), was a kind gift of Prof. Dr. K. Clemetson (Bern University, Switzerland).

After blood perfusion, platelet thrombi were rinsed with modified Tyrode's Hepes buffer (pH 7.45, 5 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 0.42 mM NaH_2PO_4 , 1 mg/ml glucose, 1 mg/ml bovine serum albumin, 2 mM $CaCl_2$, 2 mM $MgCl_2$ and 1 U/ml heparin), and then stained during 1.5 min flow with one to three fluorescently labeled platelet activation markers. These were: Alexa Fluor (AF)647 (or FITC) conjugated annexin A5 (AF647: 1:200, Invitrogen Life Technologies, Carlsbad, CA, USA/FITC: 1:1000, PharmaTarget, Maastricht, The Netherlands); FITC anti-mouse CD62P mAb (1:40, rat-anti-mouse, Emfret Analytics, Würzburg, Germany); and phycoerythrin (PE)-labeled JON/A mAb (1:20, Emfret Analytics); all diluted in modified Tyrode's Hepes buffer. Residual labels were removed by another perfusion for 2 min with label-free Tyrode's Hepes buffer. Multiple brightfield and (if applicable) fluorescence microscopic images were captured per surface, of which three representative images were re-analyzed in a systematic way (26).

For image recording, different fluorescence microscopic systems were used (see references in Table 1), but always containing a 60/63x oil objective and a sensitive CCD camera for capturing enhanced-contrast, brightfield images.

Microscopic Image Analysis

For all mouse strains, the recorded 16-bit or 8-bit brightfield and fluorescence images were re-analyzed using the same newly developed scripts (one per image type), written in Fiji (56). Scripts always opened a series of images one-by-one with a loop. In each loop run, background illumination was corrected using a fast Fourier transform bandpass filter, followed by manual setting of a threshold and measurement of the surface area coverage. For brightfield images, a series of Gray morphology conversions was applied to reduce striping and to improve the detection quality. Image conversion steps were as follows: a diamond large sized close, followed by a medium sized circle close and a small circle shaped dilate. Note that the first step increased the pixels that were stronger in regions with many neighboring pixels, the second step then rounded the shapes and additionally reduced straight lines, while the final step (allowing alteration by user-interface) served to obtain the best match with the original image. The Fourier transform filter served to flatten the background areas sufficiently for good analysis, with a minimal impact on the structures. For brightfield images, large structures were filtered down to 60 pixels, for the fluorescence images of annexin A5, integrin and P-selectin images large structures were filtered down to 65 pixels. Small structures were not filtered down, as these contained structures of interest within the adhered platelets (Supplementary Figure 1).

Using these scripts and by visually scoring the unprocessed brightfield images, the following parameters of thrombus formation were obtained (Table 2): surface area coverage of adhered platelets ($P1$, %SAC); platelet aggregate coverage ($P2$, %SAC); thrombus morphology score ($P3$, scale 0–5); thrombus multilayer score ($P4$, scale 0–3); and thrombus contraction score ($P5$, scale 0–3). Scoring was performed in comparison to a set of pre-defined standard images (Supplementary Figure 1). For the assessment of platelet activation, fluorescence images were analyzed for PS exposure ($P6$, %SAC), P-selectin expression ($P7$, %SAC) and integrin $\alpha_{IIb}\beta_3$ activation ($P8$, %SAC) (24).

Regardless of the image type, parameter values from three images per experiment were averaged, thus resulting in a single value per parameter and experiment. The image analysis and scoring parameters were verified by different observers, who were blinded to the experimental condition. Parameters of experiments from the same mouse strain were combined as proxy measures of platelet adhesion ($P1$), thrombus signature ($P2$ –5), and platelet activation ($P6$ –8), as described before for human platelets (26).

Network Analysis

A network of protein-protein interactions was built based on 37 investigated genes, using the STRING database (57), taking into consideration the following settings: 1st shell interactors: <20 interactors, 2nd shell interactors: <60 interactors, confidence level: medium to high. Networks were visualized in Cytoscape version 3.7.0 (58).

Network clustering analysis was performed with the Cytoscape app, MCODE to identify highly interacting nodes using the following settings: degree cutoff: 2, node score cutoff: 0.2, K-core: 2 and maximum depth: 100 (59, 60).

TABLE 1 | Overview of re-analyzed data sets on thrombus formation using the Maastricht flow chamber.

Gene	Protein	Genetic modification	Background	DB	Δ SAC%	Thrombosis phenotype	Bleeding phenotype	Remarks	References
<i>Ano1</i>	Anoctamin-1	PF4-Cre <i>Ano1</i>	C57Bl/6	07	o	n.d.	n.d.	-	(18)
<i>Ano6</i>	Anoctamin-6	<i>Ano6</i> ^{Gt(AW0382)}	C57Bl/6	07	o	n.d.	↑	Prolonged bleeding	(18)
<i>Anxa1</i>	Annexin A1	PF4-Cre <i>Anxa1</i>	C57Bl/6	22	o	n.d.	n.d.	-	(23)***
<i>ApoE</i>	Apolipoprotein E	<i>ApoE</i> ^{tm1Unc}	C57Bl/6	22	↑	n.d.	n.d.	-	(23)***
<i>Bnip2</i>	BCL2-interacting protein 2	<i>Bnip2</i> ^{tm1a}	C57Bl/6N	21	o	n.d.	n.d.	-	(23)***
<i>Capn1</i>	Calpain-1	<i>Capn1</i> ^{tm1Ahc}	C57Bl/6	10	↑	↓	n.d.	Delayed vessel occlusion	(27, 28)
<i>Cd36</i>	CD36	<i>Cd36</i> ^{tm1Mfe}	C57Bl/6	14	o	↓	o	Delayed thrombus formation, increased embolization	(29, 30)
<i>Cdc42</i>	Small GTPase CDC42	PF4-Cre <i>Cdc42</i>	C57Bl/6 x 129SV	20	o	↑	↑	Accelerated vessel occlusion, prolonged bleeding	(31)
<i>Clec1b</i>	CLEC2	INU1 Ab*	C57Bl/6	19	↓	↓	↑	Delayed thrombus formation, increased embolization	(20)
<i>Csk</i>	Tyrosine kinase CSK	PF4-Cre <i>Csk</i> ^{tm1Tara}	C57Bl/6	23	↓	↓	↑	Moderately reduced thrombus formation	(32)
<i>Dlg4</i>	Scaffold protein DLG4	<i>Dlg4</i> ^{tm1a}	C57Bl/6N	21	o	n.d.	n.d.	-	(23)***
<i>Dusp3</i>	Protein phosphatase DUSP3	<i>Dusp3</i> ^{tm1Srah}	C57Bl/6	11	↓	↓	o	Decreased pulmonary embolism, small thrombus volume	(33)
<i>Fcer1g</i>	FcR γ -chain	<i>Fcer1g</i> ^{tm1Rav}	C57Bl/6 x 129SV	04	↓	↓	n.d.	Delayed and reduced thrombus formation	(34, 35)
<i>Fpr2</i>	Formyl peptide receptor-2	<i>Fpr2</i> ^{Tg(ACTB)#Jimw}	C57Bl/6	22	↑	n.d.	n.d.	-	(23)***
<i>Gnaq</i>	Gq α -subunit	<i>Gnaq</i> ^{tm1Soff}	C57Bl/6 x 129SV	04	↓	↓	↑	Intra-abdominal bleeding, frequent postnatal death, protection thromboembolism	(34, 36)
<i>Gp6</i>	GPVI	<i>Gp6</i> ^{tm1Beni}	C57Bl/6	03	↓	↓	o	Reduced thrombus stability, enhanced embolization	(37, 38)
<i>Gp6 / Clec1b</i>	GPVI/CLEC2	JAQ1+INU1 Ab*	C57Bl/6	03	n.d.	↓	↑	Delayed thrombus formation, smaller thrombus volume	(38)**
<i>Grm8</i>	Glutamate metabotropic receptor 8	<i>Grm8</i> ^{tm1a}	C57Bl/6N	21	o	n.d.	n.d.	-	(23)***
<i>Ifnar1</i>	Interferon receptor 1	<i>Ifnar1</i> ^{tm1a}	C57Bl/6N	21	↓	n.d.	n.d.	-	(23)***
<i>Itga2</i>	Integrin α 2	LoxP-Cre <i>Itga2</i>	C57Bl/6 x 129SV	05	↓	↓	o	Reduced thrombus formation, increased embolization	(5, 39)
<i>Itgb1</i>	Integrin β 1	Mx-Cre <i>Itgb1</i>	C57Bl/6 x 129SV	04	↓	o	o	Unchanged thrombus formation	(5, 34, 40)

(Continued)

TABLE 1 | Continued

Gene	Protein	Genetic modification	Background	DB	Δ SAC%	Thrombosis phenotype	Bleeding phenotype	Remarks	References
<i>Kcnn4</i>	K-activated Ca channel-4	<i>Kcnn4</i> ^{tm1Rklr}	C57Bl/6 x 129SV	16	o	n.d.	n.d.	-	(18)
<i>Mpig6b</i>	Receptor G6B-b	<i>Mpig6b</i> ^{tm1.1Arte}	C57Bl/6	24	↓	n.d.	↑	Prolonged bleeding	(41)
<i>Orai1</i>	Calcium channel ORAI1	BMC <i>Orai1</i> ^{-/-}	C57Bl/6	01	↓	↓	o/↑	Reduced thrombus formation and stability	(42, 43)
<i>Plk3cg</i>	PI 3-kinase γ	<i>Plk3cg</i> ^{tm1Wym}	129SV	13	↓	↓	o	Protected from thromboembolic vascular occlusion	(44, 45)
<i>Plcg2</i> (GOF)	Phospholipase C- γ 2 (GOF)	<i>Plcg2</i> ^{Al/5}	C3HeB/FeJ	12	↑	↑	n.d.	Increased pulmonary thromboembolism	(46)
<i>Pld1</i>	Phospholipase D1	<i>Pld1</i> ^{tm3Mafr}	C57Bl/6	02	o	↓	o	Reduced thromboembolism, reduced thrombus stability	(14)
<i>Prkca</i>	Protein kinase C- α	<i>Prkca</i> ^{Myh6/tetO/1Jmk}	C57Bl/6	06	↓	↓	o	Delayed thrombus formation, no fecal occult blood	(47, 48)
<i>Prkcd</i>	Protein kinase C- δ	<i>Prkcd</i> ^{tm1Kin}	C57Bl/6	06	↑	o	n.d.	Unchanged thrombus formation	(47, 49)
<i>Prkcq</i>	Protein kinase C- θ	<i>Prkcq</i> ^{tm1Litt}	C57Bl/6	06	↑	n.d.	n.d.	Limited occlusion, thrombus instability	(47)
<i>Prkd2</i>	Protein kinase D2	<i>Prkd2</i> ^{tm1.1Daca}	C57Bl/6	17	↓	n.d.	o	Unchanged bleeding	(50)
<i>Ptpnj</i>	Phosphatase CD148	PF4-Cre <i>Ptpnj</i> ^{tm1.1Weis}	C57Bl/6	23	↓	↓	o	Severely compromised thrombus formation	(32)
<i>Rac1</i>	Small GTPase RAC1	Mx-Cre <i>Rac1</i>	C57Bl/6	08	↓	↓	↑	Reduced thrombus formation and volume, variable but prolonged bleeding	(51)
<i>Rhoa</i>	Small GTPase RHO-A	PF4-Cre <i>Rhoa</i>	C57Bl/6 x 129SV	08	o	↓	↑	Unstable thrombus formation, increased embolization	(13)**
<i>Stim1</i>	Regulator STIM1	BMC <i>Stim1</i> ^{-/-}	C57Bl/6	01	↓	↓	↑	Reduced thrombus stability, no vessel occlusion	(42, 52)
<i>Stim2</i>	Regulator STIM2	<i>Stim2</i> ^{tm1Beni}	C57Bl/6	01	o	n.d.	n.d.	-	(42)
<i>Syk</i>	Tyrosine kinase SYK	PF4-Cre <i>Syk</i> ^{tm1(syk)Spwa}	C57Bl/6	09	n.d.	↓	↑	Blood-filled lymphatics, impaired thrombus formation	(53)**
<i>Vps13a</i>	Vacuolar sorting protein VPS13A	<i>Vps13a</i> ^{tm1a}	C57Bl/6N	21	↓	n.d.	n.d.	-	(23)***

Shown are published changes in platelet deposition (SAC%) on collagen for mice with indicated genetic deficiencies in comparison to wild-type mice. Where indicated, GPVI and/or CLEC2 were rendered non-functional by injection of antibodies. Further indicated are published effects of the gene deficiency (same mouse strain) on in vivo arterial thrombosis, pulmonary thromboembolism and/or (tail) bleeding.

BMC, bone-marrow chimera; DB, database; GOF, gain-of-function mutation; n.d., not determined. *Antibody-mediated deficiency of CLEC2 and/or GPVI; **microfluidics data not included in reference; ***only data for M1 published; ↑ increased/prolonged; ↓ decreased/shortened; o unchanged.

TABLE 2 | Overview of microspotted surfaces (*M*) and parameters of image analysis (*P*) for the assessment of thrombus formation.

M	Microspot surface	Platelet receptors involved		
M1	Collagen type I (VWF)*	GPIb, GPVI, $\alpha_2\beta_1$		
M2	Rhodocytin + laminin + VWF-BP	GPIb, CLEC2, $\alpha_6\beta_1$		
M3	Laminin + VWF-BP	GPIb, $\alpha_6\beta_1$		
P	Parameter of analysis	Image	Range	Scaling
Platelet adhesion				
P1	Platelet surface area coverage (%SAC)	BF**	0 – 93.67	0 – 10
Thrombus signature				
P2	Platelet aggregate coverage (%SAC)	BF	0 – 49.17	0 – 10
P3	Thrombus morphology score	BF	0 – 5.0	0 – 10
P4	Thrombus multilayer score	BF	0 – 3.0	0 – 10
P5	Thrombus contraction score	BF	0 – 3.0	0 – 10
Platelet activation				
P6	PS exposure (%SAC)	FL	0 – 12.23	0 – 10
P7	P-selectin expression (%SAC)	FL	0 – 29.70	0 – 10
P8	Integrin $\alpha_{IIb}\beta_3$ activation (%SAC)	FL	0 – 28.25	0 – 10

*Binds VWF from plasma. **BF, brightfield; FL, fluorescence.

Data Processing and Statistical Analysis

For each genetically modified strain and corresponding wild-types, image data were averaged to obtain one parameter per surface, of which mean and SD values were calculated. For heatmap representation, mean values were univariate scaled from 0 to 10 per parameter (24). Gene effect heatmaps were constructed by subtracting scaled average values of the wild-type (control) strain from those of the modified strain. For statistical evaluation, a filter was applied, considering changes outside the range of composite mean $\pm$ SD as a relevant difference between modification and wild-type (26). Heatmap data were visualized by (unsupervised hierarchical) cluster analysis using the program R (61). For comparison of raw parameter values, a Kendall's tau-b correlation analysis was performed using SPSS (IBM SPSS version 24, Armonk, NY, USA).

RESULTS

Microfluidics Analysis of Thrombus Formation on Collagen of Multiple Wild-Type Mouse Datasets

Microscopic brightfield and (annexin A5) fluorescence images were collected from earlier performed whole blood perfusion experiments with blood from 38 different strains of modified mice and 22 corresponding wild-types (Table 1). Included were experiments with strains (in majority published), that were judged to be of sufficiently high quality and power to allow re-analysis by newly developed image analysis scripts (Supplementary Figure 1). From each experiment, five parameters of thrombus formation were obtained (Table 2). Platelet adhesion was quantified by the conventional analysis of platelet SAC% (*P1*). Thrombus signature (26) was composed

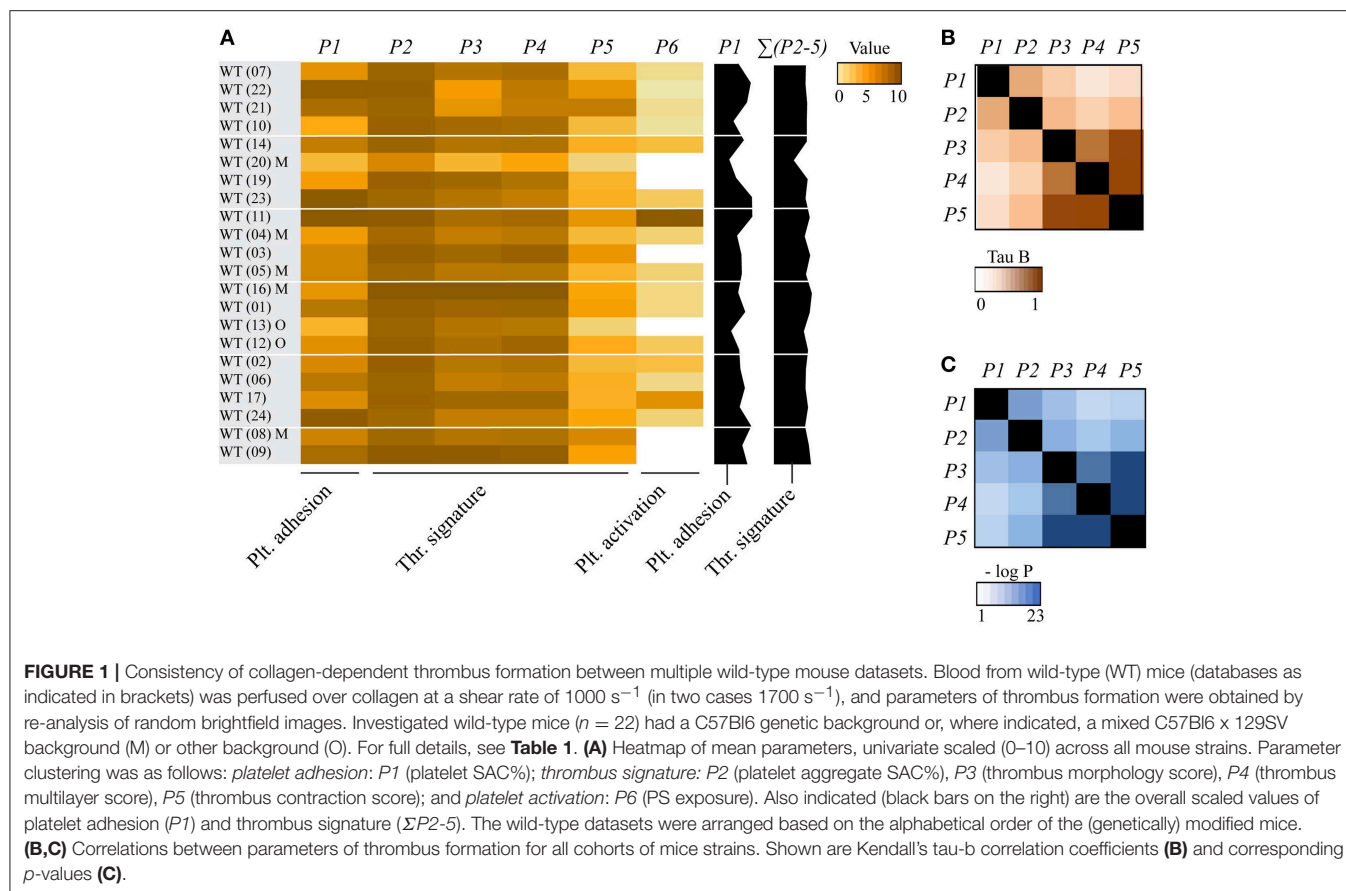
of four parameters related to the thrombus buildup ($\Sigma P2-5$), i.e., platelet aggregate SAC% (*P2*), thrombus morphology score (*P3*), thrombus multilayer score (*P4*), and thrombus contraction score (*P5*). As far as available, platelet activation was assessed from fluorescence images of PS exposure (*P6*) (see Supplementary Figure 1).

To establish the overall consistency of the combined databases of the whole blood experiments, we compared the mean values plus variation for each of the image analysis parameters *P1-5* for the 22 wild-type control datasets. The calculated coefficients of variation of means across the wild-type datasets ranged from <12% (*P3-5*) to 23–25% (*P1,2*) (Supplementary Table 1). For heatmap presentation, mean values for all wild-types (and corresponding transgenic animals) were univariate scaled from 0–10 (Figure 1A). The obtained heatmap illustrated an overall high cohesion of the data, yet also suggesting that wild-type strains with mixed C57Bl/6 x 129SV background (databases 04, 05, 08, 16, 20) had a tendency for smaller *P3-5* values. Overall, these findings point to a high degree of comparability between the various wild-type datasets.

To determine how the different parameters of collagen-mediated thrombus formation correlated within our dataset of wild-type and genetically modified mice, a correlation matrix was constructed based on the Kendall's tau-b correlation analysis (Figure 1). All parameters showed a significant moderate to strong correlation to each other (Kendall's tau-b: 0.424–0.829). Platelet adhesion (*P1*) correlated strongly to platelet aggregation (*P2*), while it correlated moderately to thrombus morphology (*P3*), multilayer (*P4*) and contraction (*P5*) (Kendall's tau-b: 0.424–0.572). Strongest associations were observed between platelet aggregation (*P2*) and the thrombus scores (*P3-5*) (Kendall's tau-b 0.55–0.65, $p < 0.001$). Furthermore, even stronger correlations were seen between *P3-5*, i.e., the thrombus morphology, multilayer and contraction scores (Kendall's tau-b 0.76–0.83, $p < 10^{-10}$).

Comparing Thrombus Formation on Collagen in Multiple Genetically Modified Mice

As detailed in Table 1, the included 38 modified mouse strains concern animals with defects of single genes, encoding for proteins implicated in GPVI- and/or GPCR-related platelet activation pathways, i.e., *Csk*, *Fcer1g* (FcR γ -chain), *Gnaq* ($G\alpha_q$), *Orai1*, *Pik3cg* (PI3K γ), *Pld1* (PLD1), *Prkca* (PKC α), *Prkcd* (PKC δ), *Prkcq* (PKC θ), *Prkd2* (PKD2), *Stim1*, *Stim2* or *Syk*. In addition, the list contains mice with genetic deficiencies of the small GTPases, *Cdc42*, *Rhoa* or *Rac1*; deficiencies of protein (tyrosine) phosphatases *Dusp3*, *Mpig6b* (G6b-B), or *Ptprj* (CD148); deficiencies of other adhesive receptors, such as *Itga2* (integrin α_2), *Itgb1* (integrin β_1) or *Cd36* (GPIV); defects linked to altered PS exposure, i.e., *Ano1* (TMEM16A), *Ano6* (TMEM16F), *Capn1* (calpain-1) or *Kcnn4*. Other mice with single gene deficiencies came from an earlier undertaking to find novel proteins implicated in thrombosis and hemostasis (23), namely *Anxa1* (annexin 1), *Apoe* (plasma lipoprotein component), *Bnip2* (CBL2-interacting protein), *Dlg4* (scaffold



protein), *Fpr2* (formyl peptide receptor), *Grm8* (glutamate receptor), *Ifnar1* (interferon receptor) or *Vps13a* (vacuolar sorting protein). In addition, a mutated mouse strain with a *Plcg2* gain-of-function (GOF, constitutive active PLC γ 2) was incorporated (46). Given the ability of antibodies JAQ1 and INU1 to specifically cause platelet depletion from GPVI or CLEC2, respectively, after *in vivo* injection into mice (5) we also included strains with such antibody-induced GPVI and/or CLEC2 deficiencies.

For assessment of the effects of (genetic) deficiency, scaled values per parameter were calculated for each of the 38 modified mouse strains and compared to those of the corresponding wild-types (**Supplementary Figure 2**). A subtraction heatmap was generated to pinpoint the effects of genetic modification, in which differences outside the range of (composite) means $\pm$ SD were considered as being relevant. The heatmap data could be ranked based on alterations in thrombus signature (**Figure 2A**) or on differences in platelet adhesion (**Figure 2B**). With either way of ranking, profound quantitative differences were observed in the majority of the thrombus formation parameters, when comparing the 38 modified mouse strains. Thrombus signatures were suppressed, in a decreasing order (**Figure 2A**), by deficiencies in *Syk*, GPVI/CLEC2, GPVI, *Fcer1g*, *Gnaq*, *Rac1*, *Prkd2*, *Prkca*, *Stim1*, *Itga2*, *Orai1*, CLEC2, *Mpig6b*, *Pik3cg*, *Csk*, and *Ptprj*. In contrast, this thrombus marker was elevated, in an increasing order, by deficiencies in

Prkcq, *Capn1*, *Plcg2* (gain-of-function mutation), *Apoe*, *Prkcd*, and *Fpr2*.

The ranking based on altered platelet adhesion revealed several similarities, but also marked differences. Defects in *Syk*, GPVI, *Rac1*, *Stim1*, and *Itga2* resulted in a strong suppression of both platelet adhesion and thrombus signature. Relative larger effects on thrombus signature—in comparison to platelet adhesion—were apparent for mice with defects in *Fcer1g*, *Prkd2*, and *Prkca* (reduced thrombus formation), as well as for mice with defective members of the PKC family, *Prkcd* and *Prkcq* (increased thrombus formation). Relative increases in platelet adhesion were only seen for mice with deficiencies in *Kcnn4*, *Ano1*, and *Capn1*. In general, these heatmaps demonstrated that the ranking based on changes in thrombus signature (**Figure 2A**) can provide additional insight into the 'thrombogenic' consequences of a gene defect, in comparison to a ranking based on altered platelet adhesion (**Figure 2B**).

Microfluidics Analysis of Thrombus Formation on Collagen and Other Surfaces

For a subset of 11 genetically modified mice strains, the same microfluidic device was used to assess whole blood thrombus formation on collagen-I (*M1*) plus two additional microspots, i.e., rhodocytin/laminin (*M2*) and laminin (*M3*), both co-coated with VWF-BP to induce shear-dependent platelet adhesion (**Table 2**).

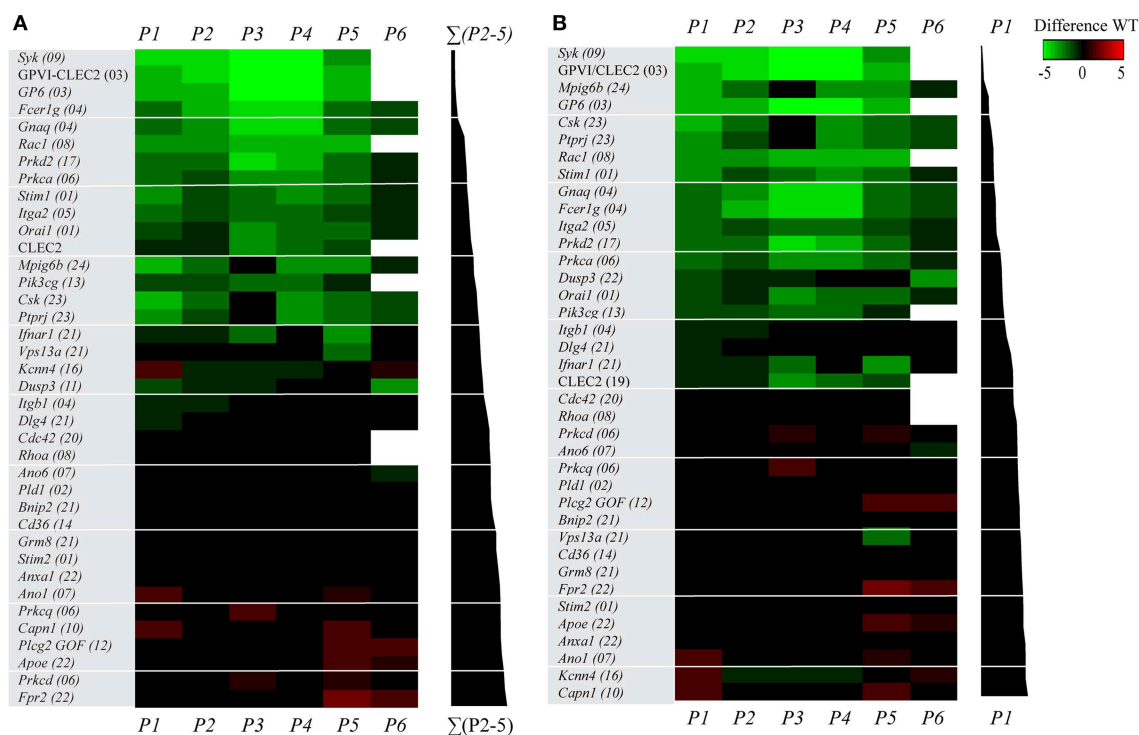


FIGURE 2 | Multi-parameter comparison of collagen-dependent thrombus formation for 38 (genetically) deficient mice. Whole blood from mice with indicated genetic or antibody-induced defects as well as from corresponding wild-type mice was perfused over collagen-I. Parameters of thrombus formation were as indicated in **Figure 1**. For detailed information on mouse strains, see **Table 1**. Mean parameters per strain were scaled (0–10) across all wild-types ($n = 22$) and (genetically) modified mice ($n = 38$), as for **Figure 1**. Subtraction heatmaps showing differences between indicated genetic (or antibody-mediated) deficiency in comparison to wild-type, after filtering for differences outside the range of (composite) mean $\pm$ SD, to select relevant changes. **(A)** Ranking of genes based on effect on thrombus signature ($\Sigma P2-5$), as shown in black bars on the right. Colors indicate unchanged (black), decreased (green) or increased (red) parameters. **(B)** Ranking of genes based on effect on platelet adhesion ($P1$), as indicated in black bars. Colors represent unchanged (black), decreased (green) or increased (red) parameters. Non-subtracted heatmap values are provided in **Supplementary Figure 2**.

As clarified before, immobilized rhodocytin triggers CLEC2-induced platelet activation (26, 54), whereas laminin surfaces allow platelet adhesion via integrin $\alpha_6\beta_1$ (22). For the same subset of mice, the formed thrombi were post-stained in three colors to quantify PS exposure ($P6$), P-selectin expression ($P7$) and integrin $\alpha_{IIb}\beta_3$ activation ($P8$), using procedures previously established for human platelet thrombi (26). Representative images from each of the microspots using wild-type blood are depicted in **Figure 3**. In comparison to collagen-I ($M1$), rhodocytin/laminin ($M2$) was less thrombogenic, with only moderately activated platelets that formed small aggregates. The laminin microspot ($M3$) only triggered adhesion of a monolayer of spreading platelets.

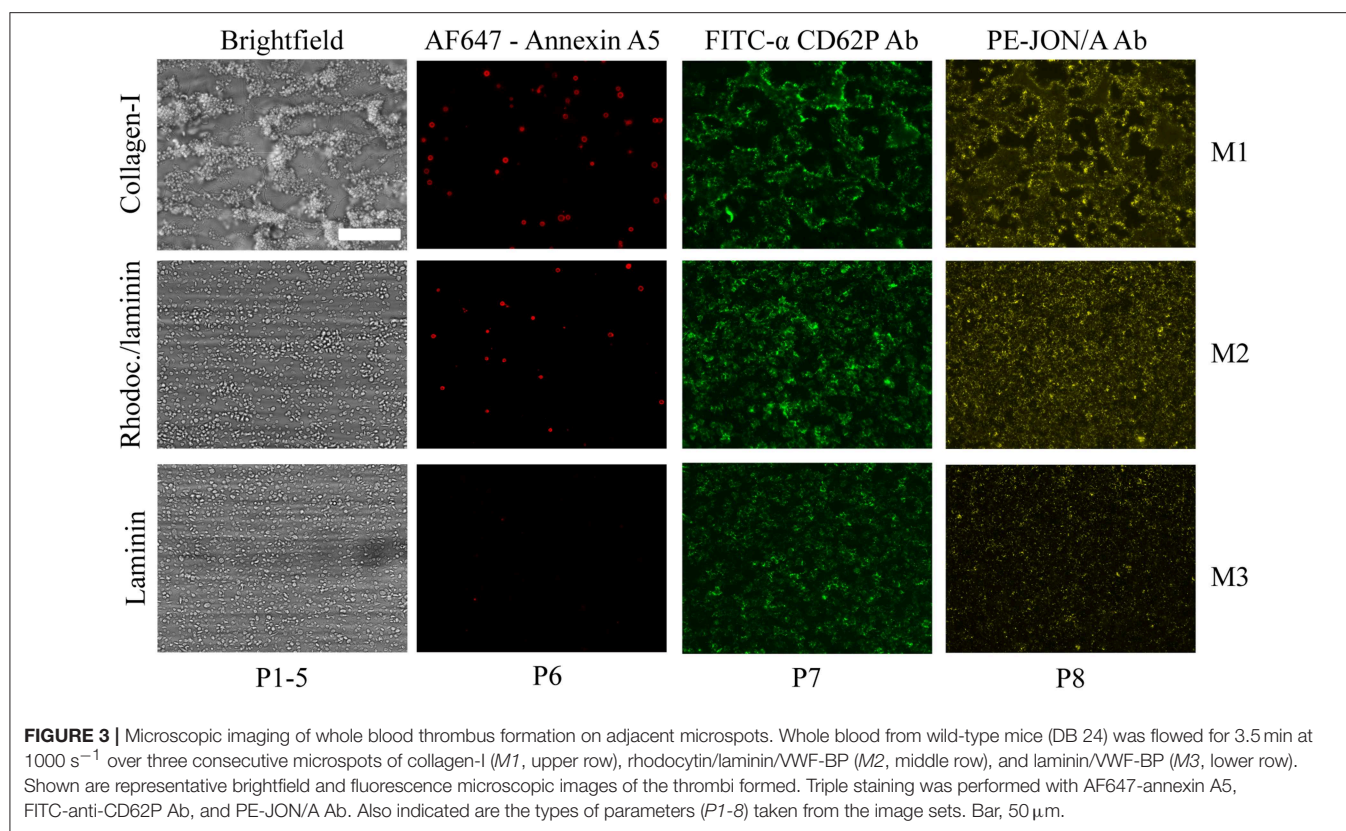
For these 11 genetically modified mouse strains plus corresponding wild-type mice, we again listed the scaled parameters ($P1-8$) for each microspot ($M1-3$) (**Supplementary Figure 3**). The ensuing subtraction heatmap revealed major reductions in platelet adhesion and thrombus signature at surface $M1$ for the animals with deficiencies in *Csk*, *Ptpnj*, *Mpig6b* (tyrosine protein kinase, phosphatases and ITIM receptor, respectively); and, to a clearly lesser extent, for deficiencies in *Ifnar1*, *Vps13a* and *Anxa1* (**Figure 4**). For the

kinase and phosphatase knockouts, this extended to a reduction in platelet activation parameters ($P6-8$) at $M1$, and furthermore to lower $P1-2$ values at the other microspots $M2-3$.

Markedly, for several of these mice, also gain-of-platelet-functions could be detected. In this case, increased parameters $P4-6$ were distributed over $M1$ (deficiencies in *Apoe* and *Fpr2*) and $M2$ (deficiency in *Grm8*). Another remarkable finding was that, for the majority of mice, parameters at the laminin surface ($M3$) were unchanged. This suggested that laminin-platelet interactions are relatively insensitive to these genetic modifications. Next to the ranking based on changes in platelet adhesion (**Figure 4B**), the ranking of genes according to changes in overall thrombus signature (**Figure 4A**) appeared to be a valuable addition in the description of the alterations in platelet properties.

Linking Microfluidics Outcomes to Thrombosis and Hemostasis *in vivo*

Recently, we described a systematic procedure to compare the consequences of genetic knockout in mice for experimentally induced (collagen-dependent) arterial thrombosis and hemostasis (23). For the 38 deficiencies, it was thus of interest



to compare the results of the current extended microfluidic assay (M1, P1-5) with the previously reported changes in arterial thrombosis tendency and tail bleeding *in vivo*. Hence, per modified mouse strain, the recorded changes (Table 1) were listed as being a reduced/unchanged/increased thrombosis phenotype and as a prolonged/unchanged/shortened bleeding time. Figure 5 gives the comparison of these *in vivo* effects with altered parameters of *in vitro* thrombus formation using microfluidics.

Strikingly, the majority of mice demonstrating an antithrombotic phenotype *in vivo* manifested with a larger or smaller reduction in collagen-dependent thrombus formation *in vitro* (Figure 5). We noted only a few exceptions: (i) mice deficient in *Cdc42* with an apparently prothrombotic phenotype, but for unknown reasons no effect *in vitro*; (ii) mice deficient in *Pld1* (where *in vitro* thrombus formation was only impaired at a higher shear rate of 1700 s^{-1}) (14); and (iii) *Cd36*-deficient mice (requiring a thrombospondin surface for altered *in vitro* thrombus formation) (29). Also, the impaired arterial thrombosis reported for *Capn1*^{-/-} mice (27) did not match with a measured higher platelet adhesion under flow, although it should be noted that the latter mice showed a complex pattern of increased and decreased platelet activation parameters *in vitro* (28).

Markedly, in the mouse strains with a reduced arterial thrombosis tendency *in vivo*, only approximately half of these were accompanied with a hemostatic defect (Figure 5). This was also reflected by comparing *in vitro* thrombus formation (by microfluidics) with alterations in bleeding time. Mice with

reduced *in vitro* thrombus formation, but unchanged or slightly prolonged bleeding times, included animals with deficiencies in the collagen receptors *Gp6* and *Itga2*; the protein kinases *Prkd2* and *Prkca*; and the protein phosphatases *Ptprj* and *Dusp3*. This may suggest that the collagen receptors (and hence collagen itself) and the (de)phosphorylating proteins within the downstream signaling pathways are not essential for hemostasis. This may suggest that the collagen receptors (and hence collagen itself) and the (de)phosphorylating proteins are not uniquely—perhaps redundantly—required for platelet functions at lower shear rates such as during hemostasis. The same mouse genes were also previously characterized as having a distinct role in arterial thrombosis and hemostasis (23).

Network Modeling to Predict Novel Proteins Implicated in Mouse Platelet Functions

Based on the 37 different mouse genes/proteins that were analyzed for effects on collagen-dependent whole blood thrombus formation, we constructed a network using the biological STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database, in order to be capable to depict additional protein-protein interactions. Accordingly, a network was established containing in total 117 nodes (37 core and 80 novel nodes) and 1142 edges (interaction score: medium to highest confidence: 0.40–0.99). Reactome pathways that were highly represented in the network were: platelet activation,

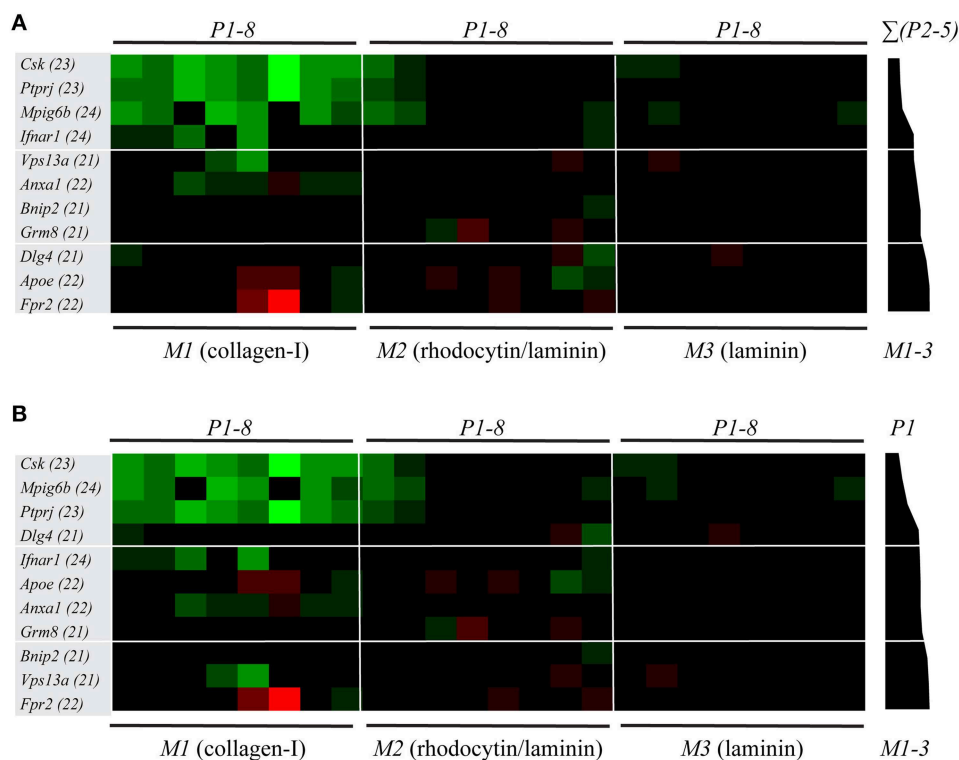


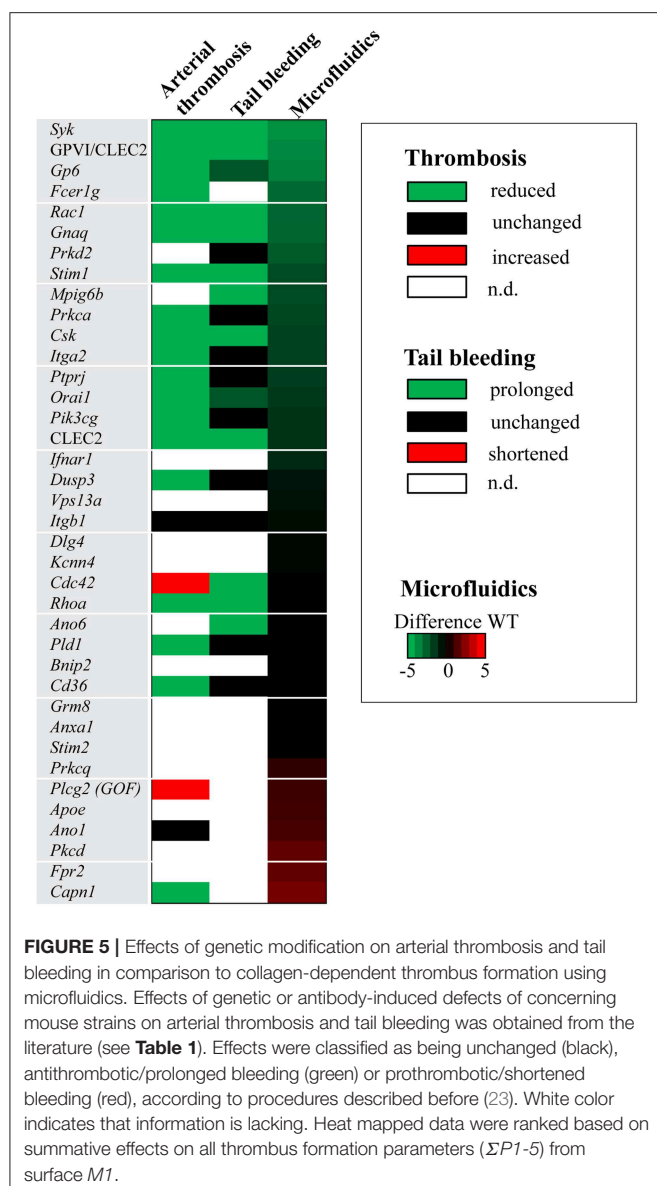
FIGURE 4 | Multi-parameter comparison of thrombus formation on three microspots for 11 mouse strains with genetic deficiencies. Whole blood from mice with indicated genetic defects or corresponding wild-types was perfused over three microspots (*M1-3*), as for **Figure 3**. Brightfield images (*P1-5*) and fluorescence images (*P6*, AF647-annexin A5; *P7*, FITC anti-CD62P Ab; *P8*, PE-JON/A Ab) were analyzed for each microspot. Per microspot, parameter values were univariate scaled (0-10) across all mouse strains. **(A)** Subtraction heatmap of differences between deficient and wild-type strains, filtered for changes outside the range of (composite) mean $\pm$ SD, to select relevant changes. Genes were ranked based on effects on thrombus signature ($\Sigma P2-5$) across microspots *M1-3*, as shown in black bars. Colors represent unchanged (black), decreased (green) or increased (red) parameters. **(B)** Subtraction heatmap with ranking based on gene effects on platelet adhesion (*P1*), as indicated in right black bars.

signaling and aggregation (count in gene set: 37 of 242; false discovery rate: $3.25e^{-39}$), hemostasis (count in gene set: 44 of 489; false discovery rate: $1.5e^{-38}$), signal transduction (count in gene set: 68 of 2430; false discovery rate: $2.14e^{-32}$), GPVI-mediated activation cascade (count in gene set: 18 of 34; false discovery rate: $2.07e^{-26}$) and G alpha (12/13) signaling events (count in gene set: 18 of 68; false discovery rate: $2.64e^{-22}$). The 37 core nodes were color- and size-coded, based on the established gene effects on thrombus signature *M1PΣ(2-5)*, and then indicated three typical clusters of genes/proteins with large size effects (**Figure 6**): (i) *Gp6* with associated receptors *Fcgr1g* and kinase *Syk* (cluster score: 7; #nodes: 19; #edges: 63); (ii) the low-molecular weight GTPases *Rac1* and *Cdc42* together with *Itga2* and *Itgb1* (cluster score: 5.24; #nodes: 22; #edges: 55); and (iii) Ca^{2+} -regulating signaling components, *Stim1*, *Stim2* and *Orail* (cluster score: 4; #nodes: 4; #edges: 6). Out of the 80 novel nodes, 12 genes have been previously shown to modify *in vivo* arterial thrombosis and/or bleeding (*Cblb*, *Ctnn*, *Gnai2*, *Gria1*, *Itga6*, *Lat*, *Lcp2*, *Ldlr*, *Pik3cb*, *Pik3r1*, *Rock2*, *Vav1*) (23). Color-coding of the same network nodes for gene effects on platelet adhesion revealed as most notable changes the above-mentioned gain-of-function effects of *Ano1*, *Kcnn4* and *Capn1* (**Supplementary Figure 4**).

DISCUSSION

In this paper, we applied standardized analysis procedures in order to allow detailed and quantitative comparison of the changes in platelet functions in multiple genetically modified mice, using microfluidic methods of collagen-dependent thrombus formation under shear conditions. For this purpose, we re-analyzed sets of microscopic brightfield and fluorescence images, using defined semi-automated scripts, resulting in quantitative parameters of platelet adhesion, platelet aggregation (defined as thrombus signature) (26), and platelet activation. The underlying rationale was that earlier captured images provide more relevant information than only a platelet surface area coverage, and thus can provide additional insight into the complex process of thrombus formation. For a subset of mice, it was also possible to extend this complex phenotyping of thrombus formation to other, non-collagen surfaces with additional parameters of platelet activation.

Recent work has shown that multiple platelet function assessment by whole blood microfluidic assays provides novel insights, for instance into the changes in human platelets linked to normal genetic variation (26, 62) and in mouse



platelets due to co-activation by interacting chemokines (63). The present comparative quantitative analysis of changes in thrombus formation in multiple genetically or antibody-induced modified mice now also allows to evaluate the assay outcomes for changes in specific platelet functions. Here, we could identify a partial distinction between genes/protein affecting platelet adhesion and those altering platelet aggregation properties (collectively termed as thrombus signature). For instance, it seems that the murine *Csk*, *Mpig6b* and *Ptprj* have a relatively large role in flow-dependent platelet adhesion. Markedly, for *Csk*, *Mpig6b*, and *Ptprj* this effect is extended to also a reduced adhesion at the non-collagen surface *M2*. On the other hand, the processes of platelet adhesion and aggregate formation are also related, as shown by an overall consistent correlation between adhesion and aggregation parameters (Kendall's tau-b = 0.42-0.68). A similar conclusion was drawn earlier from the analysis of

thrombus formation in blood samples from 94 healthy subjects, also pointing to the existence of a subject-dependent thrombus signature (26).

In comparison to the collagen surface (*M1*), for the 11 mouse strains analyzed, we observed in general smaller gene effects at the two other surfaces (*M2-3*), which mediate platelet adhesion via GPIb-V-IX and $\alpha_6\beta_1$ with or without CLEC2 (rhodocytin). Accordingly, it seems that, at least in part, distinct sets of genes/proteins are implicated in the platelet adhesion to non-collagen surfaces than to the collagen surface. Clearly *in vivo* thrombus formation is not purely mediated by collagen, but rather is the result of platelet interactions with a mixture of different extracellular matrix proteins.

The present data set also includes new findings with unpublished blood samples from mice deficient in *Rhoa* and *Syk*. The *Rhoa* defect did not appear to influence platelet adhesion nor thrombus signature parameters at the shear rate of 1000 s^{-1} , which is in support of the earlier conclusion that platelet RHO-A becomes relevant for thrombus formation at high (pathological) shear rates (13). In contrast, genetic deficiency in *Syk* resulted in a strong reduction of all platelet and thrombus parameters on collagen. This highlights the importance of SYK signaling in collagen-dependent thrombus formation.

Given the earlier established correlation between the outcome of microfluidic tests (in terms of platelet surface area coverage) and experimental arterial thrombosis in mice (23), it was of interest to evaluate for the current mouse strains how a more detailed analysis (considering more thrombus parameters) contributes to this relationship. Such a comparison clearly has limitations, such as the wide variety of methods and test outcomes of the *in vivo* thrombosis measurements, making a clear differentiation between moderate and strong phenotypes difficult (23); and furthermore, the absence of coagulation and vessel wall components other than collagen in the *in vitro* approach. Nevertheless, a ranking of the investigated mouse strains according to overall changes in thrombus parameters (including platelet adhesion and aggregation) showed a good reflection with published changes in arterial thrombosis *in vivo*. On the other hand, for a considerable set of genes, a changed thrombus formation *in vitro* (and mostly *in vivo*) was not associated with an altered bleeding time. In several cases, a discrepancy is well explainable. For instance, defects in platelet *Clec1b* (as a non-collagen receptor) (64) or in *Ano6* (an isolated defect of platelet-dependent coagulation) (18), will not be picked up by flow assays over collagen with anticoagulated blood. A striking example is *Gp6*. Whereas, GPVI deficiency overall impairs thrombus formation *in vitro* as well as arterial thrombosis *in vivo*, mouse tail bleeding times are only moderately prolonged (37, 38). In line with that, mild bleeding symptoms have been reported in patients with a defect in the *GP6* gene (65, 66). Hence, this gene does likely have a restricted role, which is in line with the constructed network indicating multiple GPVI-linked proteins that contribute to both thrombosis and hemostasis.

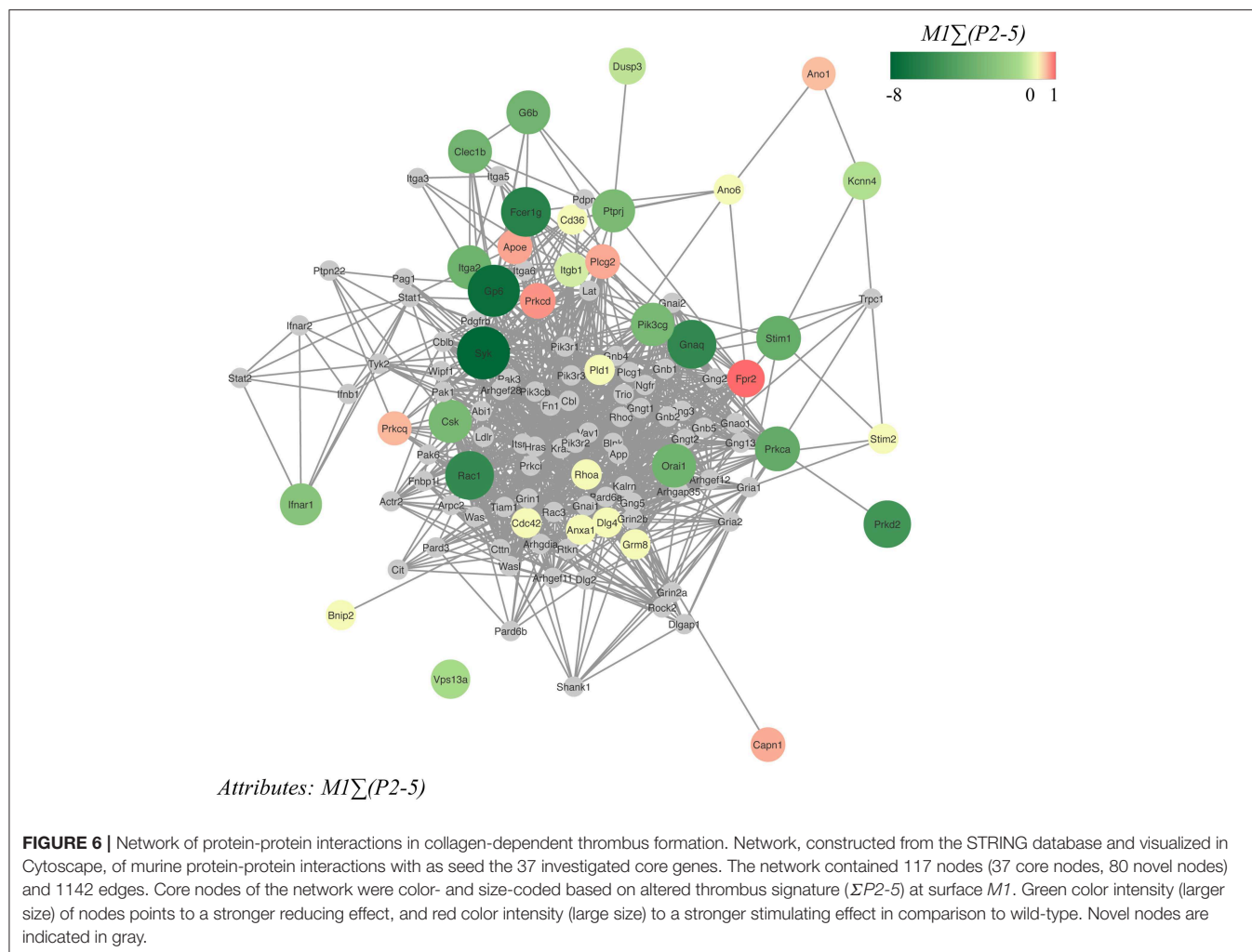


FIGURE 6 | Network of protein-protein interactions in collagen-dependent thrombus formation. Network, constructed from the STRING database and visualized in Cytoscape, of murine protein-protein interactions with as seed the 37 investigated core genes. The network contained 117 nodes (37 core nodes, 80 novel nodes) and 1142 edges. Core nodes of the network were color- and size-coded based on altered thrombus signature ($\Sigma P2-5$) at surface $M1$. Green color intensity (larger size) of nodes points to a stronger reducing effect, and red color intensity (large size) to a stronger stimulating effect in comparison to wild-type. Novel nodes are indicated in gray.

Translation of the current evaluation of genes in murine thrombus formation to human pathophysiology can—taking into account the above—provide better insight into the genetic background of human platelet function abnormalities and how these can relate to thrombotic and bleeding disorders. Indeed, for homologs of several of the genes analyzed in this paper, e.g., human *ORAI1* and *STIM1*, mutations have been identified that link to defective collagen-dependent thrombus formation *in vitro* (67). By applying an extended image analysis, a detailed description of the formed thrombi can be generated. This can aid in the identification of the defective part (platelet adhesion, aggregation or procoagulant response) of the process of thrombus formation in patients with an unexplained increased risk for either thrombosis or bleeding. Extended analysis of the available mouse data and summation in the form of a network can also contribute to a more targeted approach to select novel candidate genes and proteins, possibly affecting platelet functions in a positive or negative way.

Moreover, by applying new knowledge and techniques, in the present paper, new insights were gained from previously performed mouse experiments. In such a manner, our study may contribute to the 3R approaches by reducing, refining and replacing animal experiments.

Taken together, we conclude that the presently developed multi-parameter analysis of thrombus formation on microspots using microfluidics can be used to: (i) determine the severity of platelet abnormalities; (ii) distinguish between altered platelet adhesion, aggregation and activation; and (iii) elucidate both collagen and non-collagen dependent platelet changes. This approach may thereby aid in the better understanding and better assessment of the changes in platelets that affect arterial thrombosis and hemostasis.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

For original data, experiments were approved by the district government of Lower Franconia (Bezirksregierung Unterfranken) and by the Animal Experimental Committee in

Hinxton/Cambridge. For previously published data, experiments were approved by the local Animal Experimental Committees as indicated in the original publications (see references in Table 1).

AUTHOR CONTRIBUTIONS

MN and JvG analyzed and interpreted the data and wrote the manuscript. SdW and MK analyzed the data. RV created scripts used for image analysis. DA, AB, ME, KK, CO, JP, IP, AP, JM, MG, YS, and CW provided the mice, other tools, and revised the manuscript. DS and BN provided the mice, original published and unpublished images, and revised the manuscript. JH and CB provided expert supervision, analyzed and interpreted data, and wrote the manuscript.

REFERENCES

- Ruggeri ZM, Mendolicchio GL. Adhesion mechanisms in platelet function. *Circ Res.* (2007) 100:1673–85. doi: 10.1161/01.RES.0000267878.97021.ab
- Stegner D, Nieswandt B. Platelet receptor signaling in thrombus formation. *J Mol Med.* (2011) 89:109–21. doi: 10.1007/s00109-010-0691-5
- Versteeg HH, Heemskerk JW, Levi M, Reitsma PH. New fundamentals in hemostasis. *Physiol Rev.* (2013) 93:327–58. doi: 10.1152/physrev.00016.2011
- Van der Meijden PE, Heemskerk JW. Platelet biology and functions: new concepts and future clinical perspectives *Nat. Rev Cardiol.* (2019) 16:166–79. doi: 10.1182/blood-2008-07-171066
- Nieswandt B, Brakebusch C, Bergmeier W, Schulte V, Bouvard D, Mokhtari-Nejad R, et al. Glycoprotein VI but not $\alpha_2\beta_1$ integrin is essential for platelet interaction with collagen. *EMBO J.* (2001) 20:2120–30. doi: 10.1093/emboj/20.9.2120
- Siljander PR, Munnix IC, Smethurst PA, Deckmyn H, Lindhout T, Ouwehand WH, et al. Platelet receptor interplay regulates collagen-induced thrombus formation in flowing human blood. *Blood.* (2004) 103:1333–41. doi: 10.1182/blood-2003-03-0889
- Auger JM, Kuijpers MJ, Senis YA, Watson SP, Heemskerk JW. Adhesion of human and mouse platelets to collagen under shear: a unifying model. *FASEB J.* (2005) 19:825–7. doi: 10.1096/fj.04-1940fje
- Varga-Szabo D, Pleines I, Nieswandt B. Cell adhesion mechanisms in platelets. *Arterioscler Thromb Vasc Biol.* (2008) 28:403–12. doi: 10.1161/ATVBAHA.107.150474
- Senis YA, Mazharian A, Mori J. Src family kinases: at the forefront of platelet activation. *Blood.* (2014) 124:2013–24. doi: 10.1182/blood-2014-01-453134
- Mammadova-Bach E, Nagy M, Heemskerk JW, Nieswandt N, Braun A. Store-operated calcium entry in blood cells in thrombo-inflammation. *Cell Calcium.* (2019) 77:39–48. doi: 10.1016/j.ceca.2018.11.005
- Harper MT, Poole AW. Diverse functions of protein kinase C isoforms in platelet activation and thrombus formation. *J Thromb Haemost.* (2010) 8:454–62. doi: 10.1111/j.1538-7836.2009.03722.x
- Guidetti GF, Canobbio I, Torti M. PI3K/Akt in platelet integrin signaling and implications in thrombosis. *Adv Biol Regul.* (2015) 59:36–52. doi: 10.1016/j.jbior.2015.06.001
- Pleines I, Hagedorn I, Gupta S, May F, Chakarova L, van Hengel J, et al. Megakaryocyte-specific RhoA deficiency causes macrothrombocytopenia and defective platelet activation in hemostasis and thrombosis. *Blood.* (2012) 119:1054–63. doi: 10.1182/blood-2011-08-372193
- Elvers M, Stegner D, Hagedorn I, Kleinschnitz C, Braun A, Kuijpers ME, et al. Impaired $\alpha_{IIb}\beta_3$ integrin activation and shear-dependent thrombus formation in mice lacking phospholipase D1. *Sci. Signal.* (2010) 3:ra1. doi: 10.1126/scisignal.2000551

FUNDING

MN, MK, and JH have received funding from the Cardiovascular Centre (HVC) MUMC⁺ Maastricht, the Interreg V Euregio Meuse-Rhine program (Poly-Valve). DS, AB, IP, and BN were supported by the Deutsche Forschungsgemeinschaft (project-number 374031971 – TRR240). CO is Senior Research Associate at the Belgian Fund for Scientific Research (F.R.S.-FNRS). YS received funding from a BHF Fellowship (FS/13/1/29894 and a Programme Grant RG/15/13/31673). CB is supported by the Alexander von Humboldt Foundation.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcvm.2019.00099/full#supplementary-material>

- Coxon CH, Geer MJ, Senis YA. ITIM receptors: more than just inhibitors of platelet activation. *Blood.* (2017) 129:3407–18. doi: 10.1182/blood-2016-12-720185
- Kunzelmann K, Nilius B, Owsianik G, Schreiber R, Ousingsawat J, Sirianant L, et al. Molecular functions of anoctamin 6 (TMEM16F): a chloride channel, cation channel, or phospholipid scramblase? *Pflugers Arch.* (2014) 466:407–14. doi: 10.1007/s00424-013-1305-1
- De Witt SM, Verdoold R, Cosemans JM, Heemskerk JW. Insights into platelet-based control of coagulation. *Thromb Res.* (2014) 133:S139–S148. doi: 10.1016/s0049-384850024-2
- Mattheij NJ, Braun A, van Kruchten R, Castoldi E, Pircher J, Baaten CC, et al. Survival protein anoctamin-6 controls multiple platelet responses including phospholipid scrambling, swelling, and protein cleavage. *FASEB J.* (2016) 30:727–37. doi: 10.1096/fj.15-280446
- Agbani EO, Poole AW. Procoagulant platelets: generation, function, and therapeutic targeting in thrombosis. *Blood.* (2017) 130:2171–9. doi: 10.1182/blood-2017-05-787259
- May F, Hagedorn I, Pleines I, Bender M, Vögtle T, Eble J, et al. CLEC-2 is an essential platelet-activating receptor in hemostasis and thrombosis. *Blood.* (2009) 114:3464–72. doi: 10.1182/blood-2009-05-222273
- Watson SP, Herbert JM, Pollitt AY. GPVI and CLEC-2 in hemostasis and vascular integrity. *J Thromb Haemost.* (2010) 8:1457–67. doi: 10.1111/j.1538-7836.2010.03875.x
- Schaff M, Tang C, Maurer E, Bourdon C, Receveur N, Eckly A, et al. Integrin $\alpha_6\beta_1$ is the main receptor for vascular laminins and plays a role in platelet adhesion, activation, and arterial thrombosis. *Circulation.* (2013) 128:541–52. doi: 10.1161/circulationaha.112.000799
- Baaten CC, Meacham S, de Witt SM, Feijge MA, Adams DJ, Akkerman JW, et al. A synthesis approach of mouse studies to identify genes and proteins in arterial thrombosis and bleeding. *Blood.* (2018) 132:e35–46. doi: 10.1182/blood-2018-02-831982
- De Witt SM, Swieringa F, Cavill R, Lamers MM, van Kruchten R, Mastenbroek T, et al. Identification of platelet function defects by multi-parameter assessment of thrombus formation. *Nat Commun.* (2014) 5:4257. doi: 10.1038/ncomms5257
- Brouns S, van Geffen JP, Heemskerk JW. High throughput measurement of platelet aggregation under flow. *Platelets.* (2018) 29:662–9. doi: 10.1080/09537104.2018.1447660
- Van Geffen JP, Brouns S, Batista J, McKinney H, Kempster CK, Sivapalaratnam S, et al. High-throughput elucidation of thrombus formation reveals sources of platelet function variability. *Haematologica.* (2019) 104:1256–67. doi: 10.3324/haematol.2018.198853

27. Kuchay SM, Kim N, Grunz EA, Fay WP, Chishti AH. Double knockouts reveal that protein tyrosine phosphatase 1 β is a physiological target of calpain-1 in platelets. *Mol Cell Biol.* (2007) 27:6038–52. doi: 10.1128/MCB.00522-07
28. Mattheij NJ, Gilio K, van Kruchten R, Jobe SM, Wieschhaus AJ, Chishti AH, et al. Dual mechanism of integrin $\alpha_{IIb}\beta_3$ closure in procoagulant platelets. *J Biol Chem.* (2013) 288:13325–13336. doi: 10.1074/jbc.M112.428359
29. Kuijpers MJ, de Witt S, Nergiz-Unal R, van Kruchten R, Korporeal SJ, Verhamme P, et al. Supporting roles of platelet thrombospondin-1 and CD36 in thrombus formation on collagen. *Arterioscler Thromb Vasc Biol.* (2014) 34:1187–92. doi: 10.1161/atvbaha.113.302917
30. Podrez EA, Byzova TV, Febbraio M, Salomon RG, Ma Y, Valiyaveetil M, et al. Platelet CD36 links hyperlipidemia, oxidant stress and a prothrombotic phenotype. *Nat Med.* (2007) 13:1086–95. doi: 10.1038/nm1626
31. Pleines I, Eckly A, Elvers M, Hagedorn I, Eliaoutou S, Bender M, et al. Multiple alterations of platelet functions demonstrated by increased secretion in mice lacking Cdc42 in platelets. *Blood.* (2010) 115:3364–73. doi: 10.1182/blood-2009-09-242271
32. Mori J, Nagy Z, Di Nunzio G, Smith CW, Geer MJ, Al Ghaithi R, et al. Maintenance of murine platelet homeostasis by the kinase Csk and phosphatase CD148. *Blood.* (2018) 131:1122–44. doi: 10.1182/blood-2017-02-768077
33. Musumeci L, Kuijpers MJ, Gilio K, Hego A, Theatre E, Maurissen L, et al. Dual-specificity phosphatase 3 deficiency or inhibition limits platelet activation and arterial thrombosis. *Circulation.* (2015) 131:656–68. doi: 10.1161/circulationaha.114.010186
34. Kuijpers MJ, Schulte V, Bergmeier W, Lindhout T, Brakebusch C, Offermanns S, et al. Complementary roles of glycoprotein VI and $\alpha_2\beta_1$ integrin in collagen-induced thrombus formation in flowing whole blood *ex vivo*. *Faseb J.* (2003) 17:685–7. doi: 10.1096/fj.02-0381fje
35. Munnix IC, Strehl A, Kuijpers MJ, Auger JM, van der Meijden PE, van Zandvoort MA, et al. The glycoprotein VI-phospholipase C γ_2 signaling pathway controls thrombus formation induced by collagen and tissue factor *in vitro* and *in vivo*. *Arterioscler Thromb Vasc Biol.* (2005) 25:2673–8. doi: 10.1161/01.ATV.0000193568.71980.4a
36. Offermanns S, Toombs CF, Hu YH, Simon MI. Defective platelet-activation in Gq α -deficient mice. *Nature.* (1997) 389:183–6. doi: 10.1038/38284
37. Nieswandt B, Schulte V, Bergmeier W, Mokhtari-Nejad R, Rackebandt K, Cazenave JP, et al. Long-term antithrombotic protection by *in vivo* depletion of platelet glycoprotein VI in mice. *J Exp Med.* (2001) 193:459–69. doi: 10.1084/jem.193.4.459
38. Bender M, May F, Lorenz V, Thielmann I, Hagedorn I, Finney BA, et al. Combined *in vivo* depletion of glycoprotein VI and C-type lectin-like receptor 2 severely compromises hemostasis and abrogates arterial thrombosis in mice. *Arterioscler Thromb Vasc Biol.* (2013) 33:926–34. doi: 10.1161/ATVBAHA.112.300672
39. Kuijpers MJ, Pozgajova M, Cosemans JM, Munnix IC, Eckes B, Nieswandt B, et al. Role of murine integrin $\alpha_2\beta_1$ in thrombus stabilization and embolization: contribution of thromboxane A $_2$. *Thromb Haemost.* (2007) 98:1072–80. doi: 10.1160/TH07-02-0089
40. Grüner S, Prostedna M, Schulte V, Krieg T, Eckes B, Brakebusch C, et al. Multiple integrin-ligand interaction synergize in shear-resistant platelet adhesion at sites of arterial injury *in vivo*. *Blood.* (2003) 102:4021–7. doi: 10.1182/blood-2003-05-1391
41. Hofmann I, Geer MJ, Vögtle T, Crispin A, Campagna DR, Barr A, et al. Congenital macrothrombocytopenia with focal myelofibrosis due to mutations in human G6b-B is rescued in humanized mice. *Blood.* (2018) 132:1399–412. doi: 10.1182/blood-2017-08-802769
42. Gilio K, van Kruchten R, Braun A, Berna-Erro A, Feijge MA, Stegner D, et al. Roles of platelet STIM1 and Orai1 in glycoprotein VI- and thrombin-dependent procoagulant activity and thrombus formation. *J Biol Chem.* (2010) 285:23629–38. doi: 10.1074/jbc.M110.108696
43. Braun A, Varga-Szabo D, Kleinschnitz C, Pleines I, Bernder M, Austinat M, et al. Orai1 (CRACM1) is the platelet SOC channel and essential for pathological thrombus formation. *Blood.* (2009) 113:2056–63. doi: 10.1182/blood-2008-07-171611
44. Cosemans JM, Munnix IC, Wetzker R, Heller R, Jackson SP, Heemsker JW. Continuous signaling via PI3K isoforms β and γ is required for platelet ADP receptor function in dynamic thrombus stabilization. *Blood.* (2006) 108:3045–52. doi: 10.1182/blood-2006-03-006338
45. Hirsch E, Bosco O, Tropel P, Laffargue M, Calvez R, Altruda F, et al. Resistance to thromboembolism in PI3K γ -deficient mice. *FASEB J.* (2001) 15:2019–21. doi: 10.1096/fj.00-0810fje
46. Elvers M, Pozgai R, Varga-Szabo D, May F, Pleines I, Kuijpers MJ, et al. Platelet hyperactivity and a prothrombotic phenotype in mice with a gain-of-function mutation in phospholipase C γ_2 . *J Thromb Haemost.* (2010) 8:1353–63. doi: 10.1111/j.1538-7836.2010.03838.x
47. Gilio K, Harper MT, Cosemans JM, Konopatskaya O, Munnix IC, Prinzen L, et al. Functional divergence of platelet protein kinase C (PKC) isoforms in thrombus formation on collagen. *J Biol Chem.* (2010) 285:23410–9. doi: 10.1074/jbc.M110.136176
48. Konopatskaya O, Gilio K, Harper MT, Zhao Y, Cosemans JM, Karim AZ, et al. PKC α regulates platelet granule secretion and thrombus formation in mice. *J Clin Invest.* (2009) 119:399–407. doi: 10.1172/JCI34665
49. Chari R, Getz T, Nagy BJ, Bhavaraju K, Mao Y, Bynagari YS, et al. Protein kinase C δ differentially regulates platelet functional responses. *Arterioscler Thromb Vasc Biol.* (2009) 29:699–705. doi: 10.1161/atvbaha.109.184010
50. Konopatskaya O, Matthews SA, Harper MT, Gilio K, Cosemans JM, Williams CM, et al. Protein kinase C mediates platelet secretion and thrombus formation through protein kinase δ . *Blood.* (2011) 118:416–24. doi: 10.1182/blood-2010-10-312199
51. Pleines I, Elvers M, Strehl A, Pozgajova M, Varga-Szabo D, May F, et al. Rac1 is essential for phospholipase C- γ_2 activation in platelets. *Pflugers Arch.* (2009) 457:1173–85. doi: 10.1007/s00424-008-0573-7
52. Varga-Szabo D, Braun A, Kleinschnitz C, Bender M, Pleines I, Pham M, et al. The calcium sensor STIM1 is an essential mediator of arterial thrombosis and ischemic brain infarction. *J Exp Med.* (2008) 205:1583–91. doi: 10.1084/jem.20080302
53. Hughes CE, Finney BA, Koentgen F, Lowe KL, Watson SP. The N-terminal SH2 domain of Syk is required for (hem)ITAM, but not integrin, signaling in mouse platelets. *Blood.* (2015) 125:144–54. doi: 10.1182/blood-2014-05-579375
54. Suzuki-Inoue K, Kato Y, Kaneko MK, Mishima K, Yatomi Y, Yamazaki Y, et al. Involvement of the snake toxin receptor CLEC-2, in podoplanin-mediated platelet activation, by cancer cells. *J Biol Chem.* (2007) 282:25993–6001. doi: 10.1074/jbc.M702327200
55. Hooley E, Papagrigoriou E, Navdaev A, Pandey AV, Clemetson JM, Clemetson KJ, et al. The crystal structure of the platelet activator aggregin reveals a novel (alpha β) $_2$ dimeric structure. *Biochemistry.* (2008) 47:7831–7. doi: 10.1021/bi800528t
56. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods.* (2012) 9:676–82. doi: 10.1038/nmeth.2019
57. Szklarczyk D, Franceschini A, Wyder S, Forslund K, Heller D, Huerta-Cepas J, et al. STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic Acid Res.* (2015) 43:D447–D452. doi: 10.1093/nar/gku1003
58. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: a software environment for integrated models of biomolecular interaction network. *Genome Res.* (2003) 13:2498–504. doi: 10.1101/gr.1239303
59. Bader GD, Hogue CW. An automated method for finding molecular complexes in large protein interaction networks. *BMC Bioinform.* (2003) 4:2. doi: 10.1186/1471-2105-4-2
60. Miryala SK, Anbarasu A, Ramaiah S. Discerning molecular interactions: a comprehensive review on biomolecular interaction databases and network analysis tools. *Gene.* (2018) 642:84–94. doi: 10.1016/j.gene.2017.11.028
61. Team RC. R: A Language and Environment for Statistical Computing. Vienna: Foundation for statistical computing. Available online at: <https://www.R-project.org> (accessed 2019 2018)
62. Petersen R, Lambourne JJ, Javierre BM, Grassi L, Kreuzhuber R, Ruklisa D, et al. Platelet function is modified by common sequence variation in megakaryocyte super enhancers. *Nat Commun.* (2017) 8:16058. doi: 10.1038/ncomms16058

63. Von Hundelshausen P, Agten SM, Eckardt V, Blanchet X, Schmitt MM, Ippel H, et al. Chemokine interactome mapping enables tailored intervention in acute and chronic inflammation. *Sci. Transl. Med.* (2017) 9:eah6650. doi: 10.1126/scitranslmed.aah6650
64. Suzuki-Inoue K, Inoue O, Ozaki Y. Novel platelet activation receptor CLEC-2: from discovery to prospects. *J. Thromb. Haemost.* (2011) 9 :44–55. doi: 10.1111/j.1538-7836.2011.04335.x
65. Matus V, Valenzuela G, Sáez CG, Hidalgo P, Lagos M, Aranda E, et al. An adenine insertion in exon 6 of human GP6 generates a truncated protein associated with a bleeding disorder in four Chilean families. *J Thromb Haemost.* (2013) 11:1751–9. doi: 10.1111/jth.12334
66. Dumont B, Lasne D, Rothschild C, Bouabdelli M, Ollivier V, Oudin C, et al. Absence of collagen-induced platelet activation caused by compound heterozygous GPVI mutations. *Blood.* (2009) 114:1900–3. doi: 10.1182/blood-2009-03-213504
67. Nagy M, Mastenbroek TG, Mattheij NJ, De Witt S, Clemetson KJ, Kirschner J, et al. Variable impairment of platelet functions in patients with severe,

genetically linked immune deficiencies. *Haematologica.* (2018) 103:540–9. doi: 10.3324/haematol.2017.176974

Conflict of Interest Statement: JH is a co-founder and shareholder of FlowChamber.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Copyright © 2019 Nagy, van Geffen, Stegner, Adams, Braun, de Witt, Elvers, Geer, Kuijpers, Kunzelmann, Mori, Oury, Pircher, Pleines, Poole, Senis, Verdoold, Weber, Nieswandt, Heemskerk and Baaten. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Platelet Function in Aging

Jessica Le Blanc^{1,2} and Marie Lordkipanidzé^{1,2*}

¹ Research Center, Montreal Heart Institute, Montreal, QC, Canada, ² Faculty of Pharmacy, Université de Montréal, Montreal, QC, Canada

Aging is associated with an increased incidence of cardiovascular disease and thrombosis. Platelets play a major role in maintaining hemostasis and in thrombus formation, making them a key player in thrombotic disorders. Whereas it is well-known that platelet aggregability is increased in vascular diseases, the contribution of age-related changes in platelet biology to cardiovascular risk is not well-understood. Several lines of evidence support that platelets from older subjects differ in their function and structure, making platelets more prone to activation and less sensitive to inhibition. These age-related changes could lead to platelet hyperactivity and to the development of a prothrombotic state in advanced age. This review will focus on platelet biochemical modifications during aging and on the mechanisms by which these alterations could lead to thrombotic disease.

Keywords: platelet function, aging, senescence, elderly, cardiovascular disease, thrombosis

OPEN ACCESS

Edited by:

Christian Schulz,
Ludwig Maximilian University of
Munich, Germany

Reviewed by:

John Hwa,
School of Medicine, Yale University,
United States
Andres Trostchansky,
University of the Republic, Uruguay

*Correspondence:

Marie Lordkipanidzé
marie.lordkipanidze@umontreal.ca

Specialty section:

This article was submitted to
Atherosclerosis and Vascular
Medicine,
a section of the journal
Frontiers in Cardiovascular Medicine

Received: 30 May 2019

Accepted: 22 July 2019

Published: 07 August 2019

Citation:

Le Blanc J and Lordkipanidzé M
(2019) Platelet Function in Aging.
Front. Cardiovasc. Med. 6:109.
doi: 10.3389/fcvm.2019.00109

INTRODUCTION

Aging is accompanied by many biological changes at molecular and cellular levels. Senescence, the most striking cellular alteration in aging, is a hyporeplicative state implicated in age-related diseases (1). Senescent cells adopt a specific secretion signature rich in pro-inflammatory cytokines called the senescence-associated secretory phenotype (SASP) (1). It has been suggested that SASP induces changes in the functions of neighboring cells (2, 3), and some authors hypothesize that it might potentially contribute to increased susceptibility to thrombosis in elders (4).

Cardiovascular disease is the first cause of death worldwide and its incidence is strongly correlated with age (5). In both normal and pathological aging, platelets show increased aggregability (6–11) which is however more pronounced in a pathological state (12, 13). Several lines of evidence suggest that platelet hyperactivity observed in advanced age could be responsible for vascular and thrombotic disease development (14–17). However, molecular mechanisms of platelet hyperactivity in aging are only partially understood. A deeper understanding of the biological mechanisms underlying the relationship between age-related changes in platelet biology and cardiovascular disease could help improve treatment and preventive strategies. This review explores biochemical changes in platelets during aging.

AGE-ASSOCIATED DECREASE IN PLATELET COUNT

Platelet count is inversely associated with age. A large study based on the Third National Health and Nutrition Examination Survey including 12,142 American subjects showed a significant decrease of 10×10^3 platelets/ μL in individuals in the 60–69 year age group as compared with those between the ages of 20–59 years, and of 20×10^3 platelets/ μL in patients aged over 69 years of age, after adjusting for many covariates such as nutritional deficiencies, medication, inflammatory conditions, autoimmune or viral illnesses and consumption

of alcohol and tobacco (18). This suggests that the drop in platelet count with age is part of the biological aging process *per se* and not only due to environmental factors.

Another large study on 33,258 subjects in France examined full blood count normal reference values by age and sex. It showed the same tendency of platelet count being lower in older adults vs. their younger counterparts (19). Many studies showed that older women presented with higher platelet counts than men of the same age (18–20). A tendency of a lower platelet count in elderly people compared with young subjects was observed in two other small studies, but was not considered statistically significant (21, 22). Despite evidence of an inverse correlation between aging and platelet count, the cause of the decline in platelet count and the physiological consequences of this phenomenon in older subjects remain to be elucidated. Two hypotheses have been put forward in an attempt to rationalize this observation: (i) older individuals have a lower stem cell reserve compared to younger subjects; and (ii) a reduced platelet count confers a biological advantage, so the individual with this characteristic may have a better chance to reach an older age (18).

Interestingly, platelet count has been associated with mortality among elders (23–25). Van der Bom et al. reported that platelet count below 100 or above $400 \times 10^9 \text{ L}^{-1}$ is associated with increased non-cardiovascular mortality such as cancer in a cohort of 5,766 American and African-American patients of 65 years and older (23). Another group has come to a similar conclusion, i.e., they found a U-shaped all-cause mortality curve associated with platelet count in 131, 308 Chinese patients of the same age (24). A third group confirmed the association across all ethnic groups (25). However, they found that the association between mortality and thrombocytosis or thrombocytopenia was stronger among non-Hispanic Whites compared with African-Americans or Hispanics, highlighting the effect of genetic background in age-related changes in platelet count.

Despite the decrease in platelet count during aging, reference ranges are rarely age-adjusted, leading to misdiagnosis of thrombocytopenia in the elderly. A study showed that a reference range adjusted for age and sex is more strongly predictive of all-cause mortality than the unadjusted platelet count reference range (26). This led the authors to propose that age-adjusted reference ranges might be useful in clinical practice to better identify thrombocytopenic patients with a truly increased risk of mortality (26).

ENHANCED PLATELET ACTIVITY IN THE ELDERLY

One of the most documented changes in platelet function during aging is platelet hyperactivity. Bleeding time decreases significantly in aging, denoting a faster clot formation and indirectly an enhanced platelet activity in the elderly (27, 28). Furthermore, platelets from older men and women have a greater sensitivity to aggregation induced by classical agonists. Platelets aggregation of older subjects occurs at a lower concentration threshold of ADP (8–11, 22, 29), epinephrine (6, 7, 10, 11), collagen (22) and arachidonic acid (10) than platelets from

younger subjects. Meade et al. reported an increase in platelet aggregability of ~8% per decade of age, calculated by the EC₅₀ of ADP in a cohort of 958 participants of all ages (8). At all ages, aggregability was found to be more pronounced in women than in men (8, 10). A recent study suggests that age-related changes in platelet behavior on von Willebrand factor are more pronounced in women than in men (30), also supporting the concept that the aging process could affect platelet function differently in both sexes.

Furthermore, β -thromboglobulin and platelet factor 4 (PF4), two proteins secreted from platelets α -granules, are both found at a significantly higher level in plasma of older compared with younger subjects (29, 31). This is consistent with the hyperaggregability observed in elderly individuals since platelets release their granule content during activation. Interestingly, PF4 has also been found to have a procoagulant effect (32), showing that the age-related prothrombotic state is probably due to a number of biological changes in the thrombotic pathway, not only occurring in platelets themselves.

The mechanisms of this age-related platelet hyperactivity remain unclear. Bastyr et al. have tested the hypothesis that modifications in phosphoinositide turnover, an important signaling mechanism of platelet activation, may be responsible for platelet hyperactivity in aging (29). They have found that platelet phosphoinositide turnover is enhanced in aging and correlates positively with platelet aggregation and plasma β -thromboglobulin levels.

It has also been suggested that there could be a functional or expressional change in platelet α and β -adrenoreceptors (33), however the reported literature is conflicting. These receptors, respectively, enhanced or inhibited platelet aggregation induced by epinephrine and noradrenaline by decreasing or increasing platelet cAMP levels. Yokoyama et al. observed an increase in α -adrenoreceptor binding capacity in platelets of elderly people without change in binding affinity (7). However, two other groups have observed the opposite, i.e., a decrease in α -adrenoreceptor binding capacity (6, 34). Finally, another study suggested a decrease of β -adrenoreceptor affinity but an unchanged binding capacity in older subjects (35).

CHANGES IN THE PLATELET-SEROTONIN SYSTEM IN ADVANCED AGE

Serotonin is a vasoactive molecule stored in platelet granules and released in plasma during platelet activation. Serotonin is also known to induce platelet shape change and boost epinephrine- and ADP-induced aggregation (33, 36). According to Gleerup et al., platelets from patients aged between 72 and 86 years old have a higher sensitivity and an increased responsiveness to serotonin than platelets from subjects 18 to 27 years of age (33). They propose that it can be an important contributing factor of increased platelet aggregability in the elderly.

On the other hand, it has been reported that platelet serotonin content decreases with age (36, 37). Diminished serotonin platelet content and an increased plasma serotonin level is also observed in patients with type 1 or type 2 diabetes and in

patients suffering from peripheral vascular diseases compared with correspondingly aged healthy volunteers (36). This leads to the suggestion that platelet hyperactivity can cause an increased release of serotonin in plasma which then feeds back to platelet hyperactivity and may thus play a role in the pathogenesis of atherothrombotic diseases.

RELATIONSHIP BETWEEN OXIDATIVE STRESS, AGING, AND PLATELET HYPERREACTIVITY

The human and animal aging process is accompanied by an increase in oxidative stress (38–40). Among biological systems modified by oxidative stress, the cardiovascular system is particularly sensitive to a misbalance in reactive oxygen species (ROS), which has been associated with an increased risk of cardiovascular events (38, 40). ROS also play a role in platelet activation by acting as a second messenger or by producing oxidized proteins important in platelet aggregation (38, 41), which could explain the greater propensity toward thrombosis in older individuals. Free radicals induce modifications of protein structures notably by carbonylation or cross-linking in many cell types. Those impaired proteins are therefore targeted by proteolytic enzyme as a mechanism of defense against oxidative damage. In aging, it is suggested that there is an accumulation of carbonylated proteins which could lead to the development of diseases such as diabetes, atherothrombosis and cancer. It has been observed that aging increases the rate of carbonylated proteins in platelets of rats (42), hamsters (42) and humans (42, 43), providing a biologically-plausible mechanism of platelet dysfunction. Platelet carbonyl content is even more sharply increased in type 2 diabetes when compared with healthy controls of the same age (42), further supporting the notion of ROS-induced platelet hyperactivity. Oxidative stress can also modify reactions involving disulfide bond formation, which can lead to activation or inactivation of a number of proteins implicated in different cellular pathways. For example, oxidative stress increases the activity of platelet membrane protein disulfide isomerase which activates several integrins by disulfide bond isomerization (44). The most important among these is the $\alpha_{IIb}\beta_3$ integrin, which undergoes partial reduction of disulfide bonds during activation (44–47). Disruption of this finely-tuned balance in $\alpha_{IIb}\beta_3$ integrin activation can thus lead to altered thrombotic susceptibility (47). This is another of many mechanisms by which oxidative stress could contribute to thrombus formation, reviewed by several authors (39, 40, 45, 48).

Nitric oxide (NO) exerts its antithrombotic effect by inducing vasodilation and by inhibiting platelet aggregation via the activation of platelet guanylate cyclase (GC) generating cGMP, which finally reduces intracellular free calcium levels (40, 49). However, NO can also contribute to oxidative stress through generation of oxidative species such as ONOO⁻, which can evolve into free radicals and cause cellular damage leading to several pathological mechanisms (50, 51). In order to investigate if age influences the platelet NO signaling system, Michimata et al. have measured GC activity in response to an exogenous NO

donor in platelets of individuals of different ages (52). They found a decrease of the enzyme activity in older men (mean age 51.5 years) compared with younger men (mean age 29.5 years). Thus, the efficiency of the NO/GC/cGMP platelet pathway regulating aggregation may be modified in aging. Similarly, two other groups have examined the variation of cGMP levels in platelets with age (50, 53). Both groups showed a significant inverse correlation between cGMP platelet levels and age, suggesting a decrease in the inhibitory signaling pathway, which is consistent with Michimata's et al. results. Surprisingly, an increase in platelet nitric oxide synthase (NOS) activity in aging has been described (50), which seems contradictory with the cGMP decrease. A similar increase in platelet NOS activity and decrease in cGMP level have also been observed in 24-month-old rats, showing that this phenomenon is consistent in different species (54). Many hypotheses have been postulated to explain this contradiction, notably the possibility that the excess of NO produces higher amounts of oxidative reactive species such as ONOO⁻ which could potentially inhibit GC by targeting its iron/sulfur cluster (50).

A recent study suggests that hydrogen peroxide, an oxidative molecule, plays a key role in the platelet hyperactivity observed in aged mice (55). They found that platelets from old mice produce higher levels of H₂O₂ when stimulated with thrombin *in vitro* than platelets from younger mice. Furthermore, when they used PEG-catalase to selectively degrade intraplatelet H₂O₂ in 4- and 18-month-old mice, the authors observed the complete reversal of the age-dependent platelet hyperactivation; i.e., platelet activity of young and old mice were reduced to the same level (marked by a similar percentage of fibrinogen binding and integrin $\alpha_{IIb}\beta_3$ activation). These findings are consistent with previous work showing that treatment with catalase inhibits platelet activation by collagen (56). The authors proposed that the increased platelet production of peroxide observed in aged mice results from enhanced NADPH oxidase/superoxide dismutase pathway. This was further supported by qPCR data showing increased NADPH oxidase regulatory subunit and superoxide dismutase-1 mRNA in platelets of 18-month-old mice. Together, these findings suggests a critical role of this oxidant molecule in platelet hyperactivity during aging.

Surprisingly, recent human and mice data suggest that beyond a certain age (~80 years for human, ~14 months for mice), intraplatelet antioxidant reserves are increased, and consequently lead to decreased reactive oxygen species platelet content and a reduction in platelet activity and apoptosis (57). The causes of this observation are still unknown, but it could be the consequences of a biological mechanism to counteract the increase of oxidative stress burden in aging.

AGE-RELATED ALTERATIONS IN VASCULAR PROSTAGLANDINS

Vascular homeostasis relies on a balance between prostacyclin, which induces vasodilation and inhibits platelet activation, and thromboxane A₂, which does the opposite (58). While thromboxane A₂ is principally synthesized and

released by platelets, prostacyclin is essentially produced by vascular endothelium.

Giani et al. have examined the sensitivity of platelets of young and old rats to exogenous prostacyclin *in vivo* (59). They found that platelets from 11-month aged rats were more sensitive to the inhibitory effect on aggregation of prostacyclin compared with 1-month old animals. They propose that this could be a natural mechanism to counteract the enhanced aggregability observed in older rats. On the other hand, Modesti et al. studied the prostacyclin receptor by performing binding assays. They found a significant decrease of expression of the prostacyclin receptor in platelets of healthy humans with age but no changes in binding affinity (60). Interestingly, a similar decrease in the number of platelet prostacyclin receptors has been observed in patients with spontaneous angina (61), suggesting that this age-related downregulation could be the beginning of an imbalance in vascular homeostasis leading eventually to a pathological phenotype.

In order to explore the effect of increasing age on thromboxane A₂ and prostacyclin production, Reilly et al. have studied the urinary excretion of 2,3-dinor-thromboxane B₂ and 2,3-dinor-6-keto-PGF1 α , metabolites of thromboxane A₂ and prostacyclin, respectively (27). They found that both metabolites were significantly higher in individuals aged over 50 years old than in younger individuals, suggesting that prostacyclin and thromboxane A₂ biosynthesis is increased with age. This observation has been confirmed later by a second group who found a positive correlation between the urinary excretion of 11-dehydro-thromboxane B₂, another stable metabolite of thromboxane A₂, and age in 833 patients with atrial fibrillation (62). Furthermore, in this study, patients with elevated 11-dehydro-thromboxane B₂ excretion experienced significantly more cardiovascular events during the 40 month follow-up.

CONTRIBUTION OF AGE-RELATED PLASMA MEMBRANE MODIFICATIONS TO PLATELET HYPERACTIVITY

The cell membrane composition is modified in the aging process, notably with a higher total amount of cholesterol, and those changes can affect diverse biochemical functions of the cell such as transcellular signalization or cell membrane ion transport (63–65). An increase in the lipid structural order, and consequently a decrease in membrane fluidity, has been shown in human lymphocyte membranes and in brain cells of mice and rats (63, 66). Cohen et al. were the first to observe similar membrane structural changes in human platelets during aging (67). Others have also seen a positive correlation between membrane structural order in human platelets, increasing age and serum cholesterol levels (67–69). An increase in lipid peroxide membrane composition, which promotes crosslink lipid-protein interactions in the membrane, has also been reported in platelets of aged rats and appears to contribute to decreased membrane fluidity (70). Interestingly, an increase in platelet membrane structural order *in vitro* is linked with a higher sensitivity to aggregation induced by ADP and epinephrine (71,

72) and a higher production of thromboxane A₂ (73, 74). This is consistent with the increased sensitivity to aggregating agents described above.

PLATELET TRANSCRIPTOME MODIFICATIONS IN AGING

The platelet transcriptome, which includes thousands of mRNAs, miRNAs, long non-coding RNAs and other types of transcripts, has long been considered static (75). However, recent advances have rather shown that the platelet transcriptome changes dynamically in response to inflammation, cancer and other pathological states (76). Age-related modifications in the platelet transcriptome have been suggested to govern platelet function changes in aging (77, 78). RNA-sequencing of platelets from young (aged < 45 years) and older individuals (aged > 64 years) revealed a total of 514 transcripts that are expressed differently in both groups (78). It is also suggested that the platelet proteome of children, adults and elders differs, but little is known about age-related changes in the expression of those proteins and their biological consequences (78, 79). Interestingly, it has been shown that the transcriptome of mouse cardiac cells varies in the presence of hypertension and in function of age, leading to variation in regulation of platelet signaling and proinflammatory mechanisms (80). This suggests that age-related changes in the transcriptome of other cell types can also modify platelet function indirectly, highlighting the complexity of transcriptome regulation in aging.

Campbell et al. have examined the changes in the platelet-monocyte relationship with increasing age (78). They observed that monocytes produce increased levels of interleukin-8 (IL-8) and monocyte chemotactic protein 1 (MCP-1) when co-incubated with platelets from older patients compared with platelets from younger patients. They suggest that granzyme A, a serine protease with increased intraplatelet expression in older patients, is responsible for excessive production of these two pro-inflammatory cytokines in monocytes. This highlights the cross-talk between platelets and immune cells as a possible mechanism by which alterations in the platelet transcriptome and proteome could contribute to inflammatory diseases in aging.

AGE-RELATED HORMONAL CHANGES ON PLATELET FUNCTION IN WOMEN

Although men are more susceptible to suffer from cardiovascular disease than women (81), this difference of morbidity between the sexes is reduced after the age of menopause. Despite this, hormone replacement therapy (as well as hormonal contraception) does not mitigate the increased cardiovascular risk in women and may even worsen it (82–84). A number of investigators have studied the role of estrogen level changes in blood on platelet function and the link with the normal aging process in women.

Platelets and megakaryocytes express both estrogen receptors ER α and ER β (ER β being predominant), on which estrogen can bind and modulate gene transcription of some proteins

and enzymes required in platelet function such as integrin β_3 , CD34, platelet-derived growth factor, and NOS (85). Estrogen is also known to inhibit platelet aggregation *in vitro* via the NO/GC/cGMP pathway (86, 87).

Animal studies have shown that decreased estrogen levels *per se* directly affect platelet function. According to Jayachandran et al. a surgically-induced menopause in pigs increases significantly the expression of estrogen receptors on platelets (88). In this model, they also observed an increased expression of heat shock proteins 70 and 90 in platelets, which act as ER chaperones to maintain their conformation until they bind to their ligand. An increase in NOS activity, eNOS expression and cGMP concentration have also been observed in ovariectomized pigs (88).

It has been suggested that the hormonal cycle in women of procreating age may influence platelet activity. Some authors have observed an increased platelet function during the luteal phase compared with the follicular phase and have found a negative correlation between progesterone levels and platelet reactivity (89–91), while others observed no significant differences in platelet aggregation responses during the menstrual cycle (92). Moreover, women taking oral contraception (which contains estrogen and progestogen) have greater platelet count and less spontaneous platelet aggregation than those not taking an anovulant (92). Thus, the effect of hormones on platelet reactivity is not clear.

Few well-controlled aging studies have considered the hormonal status of women as a covariate of platelet function, and therefore it is difficult to ascertain the contribution of hormonal changes on the increased thrombotic risk observed with aging (85). Indeed, when looking at platelet function in perimenopausal women, it may be difficult to dissociate hormonal factors that vary with age from the aging process in platelets themselves. To examine the effect of menopause on platelet function with minimal impact of age, Lundberg Slingsby et al. designed a study in which they formed two groups of women: late premenopausal women and recent postmenopausal women with a mean difference age between groups of only 4 years (93). They found that platelets from postmenopausal women have a greater propensity to aggregation by classical agonists (ADP, TRAP, collagen, epinephrine) than premenopausal women. Thus, the menopause-induced drop in estrogen levels may contribute to platelet hyperactivity in women undergoing the natural aging process. Further investigations are required to fully understand the influence of estrogen and the consequences of menopause on platelet function of women, and their interaction with aging.

AGING, PLATELET HYPERACTIVITY AND COEXISTING DISEASES

Most studies have reported direct associations between biochemical age-related changes in platelets and their hyperaggregability. However, causality is difficult to establish in observational studies, and inverse causality has also been suggested. Kurabayashi et al. have suggested that

the enhanced platelet activity observed in the elderly is caused by underlying atherosclerosis rather than by the aging process (94). They have examined shape change and peroxidase content of platelets from a small group of young and old subjects with or without atherosclerosis. They have observed no significant differences in these parameters between young and old healthy individuals. In elderly subjects with atherosclerosis on the other hand, platelets presented more frequently in an activated state (formation of pseudopods) and their peroxidase content was decreased. Although the small sample size and absence of longitudinal follow-up somewhat mitigates their conclusion, Kurabayashi et al. raise an important question by asking whether platelet hyperactivity in the elderly is caused by aging or by a coexisting onset of an atherosclerotic state. Adequately controlled longitudinal studies are thus required to answer this important question.

TABLE 1 | Summary of age-related changes in platelets.

	Change during aging	References
Platelet count	↓	(18–20)
PLATELET ACTIVITY		
Bleeding time	↓	(27, 28)
Platelet sensitivity to agonists	↑	(6–11, 22, 29)
β -thromboglobulin plasma level	↑	(29, 31)
PF4 plasma level	↑	(29, 31)
Platelet phosphoinositide turnover	↑	(29)
Platelet α -adrenoreceptor binding capacity	Controversial	(7) (↑), (6, 34) (↓)
Platelet β -adrenoreceptor affinity	↓	(35)
PLATELET SEROTONIN SYSTEM		
Platelet sensitivity to serotonin	↑	(33)
Platelet serotonin content	↓	(36, 37)
PLATELETS AND OXIDATIVE STRESS		
Rate of intraplatelet carbonylated proteins	↑	(42, 43)
Intraplatelet GC activity	↓	(52)
Intraplatelet cGMP levels	↓	(50, 53, 54)
Intraplatelet NOS activity	↑	(50, 54)
Intraplatelet production of H_2O_2	↑	(55)
PLATELETS AND PROSTAGLANDIN PATHWAYS		
Platelet sensitivity to prostacyclin	↑	(59)
Platelet expression of prostacyclin receptor	↓	(60)
Thromboxane A2 production	↑	(27, 62)
Prostacyclin production	↑	(27)
PLASMA MEMBRANE MODIFICATION		
Platelet membrane lipid structural order	↑	(67–69)
Platelet lipid peroxide membrane composition	↑	(70)

CONCLUSION

In conclusion, aging is associated with an increase in platelet activity and a higher rate of vascular and thrombotic disease. Platelets of elderly and young humans significantly differ in terms of number, activity and structure (see **Table 1**). These age-related differences are closely linked to the prothrombotic state observed in the elderly population; however, the nature of this link remains only partially defined. Indeed, it is still not clear whether platelet hyperactivity and vascular disease develop concomitantly or if one is the consequence of the other. Moreover, in addition to age, many other environmental and genetic factors can influence platelet function such as elevated plasma cholesterol, tobacco and alcohol consumption, diabetes, hypertension and sexual hormones (11). Although some studies have taken into account these covariates in their analyses, it is impossible to rule out the presence of a confounding variable in such complex traits. Therefore, additional studies are required to fully elucidate the connection between molecular changes in platelets and pathophysiological changes in the vascular

system during aging. A better understanding of the mechanisms underlying modifications of platelet function in aging could lead to the development of better-tailored treatments to curb thrombosis in this at-risk population.

AUTHOR CONTRIBUTIONS

JL and ML conceived the paper. JL wrote the first draft of the manuscript. ML extensively reviewed the manuscript.

FUNDING

JL was supported by a scholarship from the Faculté de pharmacie de l'Université de Montréal. ML was a Fonds de Recherche du Québec en santé (FRQS) Junior 1 Research Scholar, and was supported by research grants from the FRQS, the Canadian Institutes for Health Research, the Canada Foundation for Innovation, Diabetes Quebec, Heart and Stroke Foundation of Canada, and by the Fondation de l'Institut de Cardiologie de Montréal.

REFERENCES

- He S, Sharpless NE. Senescence in health and disease. *Cell*. (2017) 169:1000–11. doi: 10.1016/j.cell.2017.05.015
- Parrinello S, Coppe JP, Krtolica A, Campisi J. Stromal-epithelial interactions in aging and cancer: senescent fibroblasts alter epithelial cell differentiation. *J Cell Sci*. (2005) 118 (Pt 3):485–96. doi: 10.1242/jcs.01635
- Coppe JP, Desprez PY, Krtolica A, Campisi J. The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annu Rev Pathol*. (2010) 5:99–118. doi: 10.1146/annurev-pathol-121808-102144
- Valenzuela CA, Quintanilla R, Moore-Carrasco R, Brown NE. The potential role of senescence as a modulator of platelets and tumorigenesis. *Front Oncol*. (2017) 7:188. doi: 10.3389/fonc.2017.00188
- Global Health Estimates 2016: Deaths by Cause, Age, Sex, by Country and by Region, 2000–2016. Geneva: World Health Organization (2016).
- Winther K, Naesh O. Platelet alpha-adrenoceptor function and aging. *Thromb Res*. (1987) 46:677–84.
- Yokoyama M, Kusui A, Sakamoto S, Fukuzaki H. Age-associated increments in human platelet alpha-adrenoceptor capacity. Possible mechanism for platelet hyperactivity to epinephrine in aging man. *Thromb Res*. (1984) 34:287–95.
- Meade TW, Vickers MV, Thompson SG, Stirling Y, Haines AP, Miller GJ. Epidemiological characteristics of platelet aggregability. *Br Med J*. (1985) 290:428–32.
- Gleerup G, Winther K. The effect of ageing on platelet function and fibrinolytic activity. *Angiology*. (1995) 46:715–8. doi: 10.1177/000331979504600810
- Kasjanovova D, Adameckova D, Gratzlova J, Hegyi L. Sex-related and age-related differences in platelet function *in vitro*: influence of hematocrit. *Mech Age Dev*. (1993) 71:103–9.
- O'Donnell CJ, Larson MG, Feng D, Sutherland PA, Lindpaintner K, Myers RH, et al. Genetic and environmental contributions to platelet aggregation: the Framingham heart study. *Circulation*. (2001) 103:3051–6. doi: 10.1161/01.cir.103.25.3051
- Frossard M, Fuchs I, Leitner JM, Hsieh K, Vlcek M, Losert H, et al. Platelet function predicts myocardial damage in patients with acute myocardial infarction. *Circulation*. (2004) 110:1392–7. doi: 10.1161/01.Cir.0000141575.92958.9c
- Willoughby S, Holmes A, Loscalzo J. Platelets and cardiovascular disease. *Eur J Cardiovasc Nurs*. (2002) 1:273–88. doi: 10.1016/s1474-51510200038-5
- Davi G, Patrono C. Platelet activation and atherothrombosis. *N Engl J Med*. (2007) 357:2482–94. doi: 10.1056/NEJMra071014
- Breddin HK, Lippold R, Bittner M, Kirchmaier CM, Krzywanek HJ, Michaelis J. Spontaneous platelet aggregation as a predictive risk factor for vascular occlusions in healthy volunteers? Results of the HAPARG Study. Haemostatic parameters as risk factors in healthy volunteers. *Atherosclerosis*. (1999) 144:211–9.
- Jackson SP. Arterial thrombosis—insidious, unpredictable and deadly. *Nat Med*. (2011) 17:1423–36. doi: 10.1038/nm.2515
- Puurunen MK, Hwang SJ, Larson MG, Vasan RS, O'Donnell CJ, Tofler G, et al. ADP platelet hyperreactivity predicts cardiovascular disease in the FHS (Framingham Heart Study). *J Am Heart Assoc*. (2018) 7:e008522. doi: 10.1161/jaha.118.008522
- Segal JB, Moliterno AR. Platelet counts differ by sex, ethnicity, and age in the United States. *Ann Epidemiol*. (2006) 16:123–30. doi: 10.1016/j.annepidem.2005.06.052
- Troussard X, Vol S, Cornet E, Bardet V, Couaillat JP, Fossat C, et al. Full blood count normal reference values for adults in France. *J Clin Pathol*. (2014) 67:341–4. doi: 10.1136/jclinpath-2013-201687
- Robbins J, Wahl P, Savage P, Enright P, Powe N, Lyles M. Hematological and biochemical laboratory values in older cardiovascular health study participants. *J Am Geriatr Soc*. (1995) 43:855–9.
- Emery JD, Leifer DW, Moura GL, Southern P, Morrissey JH, Lawrence JB. Whole-blood platelet aggregation predicts *in vitro* and *in vivo* primary hemostatic function in the elderly. *Arterioscler Thromb Vasc Biol*. (1995) 15:748–53.
- Kasjanovova D, Balaz V. Age-related changes in human platelet function *in vitro*. *Mech Ageing Dev*. (1986) 37:175–82.
- van der Bom JG, Heckbert SR, Lumley T, Holmes CE, Cushman M, Folsom AR, et al. Platelet count and the risk for thrombosis and death in the elderly. *J Thromb Haemost*. (2009) 7:399–405. doi: 10.1111/j.1538-7836.2008.03267.x
- Tsai MT, Chen YT, Lin CH, Huang TP, Tarnag DC. U-shaped mortality curve associated with platelet count among older people: a community-based cohort study. *Blood*. (2015) 126:1633–5. doi: 10.1182/blood-2015-06-654764
- Msaouel P, Lam AP, Gundabolu K, Chrysosfakis G, Yu Y, Mantzaris I, et al. Abnormal platelet count is an independent predictor of mortality in the elderly and is influenced by ethnicity. *Haematologica*. (2014) 99:930–6. doi: 10.3324/haematol.2013.101949
- Bonaccio M, Di Castelnuovo A, Costanzo S, De Curtis A, Donati MB, Cerletti C, et al. Age-sex-specific ranges of platelet count and all-cause mortality:

- prospective findings from the MOLI-SANI study. *Blood*. (2016) 127:1614–6. doi: 10.1182/blood-2016-01-692814
27. Reilly IA, FitzGerald GA. Eicosenoid biosynthesis and platelet function with advancing age. *Thromb Res*. (1986) 41:545–54.
 28. Jorgensen KA, Dyerberg J, Olesen AS, Stoffersen E. Acetylsalicylic acid, bleeding time and age. *Thromb Res*. (1980) 19:799–805.
 29. Bastyr EJ, 3rd, Kadrofske MM, Vinik AI. Platelet activity and phosphoinositide turnover increase with advancing age. *Am J Med*. (1990) 88:601–6.
 30. Cowman J, Dunne E, Oglesby I, Byrne B, Ralph A, Voisin B, et al. Age-related changes in platelet function are more profound in women than in men. *Sci Rep*. (2015) 5:12235. doi: 10.1038/srep12235
 31. Zahavi J, Jones NA, Leyton J, Dubiel M, Kakkar VV. Enhanced *in vivo* platelet “release reaction” in old healthy individuals. *Thromb Res*. (1980) 17:329–36.
 32. Kowalska MA, Rauova L, Poncz M. Role of the platelet chemokine platelet factor 4 (PF4) in hemostasis and thrombosis. *Thrombo Res*. (2010) 125:292–6. doi: 10.1016/j.thromres.2009.11.023
 33. Gleerup G, Winther K. The effect of ageing on human platelet sensitivity to serotonin. *Eur J Clin Invest*. (1988) 18:504–6.
 34. Brodde OE, Anlauf M, Graben N, Bock KD. Age-dependent decrease of alpha 2-adrenergic receptor number in human platelets. *Eur J Pharmacol*. (1982) 81:345–7.
 35. Winther K, Naesh O. Aging and platelet beta-adrenoceptor function. *Eur J Pharmacol*. (1987) 136:219–23.
 36. Barradas MA, Gill DS, Fonseca VA, Mikhailidis DP, Dandona P. Intraplatelet serotonin in patients with diabetes mellitus and peripheral vascular disease. *Eur J Clin Invest*. (1988) 18:399–404.
 37. Flachaire E, Beney C, Berthier A, Salandre J, Quincy C, Renaud B. Determination of reference values for serotonin concentration in platelets of healthy newborns, children, adults, and elderly subjects by HPLC with electrochemical detection. *Clin Chem*. (1990) 36:2117–20.
 38. Fuentes F, Palomo I, Fuentes E. Platelet oxidative stress as a novel target of cardiovascular risk in frail older people. *Vasc Pharmacol*. (2017) 93–95:14–9. doi: 10.1016/j.vph.2017.07.003
 39. Violi F, Loffredo L, Carnevale R, Pignatelli P, Pastori D. Atherothrombosis and oxidative stress: mechanisms and management in elderly. *Antioxid Redox Signal*. (2017) 27:1083–124. doi: 10.1089/ars.2016.6963
 40. Fuentes E, Palomo I. Role of oxidative stress on platelet hyperreactivity during aging. *Life Sci*. (2016) 148:17–23. doi: 10.1016/j.lfs.2016.02.026
 41. Jang JY, Min JH, Chae YH, Baek JY, Wang SB, Park SJ, et al. Reactive oxygen species play a critical role in collagen-induced platelet activation via SHP-2 oxidation. *Antioxid Redox Signal*. (2014) 20:2528–40. doi: 10.1089/ars.2013.5337
 42. Alexandru N, Constantin A, Popov D. Carbonylation of platelet proteins occurs as consequence of oxidative stress and thrombin activation, and is stimulated by ageing and type 2 diabetes. *Clin Chem Lab Med*. (2008) 46:528–36. doi: 10.1515/cclm.2008.104
 43. Goswami K, Koner BC. Level of sialic acid residues in platelet proteins in diabetes, aging, and Hodgkin's lymphoma: a potential role of free radicals in desialylation. *Biochem Biophys Res Commun*. (2002) 297:502–5. doi: 10.1016/s0006-291x(02)02241-6.
 44. Gaspar RS, Trostchansky A, Paes AM. Potential role of protein disulfide isomerase in metabolic syndrome-derived platelet hyperactivity. *Oxid Med Cell Longev*. (2016) 2016:2423547. doi: 10.1155/2016/2423547
 45. Essex DW. Redox control of platelet function. *Antioxid Redox Signal*. (2009) 11:1191–225. doi: 10.1089/ars.2008.2322
 46. Kim K, Hahn E, Li J, Holbrook LM, Sasikumar P, Stanley RG, et al. Platelet protein disulfide isomerase is required for thrombus formation but not for hemostasis in mice. *Blood*. (2013) 122:1052–61. doi: 10.1182/blood-2013-03-492504
 47. Levin L, Zelzion E, Nachliel E, Gutman M, Tsfadia Y, Einav Y. A single disulfide bond disruption in the beta3 integrin subunit promotes thiol/disulfide exchange, a molecular dynamics study. *PLoS ONE*. (2013) 8:e59175. doi: 10.1371/journal.pone.0059175
 48. Butera D, Cook KM, Chiu J, Wong JW, Hogg PJ. Control of blood proteins by functional disulfide bonds. *Blood*. (2014) 123:2000–7. doi: 10.1182/blood-2014-01-549816
 49. Galley HF, Webster NR. Physiology of the endothelium. *Br J Anaesth*. (2004) 93:105–13. doi: 10.1093/bja/ae163
 50. Kawamoto EM, Munhoz CD, Glezer I, Bahia VS, Caramelli P, Nitrini R, et al. Oxidative state in platelets and erythrocytes in aging and Alzheimer's disease. *Neurobiol Aging*. (2005) 26:857–64. doi: 10.1016/j.neurobiolaging.2004.08.011
 51. Bartesaghi S, Radi R. Fundamentals on the biochemistry of peroxynitrite and protein tyrosine nitration. *Redox Biol*. (2018) 14:618–25. doi: 10.1016/j.redox.2017.09.009
 52. Michimata T, Imamura M, Mizuma H, Murakami M, Iriuchijima T. Sex and age differences in soluble guanylate cyclase activity in human platelets. *Life Sci*. (1996) 58:415–9.
 53. Origlia C, Pescarmona G, Capizzi A, Cogotti S, Gambino R, Cassader M, et al. Platelet cGMP inversely correlates with age in healthy subjects. *J Endocrinol Invest*. (2004) 27:Rc1–4. doi: 10.1007/bf03346251
 54. Kawamoto EM, Vasconcelos AR, Degaspari S, Bohmer AE, Scavone C, Marcourakis T. Age-related changes in nitric oxide activity, cyclic GMP, and TBARS levels in platelets and erythrocytes reflect the oxidative status in central nervous system. *Age*. (2013) 35:331–42. doi: 10.1007/s11357-011-9365-7
 55. Dayal S, Wilson KM, Motto DG, Miller FJ, Jr, Chauhan AK, Lentz SR. Hydrogen peroxide promotes aging-related platelet hyperactivation and thrombosis. *Circulation*. (2013) 127:1308–16. doi: 10.1161/circulationaha.112.000966
 56. Pignatelli P, Pulcinelli FM, Lenti L, Gazzaniga PP, Violi F. Hydrogen peroxide is involved in collagen-induced platelet activation. *Blood*. (1998) 91:484–90.
 57. Jain K, Tyagi T, Patell K, Xie Y, Kadado AJ, Lee SH, et al. Age associated non-linear regulation of redox homeostasis in the anucleate platelet: Implications for CVD risk patients. *EBioMedicine*. (2019) 44:28–40. doi: 10.1016/j.ebiom.2019.05.022
 58. Menconi M, Taylor L, Martin B, Polgar P. A review: prostaglandins, aging, and blood vessels. *J Am Geriatr Soc*. (1987) 35:239–47.
 59. Giani E, Masi I, Galli C. Platelets from aged rats aggregate more, but are more sensitive to prostacyclin. *Prostaglandins Leukot Med*. (1985) 20:237–46.
 60. Modesti PA, Fortini A, Abbate R, Gensini GF. Age related changes of platelet prostacyclin receptors in humans. *Eur J Clin Invest*. (1985) 15:204–8.
 61. Neri Serneri GG, Modesti PA, Fortini A, Abbate R, Lombardi A, Gensini GF. Reduction in prostacyclin platelet receptors in active spontaneous angina. *Lancet*. (1984) 2:838–41.
 62. Pastori D, Pignatelli P, Farcomeni A, Nocella C, Bartimoccia S, Carnevale R, et al. Age-related increase of thromboxane B2 and risk of cardiovascular disease in atrial fibrillation. *Oncotarget*. (2016) 7:39143–7. doi: 10.18632/oncotarget.9826
 63. Igbavboa U, Avdulov NA, Schroeder F, Wood WG. Increasing age alters transbilayer fluidity and cholesterol asymmetry in synaptic plasma membranes of mice. *J Neurochem*. (1996) 66:1717–25.
 64. Schootemeijer A, Gorter G, Tertoolen LG, De Laat SW, Akkerman JW. Relation between membrane fluidity and signal transduction in the human megakaryoblastic cell line MEG-01. *Biochim et Biophys Acta*. (1995) 1236:128–34.
 65. Kitagawa S, Endo J, Kametani F. Effects of long-chain cis-unsaturated fatty acids and their alcohol analogs on aggregation of bovine platelets and their relation with membrane fluidity change. *Biochim et Biophys Acta*. (1985) 818:391–7.
 66. Rivnay B, Bergman S, Shinitzky M, Globerson A. Correlations between membrane viscosity, serum cholesterol, lymphocyte activation and aging in man. *Mechan Ageing Dev*. (1980) 12:119–26.
 67. Cohen BM, Zubenko GS. Aging and the biophysical properties of cell membranes. *Life Sci*. (1985) 37:1403–9.
 68. Prisco D, Rogasi PG, Matucci M. Age related changes in platelet lipid composition. *Thromb Res*. (1986) 44:427–37. doi: 10.1016/0049-3848(86)90321-X
 69. Marinho CFM, Costa-Maia J, Pinto-de-Barros J, Oliveira CR. Correlation between human platelet cytoplasmic membrane outer leaflet fluidity, Na⁺H⁺exchanger activity and aging. *Eur Arch Psychiatry Clin Neurosci*. (1997) 247:275–7.
 70. Hossain MS, Hashimoto M, Gamoh S, Masumura S. Association of age-related decrease in platelet membrane fluidity with platelet lipid peroxide. *Life Sci*. (1999) 64:135–43.
 71. Insel PA, Nirenberg P, Turnbull J, Shattil SJ. Relationships between membrane cholesterol, alpha-adrenergic receptors, and platelet function. *Biochemistry*. (1978) 17:5269–74.

72. Shattil SJ, Anaya-Galindo R, Bennett J, Colman RW, Cooper RA. Platelet hypersensitivity induced by cholesterol incorporation. *J Clin Invest.* (1975) 55:636–43. doi: 10.1172/jci107971
73. Stuart MJ, Gerrard JM, White JG. Effect of cholesterol on production of thromboxane b2 by platelets *in vitro*. *N Engl J Med.* (1980) 302:6–10. doi: 10.1056/nejm198001033020102
74. Tremoli E, Maderia P, Colli S, Morazzoni G, Sirtori M, Sirtori CR. Increased platelet sensitivity and thromboxane B2 formation in type-II hyperlipoproteinaemic patients. *Eur J Clin Invest.* (1984) 14:329–33.
75. Clancy L, Freedman JE. The role of circulating platelet transcripts. *J Thromb Haemost.* (2015) 13 Suppl 1:S33–9. doi: 10.1111/jth.12922
76. Best MG, Vancura A, Wurdinger T. Platelet RNA as a circulating biomarker trove for cancer diagnostics. *J Thromb Haemost.* (2017) 15:1295–306. doi: 10.1111/jth.13720
77. Simon LM, Edelstein LC, Nagalla S, Woodley AB, Chen ES, Kong X, et al. Human platelet microRNA-mRNA networks associated with age and gender revealed by integrated plateletomics. *Blood.* (2014) 123:e37–45. doi: 10.1182/blood-2013-12-544692
78. Campbell RA, Franks Z, Bhatnagar A, Rowley JW, Manne BK, Supiano MA, et al. Granzyme A in human platelets regulates the synthesis of proinflammatory cytokines by monocytes in aging. *J Immunol.* (2018) 200:295–304. doi: 10.4049/jimmunol.1700885
79. Cini C, Yip C, Attard C, Karlaftis V, Monagle P, Linden M, et al. Differences in the resting platelet proteome and platelet releasate between healthy children and adults. *J Proteom.* (2015) 123:78–88. doi: 10.1016/j.jprot.2015.04.003
80. Marques FZ, Chu PY, Ziemann M, Kaspi A, Kiriazis H, Du XJ, et al. Age-related differential structural and transcriptomic responses in the hypertensive heart. *Front Physiol.* (2018) 9:817. doi: 10.3389/fphys.2018.00817
81. Mosca L, Barrett-Connor E, Wenger NK. Sex/gender differences in cardiovascular disease prevention: what a difference a decade makes. *Circulation.* (2011) 124:2145–54. doi: 10.1161/circulationaha.110.968792
82. de Bastos M, Stegeman BH, Rosendaal FR, Van Hylckama Vlieg A, Helmerhorst FM, Stijnen T, et al. Combined oral contraceptives: venous thrombosis. *Cochrane Database Syst Rev.* (2014) 3:Cd010813. doi: 10.1002/14651858.CD010813
83. Boardman HM, Hartley L, Eisinga A, Main C, Roque i Figuls M, Bonfill Cosp X, et al. Hormone therapy for preventing cardiovascular disease in post-menopausal women. *Cochrane Database Syst Rev.* (2015) 3:Cd002229. doi: 10.1002/14651858.CD002229
84. Mosca L, Collins P, Herrington DM, Mendelsohn ME, Pasternak RC, Robertson RM, et al. Hormone replacement therapy and cardiovascular disease: a statement for healthcare professionals from the American Heart Association. *Circulation.* (2001) 104:499–503. doi: 10.1161/hc2901.092200
85. Miller VM, Jayachandran M, Owen WG. Ageing, oestrogen, platelets and thrombotic risk. *Clin Exp Pharmacol Physiol.* (2007) 34:814–21. doi: 10.1111/j.1440-1681.2007.04685.x
86. Bar J, Lahav J, Hod M, Ben-Rafael Z, Weinberger I, Brosens J. Regulation of platelet aggregation and adenosine triphosphate release *in vitro* by 17beta-estradiol and medroxyprogesterone acetate in postmenopausal women. *Thromb Haemost.* (2000) 84:695–700. doi: 10.1055/s-0037-1614089
87. Wu GJ, Lee JJ, Chou DS, Jayakumar T, Hsiao G, Chen WF, et al. Inhibitory signaling of 17beta-estradiol in platelet activation: the pivotal role of cyclic AMP-mediated nitric oxide synthase activation. *Eur J Pharmacol.* (2010) 649:140–9. doi: 10.1016/j.ejphar.2010.09.055
88. Jayachandran M, Miller VM. Ovariectomy upregulates expression of estrogen receptors, NOS, and HSPs in porcine platelets. *Am J Physiol Heart Circul Physiol.* (2002) 283:H220–6. doi: 10.1152/ajpheart.00950.2001
89. Feuring M, Christ M, Roell A, Schueller P, Losel R, Dempfle CE, et al. Alterations in platelet function during the ovarian cycle. *Blood Coagul Fibrinol.* (2002) 13:443–7. doi: 10.1097/00001721-200207000-00009
90. Roell A, Schueller P, Schultz A, Losel R, Wehling M, Christ M, et al. Effect of oral contraceptives and ovarian cycle on platelet function. *Platelets.* (2007) 18:165–70. doi: 10.1080/09537100600936224
91. Skouby SO, Sidelmann JJ. Impact of progestogens on hemostasis. *Horm Mol Biol Clin Invest.* (2018) 37:20180041. doi: 10.1515/hmbci-2018-0041
92. Berlin G, Hammar M, Tapper L, Tynngard N. Effects of age, gender and menstrual cycle on platelet function assessed by impedance aggregometry. *Platelets.* (2019) 30:473–9. doi: 10.1080/09537104.2018.1466387
93. Lundberg Slingsby MH, Nyberg M, Egelund J, Mandrup CM, Frikke-Schmidt R, Kirkby NS, et al. Aerobic exercise training lowers platelet reactivity and improves platelet sensitivity to prostacyclin in pre- and postmenopausal women. *J Thromb Haemost.* (2017) 15:2419–31. doi: 10.1111/jth.13866
94. Kurabayashi H, Kubota K, Hishinuma A, Majima M. Platelet activation is caused not by aging but by atherosclerosis. *Arch Gerontol Geriatr.* (2010) 51:205–8. doi: 10.1016/j.archger.2009.10.009

Conflict of Interest Statement: ML has received speaker fees from Bayer for unrelated work; has participated in industry-funded trials from Idorsia for unrelated work; and has received in-kind and financial support for investigator-initiated grants from Roche Diagnostics and AggreGlyne, also for unrelated work.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Le Blanc and Lordkipanidzé. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Platelet Function During Extracorporeal Membrane Oxygenation in Adult Patients

Camilla Mains Balle¹, Anni Nørgaard Jeppesen², Steffen Christensen² and Anne-Mette Hvas^{1,3*}

¹ Department of Clinical Biochemistry, Aarhus University Hospital, Aarhus, Denmark, ² Department of Anesthesiology and Intensive Care Medicine, Aarhus University Hospital, Aarhus, Denmark, ³ Department of Clinical Medicine, Aarhus University, Aarhus, Denmark

OPEN ACCESS

Edited by:

Vera Ignjatovic,
Murdoch Childrens Research
Institute, Australia

Reviewed by:

Gail Mary Annich,
Hospital for Sick Children, Canada
Scott James Cameron,
University of Rochester, United States

*Correspondence:

Anne-Mette Hvas
am.hvas@dadlnet.dk

Specialty section:

This article was submitted to
Atherosclerosis and Vascular
Medicine,
a section of the journal
Frontiers in Cardiovascular Medicine

Received: 30 April 2019

Accepted: 26 July 2019

Published: 08 August 2019

Citation:

Balle CM, Jeppesen AN,
Christensen S and Hvas A-M (2019)
Platelet Function During
Extracorporeal Membrane
Oxygenation in Adult Patients.
Front. Cardiovasc. Med. 6:114.
doi: 10.3389/fcvm.2019.00114

Objective: Hemorrhagic and thromboembolic complications are common during support with extracorporeal membrane oxygenation (ECMO). As platelets play a pivotal role in hemostasis, we aimed to clarify how ECMO support affects platelet function.

Methods: We included 33 adult patients undergoing ECMO support at a tertiary ECMO referral center at Aarhus University Hospital, Denmark. Blood samples were collected on the first morning following ECMO initiation, and subsequently every morning until the 7th (± 1) day. Platelet aggregation was evaluated by whole blood impedance aggregometry (Multiplate[®] Analyzer) using adenosine diphosphate (ADPtest), arachidonic acid (ASPItest), and thrombin-receptor-agonist-peptide-6 (TRAPtest) as agonists. A new model was applied, taking platelet count into consideration in interpretation of impedance aggregometry analyses. On the 1st and 3rd day, platelet activation was assessed by flow cytometry (Navios) using collagen-related peptide, ADP, TRAP, and arachidonic acid as agonists.

Results: Blood samples from all 33 patients were analyzed on day 1 of ECMO support; 24 patients were still receiving ECMO and analyzed on day 3; 12 patients were analyzed on day 7 (± 1). After ECMO initiation, platelet counts decreased significantly ($p < 0.002$) and remained low during ECMO support. ECMO patients demonstrated significantly reduced platelet aggregation on day 1 compared with healthy controls (all $p < 0.001$). However, when taking platelet count into consideration, platelet aggregation relative to platelet count did not differ from healthy controls. Flow cytometry analyses demonstrated impaired platelet activation in ECMO patients on day 1 compared with healthy controls (all $p < 0.03$). No substantial difference was found in platelet activation from day 1 to day 3 on ECMO support.

Conclusions: Employing impedance aggregometry and flow cytometry, we found both impaired platelet aggregation and decreased platelet activation on day 1 of ECMO support compared with healthy controls. However, platelet aggregation was not impaired, when interpreted relative to the low platelet counts. Furthermore, levels of

bound fibrinogen, on the surface of activated platelets in ECMO patients, were higher than in healthy controls. Together, these findings suggestively oppose that platelets are universally impaired during ECMO support. No marked difference in activation from day 1 to day 3 was seen during ECMO support.

Keywords: blood platelets, extracorporeal membrane oxygenation, extracorporeal life support, platelet activation, platelet aggregation, platelet function tests

INTRODUCTION

Extracorporeal membrane oxygenation (ECMO) is used to treat critically ill patients suffering from acute respiratory and/or cardiac failure unresponsive to conventional treatment (1). Recent years have seen major advances in the management and technology of ECMO support (2). Nevertheless, mortality rates remain as high as 38–42% in veno-venous (3, 4) and 59–71% in veno-arterial ECMO cohorts (3).

Bleeding and thromboembolic events frequently occur during ECMO support, greatly affecting patient outcomes (5, 6). Major bleeding is reported to occur in up to one third of adult ECMO patients (4, 7, 8), and reports of intracranial hemorrhage range from 5 to 20% (3, 9, 10). Thromboembolic complications are generally less common with intracranial infarction reported to occur in up to 5% (3, 7, 9) and venous thrombosis in 7–10% of adult ECMO patients (7, 11). The incidence of thromboembolic events might, however, be underestimated as many are subclinical and therefore overlooked (12). The high frequencies of bleeding and thromboembolic events during ECMO support emphasizes the clinical relevance of understanding the hemostatic changes occurring during ECMO support. As platelets play a pivotal role in hemostasis, it is of importance to clarify how ECMO support affects platelet function. Recent studies have investigated mechanisms *in vitro* that may affect platelet function during ECMO support, suggesting that platelets may become concurrently activated and impaired (13–15).

Only few studies have investigated platelet function during ECMO support in adult patients (16–22), of which the majority investigated platelet aggregation using whole blood impedance aggregometry (16–19). These studies reported reduced platelet aggregation during ECMO support compared with either before ECMO initiation (17, 18) or with healthy individuals (16, 19). However, none of these studies took platelet count into consideration, despite previous documentation of a strong association between platelet count and platelet aggregation, when employing impedance aggregometry (23–29). Our research group previously developed a model which estimates expected platelet aggregation at a range of platelet counts in healthy whole blood (23, 30). Thus, making it possible to take platelet count into consideration when evaluating platelet aggregation.

Flow cytometry is a method that is independent of platelet count, because it measures the characteristics of each single platelet (31, 32); a definite advantage when investigating platelet activation during ECMO support, as these patients are often thrombocytopenic.

We hypothesized that exposure to the extracorporeal circuit would result in platelet activation. Therefore, our primary aim was to investigate changes in platelet function from day 1 to day 3 on ECMO support, by measuring platelet aggregation and surface markers of platelet activation.

METHODS

The present study is a prospective single center cohort study conducted at a tertiary ECMO referral center at Aarhus University Hospital, Denmark. The study was approved as a quality assurance study by The Central Denmark Region Committees on Health and Research Ethics (enquiry 213-2016); therefore, according to Danish law, written informed consent was not required. The Danish Data Protection Agency approved the study (Ref. no. 1-16-02-712-17).

Study Population

Inclusion criteria were: Patients aged 18 years or older presenting with severe cardiac and/or pulmonary failure requiring ECMO support. Furthermore, patients were only included if a blood sample could be obtained on day 1 of ECMO support. Exclusion criteria was VA ECMO following cardiac surgery; because surgery, post-operative bleeding, and massive platelet transfusion may have influenced platelet function analysis.

ECMO Management

Patients were connected to the ECMO circuit by 17–23 Fr venous cannulas, and 15–21 Fr arterial cannulas. No patients were centrally cannulated. Mean initial blood flow rates in veno-arterial (VA) ECMO were 3.6 (± 0.5) L/min and in veno-venous (VV) ECMO 3.8 (± 0.4) L/min. Mean initial sweep flow rates in VA ECMO were 3.2 (± 1.6) and in VV ECMO 4.4 (± 2.0). The ECMO circuit was inspected for fibrin and clot formation by experienced ECMO specialists every 8 h. Post-oxygenation was evaluated, and the circuit exchanged at bedside, if fibrin or clots in the oxygenator was suspected of being the cause of the patient developing hypoxia.

Anticoagulation and Transfusions

Patients received unfractionated heparin as an intravenous bolus injection of 5,000 IU at time of ECMO cannula insertion. Following initiation of ECMO, the activated partial thromboplastin time (APTT) was kept at 40–55 s in VV ECMO patients and 50–65 s in VA ECMO patients by intravenous infusion of unfractionated heparin. Heparinization was monitored by measurement of APTT at least four times daily.

Antithrombin levels were kept above 0.80×10^3 IU/L during ECMO support.

Platelet transfusions were administered to maintain platelet counts above 50×10^9 /L. In case of bleeding complications, the transfusion threshold was increased to a platelet count of $80\text{--}100 \times 10^9$ /L. The standard threshold for transfusion of red blood cells was a hemoglobin of 6.0 mmol/L.

Clinical Data

Clinical data were extracted from the patient medical records. The following clinical data were collected: ECMO configuration; indication for support; comorbidities selected in accordance with the Charlson Comorbidity Index (33); Sequential Organ Failure Assessment (SOFA) Score modified to use the Richmond Agitation-Sedation Scale (34); Simplified Acute Physiological Score-III (SAPS-III) at time of admission to the Intensive Care Unit; 30-day mortality; transfusion of blood products; and daily medical therapy.

Blood Sampling and Laboratory Analyses

The first blood sample (denominated day 1) was obtained on the first morning following ECMO initiation. A day was defined as 24 h onward from 00:00. Blood samples were then collected on days 2 and 3. Moreover, we collected samples on day 7 (± 1), if the patients were still receiving ECMO support. A blood sample prior to ECMO initiation was possible to obtain only from patients, who had been hospitalized within the Central Region of Denmark prior to ECMO initiation. Thus, results from blood samples obtained prior to ECMO initiation could not be obtained from patients, who were put on ECMO support outside the Central Region of Denmark and subsequently transported to Aarhus University Hospital for further management. Furthermore, patients put on ECMO support for extracorporeal cardiopulmonary resuscitation (ECPR), did not routinely have blood samples taken prior to ECMO initiation.

Blood was drawn from arterial lines and anticoagulated in 3 ml hirundine tubes (Roche, Basel, Switzerland) for impedance aggregometry analyses, and in 3.5 ml sodium citrate 3.2% tubes (Terumo Europe, Leuven, Belgium) for flow cytometry analyses. Samples for platelet count, immature platelet fraction (IPF, reflecting platelet turnover), hemoglobin, and leucocytes were anticoagulated in 2 ml ethylenediaminetetra acid (EDTA) tubes (Becton Dickinson Bioscience, San Jose, CA) and estimated using an automated hematology analyzer (Sysmex XE-5000, Sysmex, Kobe, Japan). Samples for additional laboratory parameters, such as international standardized ratio (INR), APTT, and fibrinogen, were analyzed in 3.5 ml sodium citrate 3.2% tubes employing CS 5100i (Sysmex, Kobe, Japan). Samples for C-reactive protein and creatinine were collected in 1 ml lithium-heparin tubes and analyzed using Cobas 6000 (Roche, Basel, Switzerland).

Platelet Function

Whole Blood Impedance Aggregometry

Platelet aggregation was investigated by whole blood impedance aggregometry using the Multiplate[®] Analyzer (Roche, Basel, Switzerland). In short, hirudinized whole blood was diluted 1:1 with preheated (37°C) isotonic saline followed by 3 min

of incubation time at 37°C. Adenosine diphosphate (6.5 μ M, ADPtest), thrombin-receptor-agonist-peptide-6 (32 μ M, TRAPtest), and arachidonic acid (AA) (0.5 mM, ASPItest) (all from Roche, Basel, Switzerland) were used as agonists. The analysis is based on the principle that platelets become sticky following activation by an agonist, resulting in aggregation and adherence onto two metal sensor wires in the Multiplate[®] test cuvette; this increases the electrical resistance between the wires. The increase in resistance is expressed as aggregation units (AU) and plotted as an aggregation curve over time. The area under the curve (AU*min) indicates the agonist induced platelet aggregation capacity. Three different agonists were chosen to target different pathways of platelet activation: ADP stimulates the ADP receptors on the surface of the platelets, thereby inducing platelet activation; TRAP mimics thrombin by activating the platelets through the protease activated receptor-1 (PAR-1) on the platelet surface; and AA is converted by platelet enzymes to thromboxane A2 (TXA2), which activates the platelet.

Impedance aggregometry results were compared with data on healthy controls chosen to match ECMO patients on two ranked criteria: (a) gender; (b) age. Data on healthy controls for ADP and AA induced platelet aggregation were obtained from Rubak et al. (28) and data on TRAP induced platelet aggregation were obtained from Pedersen et al. (35).

To evaluate platelet aggregation relative to platelet count, we assessed platelet aggregation during ECMO support relative to a 95% prediction interval of platelet aggregation in healthy whole blood at various platelet counts. This method was established by Skipper et al. (23); after inducing varying degrees of thrombocytopenia in healthy whole blood, platelet aggregation was measured by whole blood impedance aggregometry (Multiplate[®] Analyzer), and a 95% prediction interval for platelet aggregation relative to platelet count was established (30).

Flow Cytometry

Activated platelets release the content of their intracellular granules, leading to translocation of proteins from the granule membrane to the platelet plasma membrane. Thus, platelet activation can be measured as a change in the expression of these activation-dependent markers on the surface of the platelet. P-selectin is exposed on the platelet surface following secretion of the α -granule content and CD63 is exposed following secretion of the dense granule content. Furthermore, following activation, the platelet surface receptor glycoprotein (GP) IIb/IIIa undergoes a conformational change to the active conformation with a high affinity for fibrinogen binding. Thus, bound fibrinogen on the surface of the platelet is a marker of platelet activation. In flow cytometry, fluorescently labeled antibodies directed against these activation-dependent platelet surface markers are used to measure their expression, and thereby determine the degree of platelet activation following agonist stimulation.

Flow cytometry analyses were performed after 1 h of resting and completed within 2 h of sample collection as previously described by Rubak et al. (36). In short, 35 μ l of HEPES buffer (NaCl 137 mM, KCl 2.7 mM, MgCl₂ 1 mM, 4-(2-hydroxyethyl)-1-piper-azineethanesulfonic acid (HEPES)) was pooled with 5 μ l

of each antibody and 5 μ l of agonist. Subsequently, 5 μ l of whole blood was added, and samples incubated for exactly 10 min in the dark at room temperature. Following incubation, 2 ml of fixation buffer (0.2% paraformaldehyde in phosphate-buffered saline) was added to fix the cells prior to analysis.

The expression of activation-dependent platelet surface markers was measured using a combination of the following fluorescently labeled antibodies: CD42b-PE (Clone HIP1) (AH Diagnostics); CD62p-APC (P-selectin, Clone P-sel.KO2.3) (eBioscience, San Diego, CA); CD63-PECy7 (Clone H5c6) (Beckton Dickinson Bioscience, San Jose, CA); and anti-fibrinogen-FITC (Polyclonal chicken) (Diapensia HB, Linköping, Sweden). All fluorescently labeled antibodies were diluted in HEPES buffer to reach saturating concentrations previously determined (36).

To induce platelet activation, four different agonists were chosen to target different pathways of platelet activation: ADP, TRAP and AA (as used in the platelet aggregation analyses), together with collagen-related-peptide (COL). COL mimics collagen by binding to the GPVI receptor on the platelet surface, resulting in platelet activation. The following agonists were added (final concentrations): Collagen-related peptide (COL, 0.51 μ g/ml) (University of Cambridge, UK); ADP (10.8 μ M) (Sigma Aldrich, St. Louis, MO); TRAP (28.5 μ M) (JPT, Berlin, Germany); and arachidonic acid (AA, 0.58 mM) (Sigma Aldrich, St. Louis, MO).

Analyses were performed employing a Navios flow cytometer (Beckman Coulter, Miami, USA) and processed in Navios Software version 1.3. Expressions of activation-dependent platelet surface markers were measured based on 10,000 platelets; reading times were prolonged for samples with severe thrombocytopenia in an attempt to assess as many platelets as possible. For selected patients with platelet counts below 15×10^9 /L, roughly 3,000 platelets were assessed. CD42b was used to identify the platelets followed by exclusion of platelet-platelet aggregates. The positive analysis regions (gates) were determined by negative controls. Expression of P-selectin, after addition of HEPES buffer instead of an agonist, was used to assess the level of pre-activation.

The degree of platelet activation was expressed as: (a) The percentage of positive platelets within a gate (also known as %gated); and (b) as the median fluorescence intensity (MFI) of all positive platelets within a gate. The percentage of positive platelets reflects the proportion of platelets that become activated in response to stimulation by an agonist. The MFI is a quantitative measure of the degree of expression of the activation-dependent surface markers on activated platelets.

Gates in the negative control were set to include 1.5–2.0% positive platelets for bound fibrinogen and CD63 and 0.1–0.2% positive platelets for P-selectin, as previously recommended (32, 37). When analyzing samples with platelet counts below 15×10^9 /L, gates were placed at the right edge corner of the peak in the negative control, even though the percentage of positive platelets thereby exceeded 2.0%. This was due to long reading times at such low platelet counts, resulting in the appearance of unspecific, highly fluorescent particles in the gates. The gating strategy is visualized in the supplementary data (**Supplementary Figure 1**).

Flow cytometry results were compared with data obtained from healthy controls (38) chosen to match ECMO patients on three ranked criteria: (a) gender; (b) age; and (c) level of pre-activation of the platelets.

Statistics

Primary outcome was difference in platelet activation from day 1 to day 3 on ECMO support measured by flow cytometry. Our sample size calculation was based on data from investigation of platelet activation by flow cytometry in healthy individuals (36), as no data had been published on platelet function investigated by flow cytometry in ECMO patients. The percentage of platelets positive for CD63 after addition of TRAP was 85 with a standard deviation of 7. We expected the standard deviation to be greater in ECMO patients than in healthy individuals; therefore, we increased the standard deviation to 7.5. With an estimated minimal relevant difference (MIREDIFF) of 5, a significance level (2- α) of 0.05 and a power (1- β) of 0.90, we estimated that 24 patients, analyzed by flow cytometry on days 1 and 3, had to be included in the present study.

Categorical variables are reported as frequencies and percentages. Distribution of data for continuous variables was evaluated by Q-Q plots. Normally distributed data are described as mean $\pm$ standard deviation (SD); data not following a normal distribution are described as median with interquartile range (IQR). Differences between two sets of paired data were analyzed using a paired *t*-test, if data were normally distributed, and the Wilcoxon matched-pairs signed rank test, if data did not follow a normal distribution. For repeated measurements a mixed-model analysis was performed, if data were normally or lognormally distributed. Differences between unpaired data were analyzed using an unpaired *t*-test, if data were normally distributed, and the Mann-Whitney test, if data did not follow a normal distribution. All tests of significance were two-tailed; *p* < 0.05 were considered significant. Statistical analyses were performed using GraphPad Prism version 8.0 (GraphPad, Inc., San Diego, CA).

RESULTS

We present data from 33 patients treated with ECMO between September 2017 and April 2019; among which, 24 patients had flow cytometry performed on both day 1 and day 3.

Patient demographics and clinical characteristics are presented in **Table 1**. More than twice as many patients were initially treated with VA ECMO (*n* = 23) than VV ECMO (*n* = 10). Two patients had VA ECMO converted to a VV configuration during the course of ECMO support, and another patient had VA ECMO converted to a combined VA/VV configuration. Charlson Comorbidity Index indicated low comorbidity; 18 patients scored 0–1 and only two patients scored >3. A majority of the scores were primarily driven by age above 50 years. The median duration of ECMO was 5 days (IQR: 3–8 days). Two patients had considerably longer durations of support lasting 23 and 53 days. Seven patients died and 2 patients were weaned from ECMO before day 3.

TABLE 1 | Demographic and clinical characteristics of 33 adult patients treated with extracorporeal membrane oxygenation (ECMO) on day 1 ($n = 33$), day 2 ($n = 29$), and day 3 ($n = 24$).

Variables	Value
Age (years)	49 (± 13)
Male gender	23 (70%)
ECMO configuration	
VV	10 (30%)
VA	23 (70%)
ECMO indication	
Pulmonary (Pulmonary infections, septic shock, aspiration and vasculitis)	13 (39%)
Cardiac (Cardiomyopathies, cardiogenic shock, epicarditis and hypothermia)	8 (24%)
ECPR (Cardiac arrest refractory to conventional CPR)	12 (36%)
Charlson's comorbidity index	1.5 (± 1.3)
Duration of ECMO (days)	5 [3–8]
Duration from ECMO initiation to day 1 blood sample	16 h: 33 min (± 6 h: 58 min)
SOFA score, day 1	13.2 (± 2.7)
SOFA score, day 3	13.6 (± 3.4)
SAPS-III score at ICU-admission ^a	72.4 (± 19.3)
Mortality at ECMO weaning	13 (40%)
30-day mortality	17 (52%)
Laboratory parameters, day 1 (reference interval)	
Immature platelet fraction (0.016–0.126)	0.047 [0.032–0.080]
Hemoglobin, mmol/L (7.3–10.5 ^b)	6.7 (± 0.9)
INR (<1.2)	1.8 (± 0.8)
Activated partial thromboplastin time, sec (20–29)	44 [36–59]
Fibrinogen, $\mu\text{mol/L}$ (5.5–12.0)	8.5 (± 4.7)
CRP, mg/L (<8)	139 (± 107)
Leucocytes, $10^9/\text{L}$ (3.5–10.0)	14.1 (± 8.5)
Creatinine, $\mu\text{mol/L}$ (45–105 ^b)	193 [66]
Medical treatment	
Day 1	
Unfractionated heparin ^c	20 (61%)
Antiplatelet drugs ^d	8 (24%)
[asp ($n = 4$); can ($n = 1$); asp + can ($n = 2$); asp + can + tic ($n = 1$)]	
Day 2	
Unfractionated heparin ^c	28 (97%)
Antiplatelet drugs ^d	5 (17%)
[asp ($n = 2$); asp + can ($n = 1$); tic ($n = 1$); asp + clo ($n = 1$)]	
Day 3	
Unfractionated Heparin ^c	23 (96%)
Antiplatelet drugs ^d	4 (17%)
[asp ($n = 3$); asp + clo ($n = 1$)]	

Values are expressed as n (%), mean ($\pm$ standard deviation), or median [interquartile range].

ECMO, Extracorporeal membrane oxygenation; VV, Veno-venous; VA, Veno-arterial; ECPR, extracorporeal cardiopulmonary resuscitation; h, hours; min, minutes; SOFA, Sequential Organ Failure Assessment; ICU, Intensive care unit; INR, International normalized ratio; CRP, C-reactive protein; asp, aspirin; can, cangrelor; tic, ticagrelor; clo, clopidogrel.

^aAvailable for 18 patients only.

^bCombined reference interval for men and women.

^cActive infusion at time of blood sampling.

^dAdministered within the past 24 h prior to blood sampling.

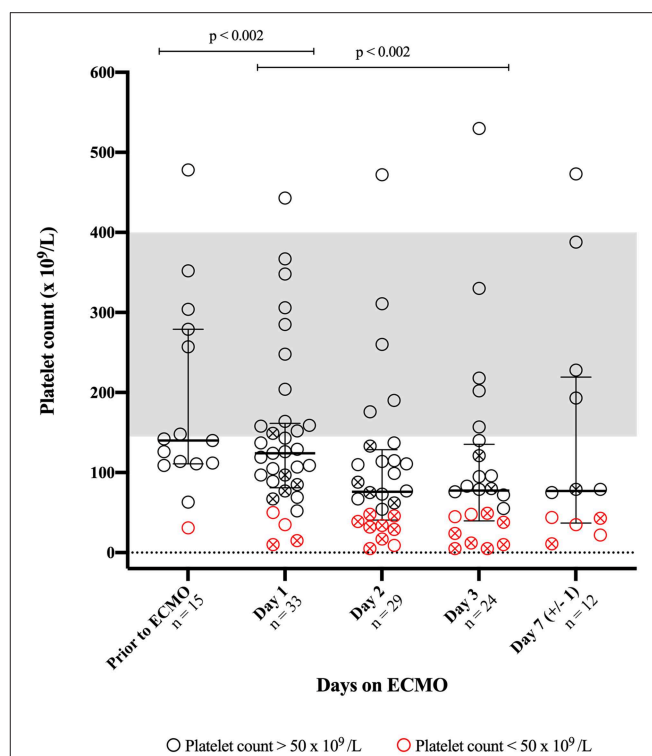


FIGURE 1 | Platelet counts obtained prior to and during support with extracorporeal membrane oxygenation (ECMO). The bars indicate median and interquartile range. The gray area represents a combined reference interval on platelet count for men and women. An X-mark in the circle indicates that the patient has received platelet concentrate within 24 h prior to blood sampling.

Laboratory parameters, obtained on day 1 of ECMO support, are presented in **Table 1**. The median APTT indicates that patients were sufficiently anticoagulated with unfractionated heparin according to clinical standard. In total, nine patients received antiplatelet therapy during ECMO support administered within the past 24 h prior to blood sampling (**Table 1**).

Platelet Count and Platelet Turnover

A platelet count prior to ECMO initiation was possible to obtain from 15 patients. Results from blood samples obtained prior to ECMO initiation could not be obtained from 10 patients, who were put on ECMO support outside the Central Region of Denmark and subsequently transported to Aarhus University Hospital for further management. Furthermore, 8 patients, put on ECMO support for extracorporeal cardiopulmonary resuscitation (ECPR), did not have blood samples taken prior to ECMO initiation.

The median duration from blood sampling prior to ECMO until ECMO initiation was 8 h 2 min (± 6 h 30 min). As seen in **Figure 1**, Platelet counts decreased significantly during ECMO support; from before ECMO initiation to day 1 ($p < 0.002$), and from day 1 to day 3 ($p < 0.002$). Platelet counts did not appear to change from day 3 to day 7 (± 1) ($p = 0.74$). Median platelet counts ($\times 10^9/\text{L}$) were 140 (prior to ECMO); 124 (day 1); 76 (day 2); 78 (day 3); and 77 (day 7 ± 1). Two patients

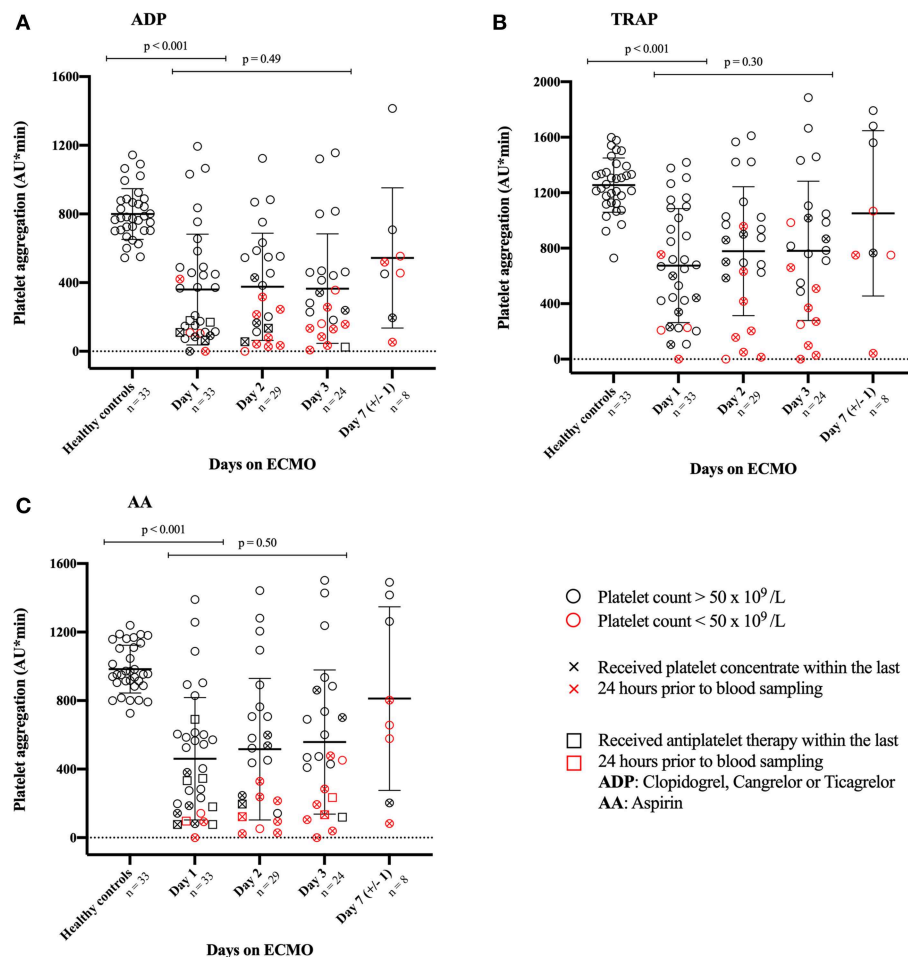


FIGURE 2 | Illustrates the platelets ability to aggregate in response to stimulation by three different agonists. Platelet aggregation was measured during support with extracorporeal membrane oxygenation (ECMO) and compared with healthy controls. Agonists used to stimulate platelet aggregation: **(A)** ADP, Adenosine diphosphate; **(C)** AA, arachidonic acid; and **(B)** TRAP, thrombin receptor activating peptide-6. The bars indicate mean with standard deviation. An X-mark in the circle indicates that the patient has received platelet concentrate within 24 h prior to blood sampling. To illustrate the association between platelet aggregation and platelet count, patients with platelet counts below 50×10^9 /L are marked red. Due to practical reasons, platelet aggregation on day 2 was not measured in 2 patients.

consistently demonstrated the highest platelet counts ($> 388 \times 10^9$ /L) throughout the course of ECMO support.

In total, 20 patients received transfusions of platelet concentrates during ECMO support, denoted by an X-mark in the circles of **Figure 1**; the median amount of transfused platelet concentrate per day was: 732 ml (IQR: 378–1018) on day 1; 372 ml (IQR: 368–758 ml) on day 2; 563 ml (IQR: 378–768 ml) on day 3; and 752 ml (IQR: 527–1064 ml) on day 7 (± 1).

Platelet turnover accelerated during ECMO support, demonstrated by a significant increase in IPF from day 1 to day 3 of ECMO support; median IPF was 0.047 (IQR 0.032–0.080) on day 1 and 0.067 (IQR 0.045–0.133) on day 3 ($p = 0.004$).

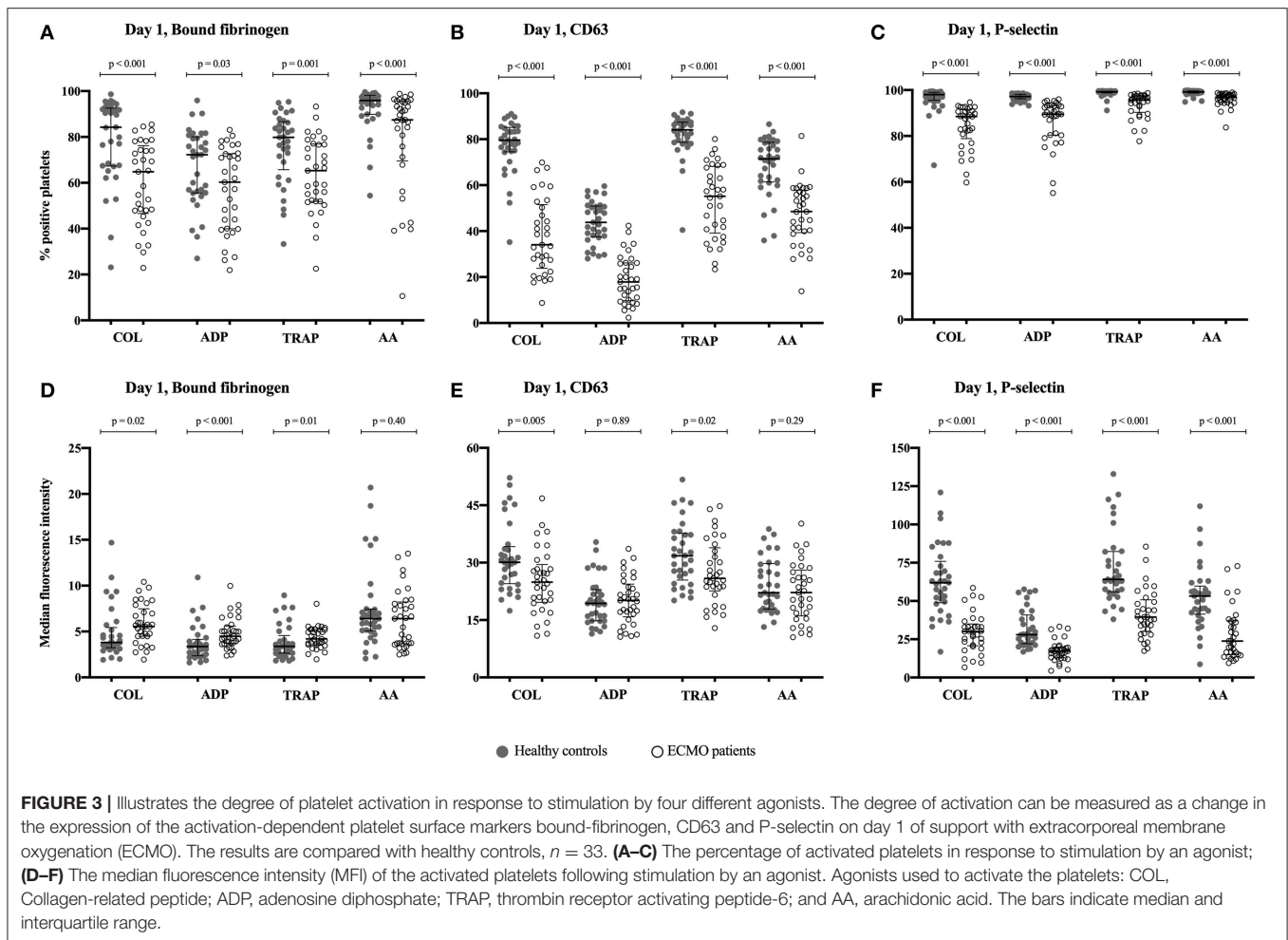
Platelet Function

Whole Blood Impedance Aggregometry

ECMO patients did not differ significantly from healthy controls on either gender or age (age: $p > 0.86$ for all agonists). As shown in **Figure 2**, ECMO patients demonstrated significantly reduced platelet aggregation on day 1 compared with healthy controls

following stimulation by all three agonists (all $p < 0.001$). As demonstrated by the red colored symbols in **Figure 2**, patients with platelet counts below 50×10^9 /L generally exhibited the lowest levels of platelet aggregation following agonist stimulation. **Figure 2** shows no significant difference in platelet aggregation from day 1 to day 3 following stimulation by any of the three agonists (ADP: $p = 0.49$; TRAP: $p = 0.30$; AA: $p = 0.50$). Overall, ECMO patients demonstrated a wide variation in platelet aggregation measured by impedance aggregometry during the course of ECMO support.

As shown in **Supplementary Figure 2**, the majority of ECMO patients demonstrated platelet aggregation within the 95% prediction interval on days 1, 2, and 3. Approximately one fourth of ECMO patients demonstrated platelet aggregation below the 95% prediction interval on day 1 after stimulation by ADP or TRAP. Following ADP stimulation on day 1, four patients with platelet counts above 200×10^9 /L demonstrated platelet aggregation below the 95% prediction interval. Of these, two patients demonstrated platelet aggregation within the 95%



prediction interval on days 2 and 3. The other two patients had received antiplatelet therapy within 24 h prior to blood sampling on day 1; they were both weaned from ECMO before day 2 due to recovery ($n = 1$) or mortality ($n = 1$). The patient with the highest platelet counts ($>470 \times 10^9/L$) demonstrated consistently low platelet aggregation on days 2 and 3. Following TRAP stimulation on day 1, the same four patients demonstrated platelet aggregation below the prediction interval, as was seen following ADP stimulation. Two additional patients fell below the 95% prediction interval on day 1 following TRAP stimulation; of these, one patient demonstrated platelet aggregation within the 95% prediction interval on days 2 and 3; the other patient demonstrated consistently low platelet aggregation on days 2 and 3 relative to the very high platelet counts ($>470 \times 10^9/L$). Of note, patients with similar platelet counts demonstrated widely varying platelet aggregation throughout all days on ECMO.

Flow Cytometry

ECMO patients did not differ significantly from healthy controls on gender or age; the mean age was $46 (\pm 11)$ years in controls and $49 (\pm 13)$ years in ECMO patients ($p = 0.34$). ECMO patients exhibited a slightly higher level of pre-activation of the platelets [$14\% (\pm 8)$] compared with healthy controls [$10\% (\pm 4)$] ($p = 0.03$).

Figure 3 illustrates the percentages of positive platelets (**Figures 3A–C**) and MFI of the platelets (**Figures 3D–F**) on day 1 of ECMO support compared with healthy controls. Following stimulation by four different agonists, the percentages of positive platelets were significantly lower in ECMO patients than in healthy controls for all platelet activation markers investigated: Bound fibrinogen (all $p < 0.03$), CD63 (all $p < 0.001$), and P-selectin (all $p < 0.001$). The MFI of bound fibrinogen was significantly higher in ECMO patients than in healthy controls following stimulation by COL, ADP, and TRAP; no difference was found in MFI of bound fibrinogen following stimulation by AA (**Figure 3D**). Conversely, the MFI of CD63 was significantly lower in ECMO patients than in healthy controls after stimulation by COL and TRAP; whereas stimulation by ADP and AA yielded no significant difference (**Figure 3E**). The MFI of P-selectin was significantly lower in ECMO patients than in healthy controls for all agonists (all $p < 0.001$) (**Figure 3F**).

Figure 4 illustrates the percentage of positive platelets (**Figures 4A–C**) and MFI (**Figures 4D–F**) of the activation-dependent platelet surface markers investigated on day 1 compared with day 3 of ECMO support. No significant difference was found in the percentages of positive platelets on day 1 compared with day 3 ($p > 0.05$); although, there were two

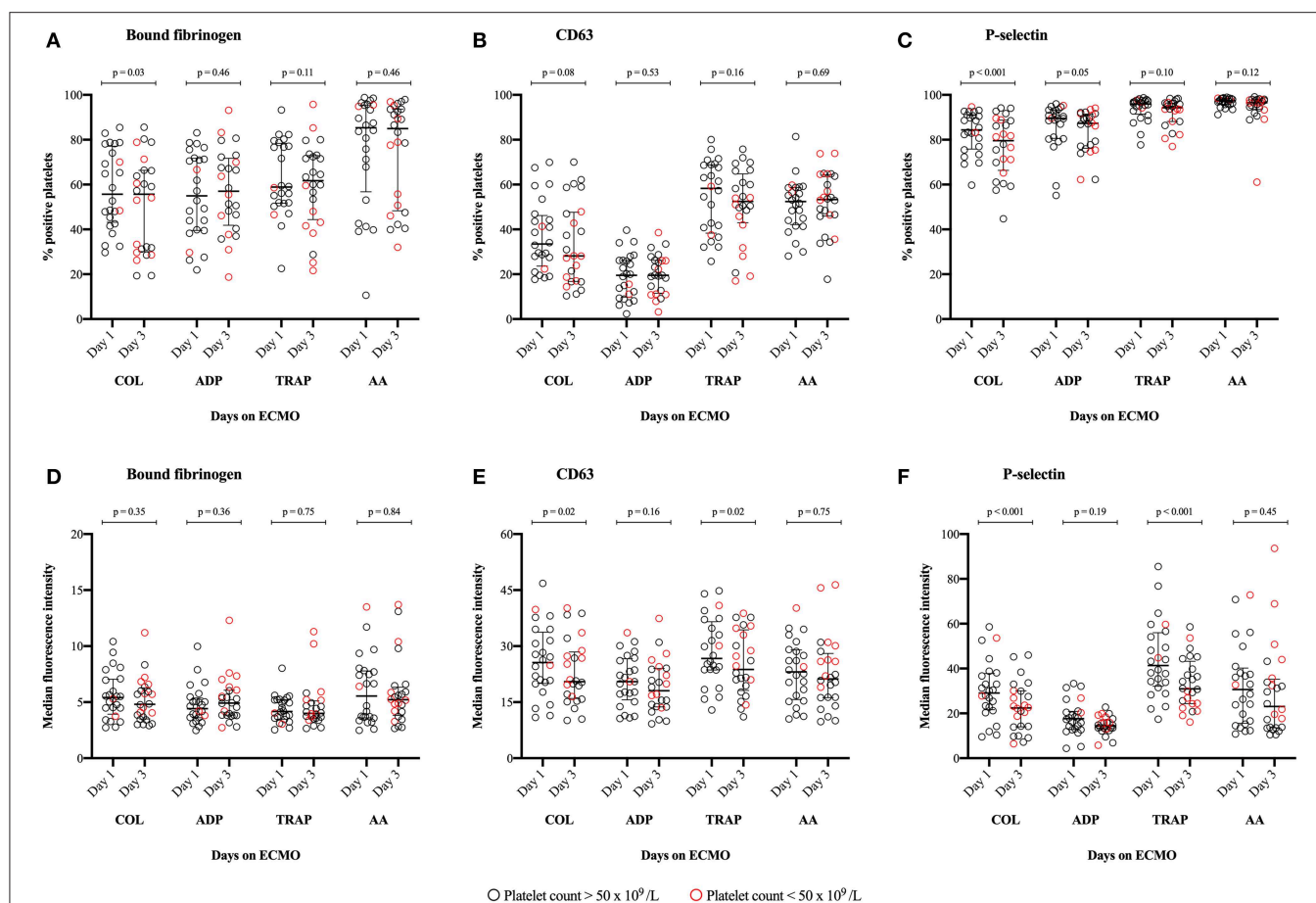


FIGURE 4 | Illustrates the degree of platelet activation in response to stimulation by four different agonists. The activation can be measured as a change in the expression of the activation-dependent platelet surface markers: Bound-fibrinogen, CD63 and P-selectin. **(A–C)** The percentage of activated platelets in response to stimulation by an agonist; **(D–F)** The median fluorescence intensity (MFI) of the activated platelets following stimulation by an agonist. Platelet activation on day 1 of support with extracorporeal membrane oxygenation (ECMO) is compared with platelet activation measured on day 3 of ECMO support, $n = 24$. Patients weaned from ECMO support prior to day 3 were excluded from this analysis on the difference between day 1 and 3. Agonists used to stimulate platelet activation: COL, Collagen-related peptide; ADP, adenosine diphosphate; TRAP, thrombin receptor activating peptide-6; and AA, arachidonic acid. The bars indicate median and interquartile range.

exceptions following stimulation with COL: Significantly reduced percentages of platelets positive for bound fibrinogen (**Figure 4A**: $p = 0.03$) and P-selectin (**Figure 4C**: $p < 0.001$) on day 3 compared with day 1. Likewise, no significant difference was found in the MFI of bound fibrinogen on activated platelets on day 1 compared with day 3 (**Figure 4D**: $p > 0.35$). Regarding CD63 and P-selectin, we found: Significantly reduced MFI on day 3 compared with day 1 following stimulation by COL and TRAP ($p < 0.02$), but no significant differences following stimulation by ADP and AA ($p > 0.16$) (**Figures 4E,F**). Overall, ECMO patients demonstrated a wide variation in expression of the activation-dependent platelet surface markers.

Clinical Endpoints

Table 2 summarizes the use of blood products during ECMO support for all patients; 91% of patients received red blood cells (RBC); 57% received fresh-frozen plasma (FFP); and 57% received platelet concentrates during ECMO support.

TABLE 2 | An overview of the use of blood products during support with extracorporeal membrane oxygenation (ECMO) for all patients ($n = 33$).

Amount, mL	RBC, n (%)	FFP, n (%)	Platelets, n (%)
None	3 (9%)	14 (43%)	14 (43%)
1–1,500	14 (43%)	13 (39%)	8 (24%)
1,500–3,000	9 (27%)	2 (6%)	7 (21%)
3,000–4,500	2 (6%)	1 (3%)	2 (6%)
>4,500	5 (15%)	3 (9%)	2 (6%)

ECMO, Extracorporeal membrane oxygenation; RBC, Red blood cells; FFP, Fresh-frozen plasma; mL, milliliter.

DISCUSSION

During ECMO support, platelet counts decreased despite administration of platelet concentrates. Moreover, platelet aggregation was impaired during ECMO support compared with healthy controls; this finding was challenged by the observation that platelet aggregation values relative to platelet count, in

ECMO patients, were within the 95% prediction interval of platelet aggregation in healthy whole blood. Flow cytometry analyses demonstrated an impaired platelet activation in ECMO patients on day 1 compared with healthy controls. However, no substantial difference was found in platelet activation from day 1 to day 3 on ECMO support.

Platelet Count and Turnover

Following ECMO initiation, platelet counts decreased significantly and remained low during ECMO support, despite administration of platelet concentrates to 57% of patients. This finding is consistent with previous studies reporting thrombocytopenia during ECMO support (17, 39). A consumptive loss of platelets—due to adherence to the artificial surfaces and formation of microthrombi in the circulation—has been proposed as a plausible mechanism for the decrease in platelet counts during ECMO support (40). Two recent studies found no association between duration of ECMO support and development of severe thrombocytopenia, but found thrombocytopenia to be associated with platelet count at ECMO cannulation, and the development of multi-organ failure while on ECMO (41, 42).

The significant increase in IPF from day 1 to day 3 on ECMO support indicates an augmented platelet turnover (43); this is consistent with a consumptive loss of platelets during ECMO support. Immature platelets exhibit an increased hemostatic potential (44, 45); they tend to contain more granules, and express a greater number of activation-dependent platelet surface markers compared with mature platelets (46). A high platelet turnover rate could theoretically yield a larger population of platelets that are more easily activated, thereby increasing thrombotic propensity and accelerating the consumptive platelet loss.

Platelet Function

Compared with healthy controls, platelet aggregation was significantly lower on day 1 of ECMO support, and the platelets ability to aggregate remained low during ECMO. Patients with the lowest platelet counts generally demonstrated the lowest platelet aggregation; this finding is consistent with the well-established association between platelet count and platelet aggregation, when employing impedance aggregometry (23–29). Our impedance aggregometry findings are in line with previous studies on platelet aggregation during ECMO support in adult patients (16–19). Importantly, these previous conclusions on platelet aggregation are hampered by not considering the effect of low platelet counts on platelet aggregation. To overcome this limitation, we assessed platelet aggregation results in relation to the 95% prediction interval of platelet aggregation in healthy whole blood with varying platelet counts (30). Employing this approach, we demonstrated that platelet aggregation in ECMO patients was largely equivalent to platelet aggregation in healthy controls, when considering the impact of low platelet counts. Thus, our findings indicate that platelet aggregation during ECMO support is impaired due to a low number of platelets.

When employing flow cytometry, no association was found between platelet count and platelet activation. On day 1 of ECMO support, the platelets demonstrated a reduced ability to

become activated, when stimulated by an exogenous agonist; this was demonstrated by the significantly lower percentage of activated platelets on day 1 in ECMO patients compared with healthy controls. The impaired ability to become activated could be due to vast activation of platelets in the ECMO circuit. Previous studies have suggested that platelets are affected by high shear forces in the circuit of blood-contacting medical devices, resulting in platelet activation and adherence to the surfaces of the circuit (14, 15, 47, 48). Furthermore, surface-induced platelet activation is thought to occur in the ECMO circuit, as platelets come into contact with the artificial surface of the circuit (13). Excessive activation, adhesion and aggregation of platelets may increase the risk of thrombotic complications due to the formation of microaggregates in the circulation. Thus, circulating activated platelets may be partially degranulated, circulating in an exhausted state, and therefore exhibit a decreased responsiveness to agonist stimulation, as demonstrated in the present study. Interestingly, recent studies have shown that high shear stress also causes the loss of platelet receptors (GPIb α and GPVI) important for platelet adhesion (20, 49, 50). Loss of these receptors result in decreased platelet adhesion, leading to impaired hemostatic capacity and an increased potential of bleeding.

The MFI of bound fibrinogen was significantly increased in ECMO patients compared with healthy controls for three out of four agonists. This suggests that activated platelets in ECMO patients express a larger quantity of bound fibrinogen on their surface, than healthy platelets do. This finding is consistent with studies reporting an increased expression of the activated fibrinogen receptor, GPIIb/IIIa, on platelets exposed to high shear stress, as in the ECMO circuit (51, 52). As high shear stress activates the platelets, a sequence of signaling events is triggered, resulting in the activation of the glycoprotein receptor GPIIb/IIIa, which then mediates platelet aggregation and thrombus formation (53).

On the contrary, the expression of P-selectin was consistently lower on platelets from ECMO patients than platelets from healthy controls measured by the MFI. This finding may be consistent with preceding platelet activation in the ECMO circuit and subsequent shedding of P-selectin into the circulation (54)—resulting in platelets expressing a lesser amount of P-selectin on their surface. Cheung et al. (55) measured soluble P-selectin levels in 10 neonates treated with ECMO and found significantly elevated levels of soluble P-selectin 4 h after ECMO initiation, indicating *in vivo* platelet activation. Further studies of soluble P-selectin, in adult ECMO patients, would be valuable in this regard.

Considerations on the possible clinical impact of the aforementioned findings led us to speculate, whether therapeutically increasing platelet counts in these patients, could enhance platelet aggregation; thereby reducing the risk of hemorrhage. Furthermore; increasing platelet counts may yield a larger population of platelets able to become activated, further reducing the risk of hemorrhage. Yet, a concern is the concurrent risk of thrombosis, and the possibility of vast platelet activation *in vivo* due to high shear stresses and adherence to the protein-coated surfaces of the circuit. A larger study, investigating the association between platelet count and incidence of bleeding

and thromboembolic events during ECMO support, would be of great interest in this regard.

Strengths and Limitations

A major strength of the present study is the use of flow cytometry to investigate platelet function in ECMO patients with varying degrees of thrombocytopenia. Furthermore, by employing a model that describes platelet aggregation in healthy thrombocytopenic whole blood, we were able to evaluate platelet aggregation, measured by impedance aggregometry, relative to the platelet count.

The purpose of the present study was to contribute to further knowledge on the hemostatic changes occurring during ECMO support. Using two different methods, whole blood impedance aggregometry and flow cytometry, we aimed to elucidate different aspects of platelet function, however, other biomarkers and methods could have been employed: i.e., soluble levels of P-selectin or β -thromboglobulin, other surface-bound markers of platelet function (i.e., GPIIb/IIIa, GPVI, CD40L), circulating platelet microparticles, circulating matrix metalloproteinases and platelet-leucocyte aggregates. Furthermore, this study is limited to the description of platelet count and function, not taking into account the wide interactions with leucocytes, nor blood-surface interactions throughout the circuit.

The results of the present study are confounded by the therapy some patients received while on ECMO support. In total, 57% of patients received platelet concentrates throughout the course of ECMO support (Table 2) (56). A time-dependent increase in platelet activation has been reported to occur during storage of platelet concentrates (57). Moreover, the response toward agonist stimulation has been shown to decrease during storage (56). Thus, our platelet function analyses are hampered by these exogenously provided platelets that have been activated during storage, and whose function is probably somewhat impaired. Additionally, therapy during ECMO support included administration of antiplatelet drugs in nine patients and continuous administration of unfractionated heparin to the vast majority of patients (Table 1). Both antiplatelet agents and unfractionated heparin alter platelet function. Previous studies have reported unfractionated heparin to increase the number of activated platelets and sensitizes the platelets to agonist stimulation (58, 59). Thus, these therapies must be considered as confounders of the results in the present study.

Despite performing a sample size calculation and achieving the required number of included patients, the study population is rather small, limiting the possibilities for sub-group analysis or more advanced statistical analysis. An additional limitation is the lack of platelet function analyses on the entire study population prior to ECMO initiation. It remains unclear whether the observed platelet dysfunction can be associated with ECMO initiation, with the underlying disease state of the patients, or a combination of both. However, it is likely that initiating ECMO has a crucial impact on platelets, as platelet counts significantly decrease after ECMO initiation compared with before ECMO.

CONCLUSIONS

We hypothesized that ECMO support would result in increased platelet function from day 1 to day 3 of support. Employing impedance aggregometry and flow cytometry, we found both impaired platelet aggregation and decreased platelet activation on day 1 of ECMO support compared with healthy controls. However, platelet aggregation was not impaired, when interpreted relative to the low platelet counts. Thus, our findings indicate that platelet aggregation during ECMO support is impaired due to a low number of platelets. Furthermore, levels of bound fibrinogen, on the surface of activated platelets in ECMO patients, were higher than in healthy controls. Together, these findings suggestively oppose that platelets are universally impaired during ECMO support. No marked difference in activation from day 1 to day 3 was seen during ECMO support.

DATA AVAILABILITY

The datasets for this manuscript are not publicly available because of a small number of included participants. Requests to access the datasets should be directed to A-MH, am.hvas@dadlnet.dk.

ETHICS STATEMENT

This study was approved as a quality assurance study by The Central Denmark Region Committees on Health and Research Ethics (enquiry 213-2016); therefore, according to Danish law, written informed consent was not required. The Danish Data Protection Agency approved the study (Ref. no. 1-16-02-712-17).

AUTHOR CONTRIBUTIONS

CB performed the laboratory analyses together with laboratory technicians Mai Stenulm Veirup and Vivi Bo Mogensen. CB extracted all clinical data from the patient medical records and took the lead in writing the manuscript. A-MH was responsible for the idea and scientific quality of the study. CB, A-MH, and AJ decided on which statistical analyses, tables and figures were to be included in the final manuscript. All authors contributed to a scientific discussion of the findings and provided critical revisions of the manuscript.

FUNDING

The present study was funded by a grant from Aarhus University, Denmark, together with private funding: Helge Peetz and Verner Peetz and wife Wilma Peetz's Foundation; Merchant L.F. Foghts Fund; P.A. Messerschmidt and wife's Fund; and The Danish Medical Association Research Fund.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcvm.2019.00114/full#supplementary-material>

REFERENCES

- Marasco SF, Lukas G, McDonald M, McMillan J, Ihle B. Review of ECMO (extra corporeal membrane oxygenation) support in critically ill adult patients. *Heart Lung Circ.* (2008) 17(Suppl. 4):S41–7. doi: 10.1016/j.hlc.2008.08.009
- Bartlett RH. ECMO: The next ten years. *Egypt J Crit Care Med.* (2016) 4:7–10. doi: 10.1016/j.ejccm.2016.01.003
- Thiagarajan RR, Barbaro RP, Rycus PT, McMullan DM, Conrad SA, Fortenberry JD, et al. Extracorporeal life support organization registry international report 2016. *ASAIO J.* (2017) 63:60–7. doi: 10.1097/MAT.0000000000000475
- Vaquer S, de Haro C, Peruga P, Oliva JC, Artigas A. Systematic review and meta-analysis of complications and mortality of veno-venous extracorporeal membrane oxygenation for refractory acute respiratory distress syndrome. *Ann Intensive Care.* (2017) 7:51. doi: 10.1186/s13613-017-0275-4
- Aubron C, Cheng AC, Pilcher D, Leong T, Magrin G, Cooper DJ, et al. Factors associated with outcomes of patients on extracorporeal membrane oxygenation support: a 5-year cohort study. *Crit Care.* (2013) 17:R73. doi: 10.1186/cc12681
- Brogan TV, Thiagarajan RR, Rycus PT, Bartlett RH, Bratton SL. Extracorporeal membrane oxygenation in adults with severe respiratory failure: a multi-center database. *Intensive Care Med.* (2009) 35:2105–14. doi: 10.1007/s00134-009-1661-7
- Arachchillage DRJ, Passariello M, Laffan M, Aw TC, Owen L, Banya W, et al. Intracranial hemorrhage and early mortality in patients receiving extracorporeal membrane oxygenation for severe respiratory failure. *Semin Thromb Hemost.* (2018) 44:276–86. doi: 10.1055/s-0038-1636840
- Kreyer S, Muders T, Theuerkauf N, Spitzhüttl J, Schellhaas T, Schewe JC, et al. Hemorrhage under veno-venous extracorporeal membrane oxygenation in acute respiratory distress syndrome patients: a retrospective data analysis. *J Thorac Dis.* (2017) 9:5017–29. doi: 10.21037/jtd.2017.11.05
- Sutter R. Acute neurologic complications during extracorporeal membrane oxygenation—a systematic review. *Crit Care Med.* (2018) 1:3223. doi: 10.1097/CCM.00000000000003223
- Fletcher Sandersjoo A, Bartek J, Jr, Thelin EP, Eriksson A, Elmi-Terander A, Broman M, et al. Predictors of intracranial hemorrhage in adult patients on extracorporeal membrane oxygenation: an observational cohort study. *J Intensive Care.* (2017) 5:27. doi: 10.1186/s40560-017-0223-2
- Zangrillo A, Landoni G, Biondi-Zoccai G, Greco M, Greco T, Frati G, et al. A meta-analysis of complications and mortality of extracorporeal membrane oxygenation. *Crit Care Resusc.* (2013) 15:172–8.
- Rastan AJ, Lachmann N, Walther T, Doll N, Gradistanac T, Gommert JF, et al. Autopsy findings in patients on postcardiotomy extracorporeal membrane oxygenation (ECMO). *Int J Artif Organs.* (2006) 29:1121–31. doi: 10.1177/039139880602901205
- Yoshimoto Y, Hasebe T, Takahashi K, Amari M, Nagashima S, Kamijo A, et al. Ultrastructural characterization of surface-induced platelet activation on artificial materials by transmission electron microscopy. *Microsc Res Tech.* (2013) 76:342–9. doi: 10.1002/jemt.22172
- Chen Z, Mondal NK, Zheng S, Koenig SC, Slaughter MS, Griffith BP, et al. High shear induces platelet dysfunction leading to enhanced thrombotic propensity and diminished hemostatic capacity. *Platelets.* (2017) 2017:1–8. doi: 10.1080/09537104.2017.1384542
- Chen Z, Mondal NK, Ding J, Koenig SC, Slaughter MS, Wu ZJ. Paradoxical effect of nonphysiological shear stress on platelets and von willebrand factor. *Artif Organs.* (2016) 40:659–68. doi: 10.1111/aor.12606
- Laine A, Niemi T, Suojäranta-Ylinen R, Raivio P, Soininen L, Lemstrom K, et al. Decreased maximum clot firmness in rotational thromboelastometry (ROTEM(R)) is associated with bleeding during extracorporeal mechanical circulatory support. *Perfusion.* (2016) 31:625–33. doi: 10.1177/0267659116647473
- Tauber H, Streif W, Fritz J, Ott H, Weigel G, Loacker L, et al. Predicting transfusion requirements during extracorporeal membrane oxygenation. *J Cardiothorac Vasc Anesth.* (2016) 30:692–701. doi: 10.1053/j.jvca.2016.01.009
- Mutlak H, Reyher C, Meybohm P, Papadopoulos N, Hanke AA, Zacharowski K, et al. Multiple electrode aggregometry for the assessment of acquired platelet dysfunctions during extracorporeal circulation. *Thorac Cardiovasc Surg.* (2015) 63:21–7. doi: 10.1055/s-0034-1383817
- Nair P, Hoechter DJ, Buscher H, Venkatesh K, Whittam S, Joseph J, et al. Prospective observational study of hemostatic alterations during adult extracorporeal membrane oxygenation (ECMO) using point-of-care thromboelastometry and platelet aggregometry. *J Cardiothorac Vasc Anesth.* (2015) 29:288–96. doi: 10.1053/j.jvca.2014.06.006
- Lukito P, Wong A, Jing J, Arthur JF, Marasco SF, Murphy DA, et al. Mechanical circulatory support is associated with loss of platelet receptors glycoprotein Ibalpha and glycoprotein VI. *J Thromb Haemost.* (2016) 14:2253–60. doi: 10.1111/jth.13497
- Chung JH, Yeo HJ, Kim D, Lee SM, Han J, Kim M, et al. Changes in the levels of beta-thromboglobulin and inflammatory mediators during extracorporeal membrane oxygenation support. *Int J Artif Organs.* (2017) 40:575–80. doi: 10.5301/ijao.5000617
- Kalbhenn J, Schlagenhauf A, Rosenfelder S, Schmutz A, Zieger B. Acquired von Willebrand syndrome and impaired platelet function during venovenous extracorporeal membrane oxygenation: rapid onset and fast recovery. *J Heart Lung Transplant.* (2018) 37:985–91. doi: 10.1016/j.healun.2018.03.013
- Skipper MT, Rubak P, Larsen OH, Hvas A-M. Thrombocytopenia model with minimal manipulation of blood cells allowing whole blood assessment of platelet function. *Platelets.* (2016) 27:295–300. doi: 10.3109/09537104.2015.1095873
- Femia EA, Scavone M, Lecchi A, Cattaneo M. Effect of platelet count on platelet aggregation measured with impedance aggregometry (Multiplate analyzer) and with light transmission aggregometry. *J Thromb Haemost.* (2013) 11:2193–6. doi: 10.1111/jth.12432
- Würtl M, Hvas A-M, Kristensen SD, Grove EL. Platelet aggregation is dependent on platelet count in patients with coronary artery disease. *Thromb Res.* (2012) 129:56–61. doi: 10.1016/j.thromres.2011.08.019
- Stissing T, Dridi NP, Ostrowski SR, Bochen L, Johansson PI. The influence of low platelet count on whole blood aggregometry assessed by Multiplate. *Clin Appl Thromb Hemost.* (2011) 17:E211–7. doi: 10.1177/1076029610397183
- Hanke AA, Roberg K, Monaca E, Sellmann T, Weber CF, Rahe-Meyer N, et al. Impact of platelet count on results obtained from multiple electrode platelet aggregometry (Multiplate). *Eur J Med Res.* (2010) 15:214–9. doi: 10.1186/2047-783x-15-5-214
- Rubak P, Villadsen K, Hvas AM. Reference intervals for platelet aggregation assessed by multiple electrode platelet aggregometry. *Thromb Res.* (2012) 130:420–3. doi: 10.1016/j.thromres.2012.06.017
- Hayward CP, Moffat KA, Pai M, Liu Y, Secharan J, McKay H, et al. An evaluation of methods for determining reference intervals for light transmission platelet aggregation tests on samples with normal or reduced platelet counts. *Thromb Haemost.* (2008) 100:134–45. doi: 10.1160/TH08-03-0183
- Skipper MT, Rubak P, Stentoft J, Hvas AM, Larsen OH. Evaluation of platelet function in thrombocytopenia. *Platelets.* (2017) 2017:1–7. doi: 10.1080/09537104.2017.1296566
- Linden MD, Frelinger AL III, Barnard MR, Przyklenk K, Furman MI, Michelson AD. Application of flow cytometry to platelet disorders. *Semin Thromb Hemost.* (2004) 30:501–11. doi: 10.1055/s-2004-835671
- Schmitz G, Rothe G, Ruf A, Barlage S, Tschöpe D, Clemetson KJ, et al. European working group on clinical cell analysis: consensus protocol for the flow cytometric characterisation of platelet function. *Thromb Haemost.* (1998) 79:885–96. doi: 10.1055/s-0037-1615088
- Charlson ME, Pompei P, Ales KL, MacKenzie CR. A new method of classifying prognostic comorbidity in longitudinal studies: development and validation. *J Chronic Dis.* (1987) 40:373–83. doi: 10.1016/0021-9681(87)90171-8
- Vasilevskis EE, Pandharipande PP, Graves AJ, Shintani A, Tsuruta R, Ely EW, et al. Validity of a modified sequential organ failure assessment score using the richmond agitation-sedation scale. *Crit Care Med.* (2016) 44:138–46. doi: 10.1097/CCM.0000000000001375
- Pedersen OH, Larsen ML, Grove EL, van Kooten Niekerk PB, Bonlokke S, Nissen PH, et al. Platelet characteristics in patients with essential thrombocythosis. *Cytometry B Clin Cytom.* (2018) 94:918–27. doi: 10.1002/cyto.b.21642

36. Rubak P, Nissen PH, Kristensen SD, Hvas AM. Investigation of platelet function and platelet disorders using flow cytometry. *Platelets*. (2016) 27:66–74. doi: 10.3109/09537104.2015.1032919
37. Ramstrom S, Sodergren AL, Tynngard N, Lindahl TL. Platelet function determined by flow cytometry: new perspectives? *Semin Thromb Hemost*. (2016) 42:268–81. doi: 10.1055/s-0035-1570082
38. Pedersen OH, Nissen PH, Hvas AM. Platelet function investigation by flow cytometry: sample volume, needle size, and reference intervals. *Platelets*. (2018) 29:199–202. doi: 10.1080/09537104.2017.1353684
39. Weingart C, Lubnow M, Philipp A, Bein T, Camboni D, Muller T. Comparison of coagulation parameters, anticoagulation, and need for transfusion in patients on interventional lung assist or veno-venous extracorporeal membrane oxygenation. *Artif Organs*. (2015) 39:765–73. doi: 10.1111/aor.12464
40. Oliver WC. Anticoagulation and coagulation management for ECMO. *Semin Cardiothorac Vasc Anesth*. (2009) 13:154–75. doi: 10.1177/1089253209347384
41. Abrams D, Baldwin MR, Champion M, Agerstrand C, Eisenberger A, Bacchetta M, et al. Thrombocytopenia and extracorporeal membrane oxygenation in adults with acute respiratory failure: a cohort study. *Intensive Care Med*. (2016) 42:844–52. doi: 10.1007/s00134-016-4312-9
42. Dzierba AL, Roberts R, Muir J, Alhammad A, Schumaker G, Clark J, et al. Severe thrombocytopenia in adults with severe acute respiratory distress syndrome: impact of extracorporeal membrane oxygenation use. *ASAIO J*. (2016) 62:710–4. doi: 10.1097/MAT.0000000000000415
43. Ault KA, Rinder HM, Mitchell J, Carmody MB, Vary CP, Hillman RS. The significance of platelets with increased RNA content (reticulated platelets). A measure of the rate of thrombopoiesis. *Am J Clin Pathol*. (1992) 98:637–46. doi: 10.1093/ajcp/98.6.637
44. Fager AM, Wood JP, Bouchard BA, Feng P, Tracy PB. Properties of procoagulant platelets: defining and characterizing the subpopulation binding a functional prothrombinase. *Arterioscler Thromb Vasc Biol*. (2010) 30:2400–7. doi: 10.1161/ATVBAHA.110.216531
45. Ibrahim H, Schutt RC, Hannawi B, DeLao T, Barker CM, Kleiman NS. Association of immature platelets with adverse cardiovascular outcomes. *J Am Coll Cardiol*. (2014) 64:2122–9. doi: 10.1016/j.jacc.2014.06.1210
46. Guthikonda S, Lev EI, Patel R, DeLao T, Bergeron AL, Dong JF, et al. Reticulated platelets and uninhibited COX-1 and COX-2 decrease the antiplatelet effects of aspirin. *J Thromb Haemost*. (2007) 5:490–6. doi: 10.1111/j.1538-7836.2007.02387.x
47. Slepian MJ, Sheriff J, Hutchinson M, Tran P, Bajaj N, Garcia JGN, et al. Shear-mediated platelet activation in the free flow: perspectives on the emerging spectrum of cell mechanobiological mechanisms mediating cardiovascular implant thrombosis. *J Biomech*. (2017) 50:20–5. doi: 10.1016/j.jbiomech.2016.11.016
48. Sheriff J, Tran PL, Hutchinson M, DeCook T, Slepian MJ, Bluestein D, et al. Repetitive hypershear activates and sensitizes platelets in a dose-dependent manner. *Artif Organs*. (2016) 40:586–95. doi: 10.1111/aor.12602
49. Chen Z, Mondal NK, Ding J, Gao J, Griffith BP, Wu ZJ. Shear-induced platelet receptor shedding by non-physiological high shear stress with short exposure time: glycoprotein Ibalpha and glycoprotein VI. *Thromb Res*. (2015) 135:692–8. doi: 10.1016/j.thromres.2015.01.030
50. Al-Tamimi M, Tan CW, Qiao J, Pennings GJ, Javadzadegan A, Yong AS, et al. Pathologic shear triggers shedding of vascular receptors: a novel mechanism for down-regulation of platelet glycoprotein VI in stenosed coronary vessels. *Blood*. (2012) 119:4311–20. doi: 10.1182/blood-2011-10-386607
51. Chen Z, Li T, Kareem K, Tran D, Griffith BP, Wu ZJ. The role of PI3K/Akt signaling pathway in non-physiological shear stress-induced platelet activation. *Artif Organs*. (2019). doi: 10.1111/aor.13465. [Epub ahead of print].
52. Chen Z, Zhang J, Kareem K, Tran D, Conway RG, Arias K, et al. Device-induced platelet dysfunction in mechanically assisted circulation increases the risks of thrombosis and bleeding. *Artif Organs*. (2019) 43:745–55. doi: 10.1111/aor.13445
53. Shattil SJ, Newman PJ. Integrins: dynamic scaffolds for adhesion and signaling in platelets. *Blood*. (2004) 104:1606–15. doi: 10.1182/blood-2004-04-1257
54. Au AE, Josefsson EC. Regulation of platelet membrane protein shedding in health and disease. *Platelets*. (2017) 28:342–53. doi: 10.1080/09537104.2016.1203401
55. Cheung PY, Sawicki G, Salas E, Etches PC, Schulz R, Radomski MW. The mechanisms of platelet dysfunction during extracorporeal membrane oxygenation in critically ill neonates. *Crit Care Med*. (2000) 28:2584–90. doi: 10.1097/00003246-200007000-00067
56. Tynngard N. Preparation, storage and quality control of platelet concentrates. *Transfus Apher Sci*. (2009) 41:97–104. doi: 10.1016/j.transci.2009.07.001
57. Vucetic D, Ilic V, Vojvodic D, Subota V, Todorovic M, Balint B. Flow cytometry analysis of platelet populations: usefulness for monitoring the storage lesion in pooled buffy-coat platelet concentrates. *Blood Transfus*. (2018) 16:83–92. doi: 10.2450/2016.0193-16
58. Xiao Z, Theroux P. Platelet activation with unfractionated heparin at therapeutic concentrations and comparisons with a low-molecular-weight heparin and with a direct thrombin inhibitor. *Circulation*. (1998) 97:251–6. doi: 10.1161/01.CIR.97.3.251
59. Gao C, Boylan B, Fang J, Wilcox DA, Newman DK, Newman PJ. Heparin promotes platelet responsiveness by potentiating alphaIIb beta3-mediated outside-in signaling. *Blood*. (2011) 117:4946–52. doi: 10.1182/blood-2010-09-307751

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Balle, Jeppesen, Christensen and Hvas. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Regenerative Effect of Platelet Concentrates in Oral and Craniofacial Regeneration

Faez Saleh Al-Hamed¹, Mohammed Mahri^{1,2}, Haider Al-Waeli¹, Jesus Torres³, Zahi Badran^{1,4} and Faleh Tamimi^{1*}

¹ Faculty of Dentistry, McGill University, Montreal, QC, Canada, ² Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Jazan University, Jazan, Saudi Arabia, ³ Faculty of Dentistry, Universidad Complutense, Madrid, Spain, ⁴ Department of Periodontology (CHU/Rmes Inserm U1229/UIC11), Faculty of Dental Surgery, University of Nantes, Nantes, France

OPEN ACCESS

Edited by:

Paul Jurasz,
University of Alberta, Canada

Reviewed by:

Aneta Radziwon-Balicka,
Herlev Hospital, Denmark
Julia Etulain,
CONICET Institute of Experimental
Medicine, National Academy of
Medicine, Argentina

*Correspondence:

Faleh Tamimi
faleh.tamimimarino@mcgill.ca

Specialty section:

This article was submitted to
Atherosclerosis and Vascular
Medicine,
a section of the journal
Frontiers in Cardiovascular Medicine

Received: 15 May 2019

Accepted: 12 August 2019

Published: 30 August 2019

Citation:

Al-Hamed FS, Mahri M, Al-Waeli H,
Torres J, Badran Z and Tamimi F
(2019) Regenerative Effect of Platelet
Concentrates in Oral and Craniofacial
Regeneration.
Front. Cardiovasc. Med. 6:126.
doi: 10.3389/fcvm.2019.00126

Platelet concentrates (PCs) are biological autologous products derived from the patient's whole blood and consist mainly of supraphysiologic concentration of platelets and growth factors (GFs). These GFs have anti-inflammatory and healing enhancing properties. Overall, PCs seem to enhance bone and soft tissue healing in alveolar ridge augmentation, periodontal surgery, socket preservation, implant surgery, endodontic regeneration, sinus augmentation, bisphosphonate related osteonecrosis of the jaw (BRONJ), osteoradionecrosis, closure of oroantral communication (OAC), and oral ulcers. On the other hand, no effect was reported for gingival recession and guided tissue regeneration (GTR) procedures. Also, PCs could reduce pain and inflammatory complications in temporomandibular disorders (TMDs), oral ulcers, and extraction sockets. However, these effects have been clinically inconsistent across the literature. Differences in study designs and types of PCs used with variable concentration of platelets, GFs, and leucocytes, as well as different application forms and techniques could explain these contradictory results. This study aims to review the clinical applications of PCs in oral and craniofacial tissue regeneration and the role of their molecular components in tissue healing.

Keywords: platelet concentrates, clinical applications, growth factors, platelets, oral tissue regeneration

INTRODUCTION

Platelet concentrates (PCs) are biological autologous products derived from the patient's whole blood that consist mainly of supraphysiological concentrations of platelets and growth factors (GFs). There are two main types of PCs: platelet-rich plasma (PRP) and platelet rich fibrin (PRF), which can be pure (i.e., P-PRP, P-PRF) or rich in leucocytes (i.e., L-PRP, L-PRF) (1, 2). PCs can also be prepared with or without red blood cells (3). PRP and P-PRF are prepared from anti-coagulated blood, whereas L-PRF is prepared from non-anti-coagulated blood. PC is prepared using gravitational centrifugation techniques, standard cell separators, or autologous selective filtration technique (plateletpheresis) (3). PCs contain high concentrations of growth factors (GFs) and cytokines that play a vital role in the healing of various tissues. PCs have been used alone or as an adjunctive treatment to enhance soft and hard tissue regeneration in dentoalveolar, and maxillofacial surgeries (4–6).

There are four phases of tissue healing: hemostasis phase, inflammatory phase, reparative phase, and remodeling phase. Blood clot serves as a matrix that allows cell movement and proliferation. Platelets are essential for blood clot formation (7). The inflammatory phase starts from the first day to the seventh day after injury (8). In this phase, platelet releases different growth factors that attract inflammatory cells: neutrophils, macrophages and lymphocytes to the injured site. These cells secrete pro-inflammatory cytokines: Tumor necrosis factor alpha (TNF- α), interleukins (IL-1, IL-6, IL-18) that enhance angiogenesis and tissue healing (9, 10). In the reparative phase, mesenchymal stem cells (MSCs) are recruited from the adjacent soft tissue or blood vessels (11). Different growth factors such as bone morphogenic proteins (BMPs) and TGF- β play a role in MSCs recruitment. They enhance bone regeneration as they induce osteoblast differentiation (12). The remodeling phase is characterized by replacement of woven bone with lamellar bone. GFs are also involved in this phase (10, 12).

This study aims to review the clinical applications of PCs in oral and maxillofacial surgery procedures including maxillary sinus augmentation, alveolar ridge augmentation, implant surgery, jaw cysts, periodontal surgery, socket preservation, endodontic surgery, alveolar clefts, cleft lip and palate, oroantral communication (OAC), oral ulcers, osteoradionecrosis, bisphosphonate related osteonecrosis of the jaw (BRONJ), and temporomandibular joint disorders. In addition, the main cellular and molecular components of PCs were discussed with an emphasis on their regenerative properties.

TYPES OF PLATELET CONCENTRATES USED IN ORAL AND CRANIOFACIAL RECONSTRUCTION

Platelet-Rich Plasma (PRP)

PRP is the first generation of PCs. It can be prepared using centrifugation or plasmapheresis. The centrifugation can be done in one or two steps (i.e., single or double spin) using various forces (g) and centrifugation times. The centrifugation time ranges from 8 to 30 min (13). The most commonly used centrifugation forces (g) range from 100 to 1,000 g (14, 15), however, some protocols used forces (g) as high as 3,731 g (16). The most common described one step centrifugation protocol is plasma rich in growth factors (*Anitua's PRGF*). In this protocol, plasma is divided into two fractions, the top fraction (platelet poor plasma) and the bottom fraction, including the buffy coat, contains higher concentration of platelets. For the two-step centrifugation protocol, first spin separates the blood in the centrifugation tubes into three layers: red blood cells (RBCs) in the bottom, a buffy coat (BC) rich in platelets and leukocytes in the middle, and platelet poor plasma (PPP) in the top. To produce P-PRP, the PPP layer, and the upper part of the BC layer are transferred to another tube and exposed to a second centrifugation. Then, most of the PPP layer is removed and the platelet pellet concentration in the bottom of the centrifugation tube is resuspended in a small volume of plasma to produce P-PRP. In this protocol, most

of leukocytes are discarded. To produce L-PRP, the PPP, the whole BC, and part of RBCs layer are transferred to another tube and centrifuged. The L-PRP consists of the platelet and leukocyte concentration and some RBCs suspended in a small fraction of plasma (1). The increase in platelet count achieved with different PRP methods can range from 2.6 to 7.3 folds, but the two spin protocols the ones that yield higher platelet concentration (13).

PRP can be applied without activation to the recipient site as the exposed collagen or thrombin produced as a result of tissue injury activates platelets (17). However, an activator such as calcium chloride (CaCl₂), thrombin, collagen, calcium gluconate, lysis by freezing, photoactivation, or a mixture of CaCl₂ and thrombin is commonly added before the application. Calcium chloride is the most commonly used activator to counteract the effect of the anticoagulants. The purpose of platelet activation is to enhance the release of GFs from α -granules and to form the gel (17–19) (Table 1).

Platelet-Rich Fibrin (PRF)

PRF is the second generation of PCs. L-PRF is more commonly used than P-PRF. It can be prepared with a simple inexpensive process without the addition of anticoagulants by using a single spin protocol [3,000 rpm (~700 g) for 10 min] that results in 1–2 folds increase in platelet count (20). The slow polymerization during PRF preparation generates a fibrin network that enhances cell migration and proliferation. In addition, this network acts as a reservoir of platelets, leukocytes, and GFs (21). L-PRF forms a gel that can be applied directly to the surgical site or can be compressed with a special kit to form a membrane that used to cover bone grafts in different augmentation procedures (22).

P-PRF can be prepared only by one method (*Fibrinet PRFM kit*) using two spin protocol. Blood is collected in specified tubes containing tri-sodium citrate and a separator gel, and then centrifuged at 1,100 rpm for 6 min. This separates the blood into three layers: RBCs, BC, and PPP. The BC and the PPP layers are collected and activated with CaCl₂, followed by second centrifugation at 4,500 rpm for 15 min. Then, platelet rich fibrin matrix (PRFM) clot can be collected and applied to the surgical site (23) (Table 1).

PCs consist of different concentration of platelets, white blood cells, plasma proteins, and GFs. Each component may affect tissue regeneration.

Platelets

Platelets are ~2.5 μ m long cell fragments derived from bone marrow megakaryocytes (24). The normal blood platelet count in healthy individuals ranges from 150,000 to 450,000 platelets/ μ l. Although platelets lack nuclei, they contain other organelles including mitochondria, microtubules, α -granules, dense granules, and lysosomes (25).

Platelet ultrastructure can be divided into four zones: the peripheral zone, the sole-gel zone, the organelle zone, and the membrane system. The peripheral zone contains the plasma membrane, a smooth membrane rich in glycocalyx [glycoprotein (GP)], lipid bilayer area, and actin filaments. The

TABLE 1 | Types of platelet concentrates (PCs) (1–3, 19).

Type of PCs	Type of preparation protocol	Platelet concentration	Leukocytes, + = > 1%	Red blood cells, + = > 10%	Activation	Commercial kits
PRP	1	A	–	–	I, II, III, IV	Cell separator PRP, Vivostat PRF, Anitua's PRGF
Red PRP	2	B		+		
	3	C				
L-PRP	1	A	+	–	I, II, III, IV	Curasan, Regen, Plateltex, SmartPreP, PCCS, Magellan, Angel, GPS PRP
Red-L-PRP	2	B		+		
	3	C				
PRF	1	A	–	–	I, II, III, IV	Fibrinet PRFM
Red-PRF	2	B		+		
	3	C				
L-PRF	1	A	+	–	I	Choukroun's PRF
Red-L-PRF	2	B		+		
	3	C				

ISTH classification of PCs: Type of PC preparation protocols: (1) gravitational centrifugation techniques; (2) standard cell separators; and (3) autologous selective filtration technology (plateletpheresis). Platelet concentration: A, platelet count of $< 900 \times 10^3/\mu\text{L}$; B, platelet count of $900\text{--}1,700 \times 10^3/\mu\text{L}$; C, platelet count of $> 1,700 \times 10^3/\mu\text{L}$. Activation protocol: I, no activation; II, the use of PRP with activation; III, use of frozen–thawed preparations; IV, photoactivated PRP (not mentioned in ISTH classification).

L-PRF, leukocyte-rich platelet-rich fibrin; L-PRP, leukocyte-rich PRP; PRF, platelet-rich fibrin; Red-L-PRF, red blood cell-rich and leukocyte rich platelet-rich fibrin; Red-L-PRP, red blood cell-rich and leukocyte-rich PRP; Red-PRF, red blood cell-rich platelet-rich fibrin; Red-PRP, red blood cell-rich PRP. + or – defines whether (+) or not (–) there are leukocytes equal or greater than ($\geq$) 1% and/or Red cells equal or greater than ($\geq$) 10% of the total cell counts.

glycocalyx is essential for platelet adhesion to subendothelial cells, platelet activation, and aggregation (25). The sole gel zone consists of actin microfilaments, a matrix in which all the platelet organelles are suspended, and microtubules (25). The organelle zone contains the secretory organelles: α -granules, dense-granules and lysosomes. The α -granules are the most abundant platelet organelles, each platelet has 50–80 α -granules. The α -granules are round or oval in shape, and 200 to 500 nm in diameter. They contain adhesive proteins (von Willebrand factor (VWF), fibrinogen, and thrombospondin), GFs, microbicidal proteins (thymosin- β 4, thrombospondins 1 and 2), immune mediators (complement C3 and C4 precursors), as well as coagulant and fibrinolytic proteins (Factors V, IX, XIII, antithrombin, and plasminogen) (26). The dense granules are smaller than the α -granules. Each platelet contains 3–5 dense-granules. Dense granules contain adenosine triphosphate (ATP), adenosine diphosphate (ADP), serotonin, histamine, polyphosphate, pyrophosphate, calcium, magnesium and potassium. Lysosomes are spherical and each platelet contains <3 lysosomes that contain degrading enzymes (26). The membrane system, besides the plasma membrane, contains the Golgi apparatus, the canalicular system, dense tubules, and the endoplasmic reticulum (27).

The primary function of platelet is to enhance hemostasis through four steps: platelet adhesion, activation, secretion and aggregation. Also, platelets play a vital

role in inflammation, tissue healing, and antimicrobial host defense (27).

REGENERATIVE PROPERTIES OF PLATELET GRANULES

α -granules

Platelet α -granules store GFs, adhesion proteins, coagulation and fibrinolytic proteins that are secreted upon activation. These bioactive molecules have bone regenerative properties as they enhance osteogenesis and angiogenesis (28–30). GFs include transforming growth factor-beta (TGF- β), fibroblast growth factors (FGF), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), platelet derived growth factors AB (PDGF-AB, PDGF-BB), and brain-derived neurotrophic factor (BDNF) (31). GFs play a role in chemotaxis, proliferation, cell differentiation, the formation of extracellular matrix, osteogenesis, and prevention of bone resorption (29, 30, 32) (Table 2). The clinical applications of GFs are limited due to protein instability, short duration of action, high costs, and they required high doses. This is why PCs are very practical alternatives to deliver GFs (55).

Dense Granules

Platelet dense granules contain ADP, ATP, serotonin, histamine, polyphosphate, pyrophosphate, calcium, magnesium, and potassium. ATP was found to increase

TABLE 2 | Main components of platelets and plasma proteins and their effect on bone healing.

Source	Effect on tissue regeneration	References
PLATELETS		
α-Granules		
IGF	Enhances bone growth, BMD, bone mass and implant osseointegration	(28, 33)
BMP	Plays a role in osteoblast and chondrocyte differentiation and bone regeneration	(29)
PDGF	Improves bone healing and implant osseointegration	(33)
TGF- β	Enhances bone regeneration	(30)
VEGF	Promotes angiogenesis and endochondral ossification	(34, 35)
FGF	Regulates osteogenesis, chondrogenesis, and bone mineral homeostasis	(32)
BDNF	Upregulated in granulation tissue of fractured bone and this suggest its involvement in angiogenesis	(36)
IL-1	Regulates bone metabolism	(37)
IL-6	Modulates inflammation and induces bone resorption	(38)
IL-8	Induces mesenchymal stem cell migration	(39)
Dense granules		
Calcium	Increased cytoplasmic Ca ²⁺ concentration may induce osteoblast apoptosis	(40)
ATP	Increases bone mineralization at low concentration, but decreases bone mineralization at high concentration	(41)
Serotonin	Reduces bone mineral density and inhibits osteoblast differentiation and proliferation. Inhibits FXIII that mediate the assembly of plasma fibronectin in cell cultures	(42, 43)
Polyphosphate	Inhibits mineralization at low concentration, whereas enhances mineralization at higher concentration in cell cultures	(44, 45)
BLOOD PLASMA		
Albumin		
	Enhances bone remodeling when used as adjunctive to bone graft material	(46)
Globulins		
β -Globulins	Fibronectin, a β -globulin derived protein, enhances re-epithelization and extracellular matrix formation (<i>in vitro</i>). Fibronectin combined with β -TCP was slightly better than β -TCP alone in bone regeneration in a rat bone defect model	(47, 48)
Coagulation proteins		
Thrombin	Enhances cell migration, osteoblast function, and bone repair	(49)
Factor VIII	Mice lacking F-VIII showed reduced bone healing scores	(50)
Plasminogen	Enhances angiogenesis	(51)
Factor XIII	In osteoblast cell cultures, the inhibition of FXIII-A transglutaminase, resulted in reduction in fibronectin, collagen matrix assembly and mineralization	(43)
Fibrinogen	Fibrinogen 3D scaffolds enhances bone regeneration and increases TGF- β	(52)
Complement system		
C1–C9	Maintains cell proliferation and turnover and enhances angiogenesis and tissue healing	(53)
Other molecules		
Glucose	High serum glucose (in diabetic patients) reduces bone mineral density and bone turnover by altering osteoblast function and collagen properties	(54)

PF4, Platelet factor 4; TGF- β , Transforming growth factor beta; FGF, fibroblast growth factors; VEGF, Vascular endothelial growth factors; IGF, Insulin-like growth factor; PDGF, Platelet derived growth factor; BDNF, Brain-derived neurotrophic factor; TGF- β , Transforming growth factor-beta; BMP, bone morphogenetic proteins; F-XIII, Fibrin stabilizing factor; BMD, bone mineral density; NR, not reported.

bone mineralization at low concentration but decrease bone mineralization at high concentration (41). On the other hand, polyphosphate at low concentration (10 μ M) inhibits matrix mineralization in osteoblast cell cultures (44), whereas at higher concentration (100 μ M), it enhances osteoblast function and mineralization (45). Serotonin is the fastest-released molecule from platelet after activation, regardless of the type of activation material (56). It reduces bone mineral density and inhibits osteoblast differentiation and proliferation (42). Also, it inhibits FXIII-A, which mediates the assembly of plasma fibronectin in cell cultures (43).

Regenerative Properties of Plasma Proteins

Plasma proteins are one of main components of blood plasma. They include albumin, globulins (i.e., fibronectin), coagulation proteins (i.e., fibrinogen, prothrombin, thrombin, III, IV, V, VI, VII, VIII, IX, X, XI, XII, XIII), and the complement system. Albumin was reported to enhance bone healing when used as adjunctive to freeze-dried cancellous bone grafts compared to bone graft alone (46). Fibronectin is an adhesive protein that plays a key role in wound healing: enhancing re-epithelization and extracellular matrix formation (ECM) (47). Fibronectin can interact with different types of cells and cytokines to form the

ECM through its specific function domains and binding sites (47). In addition, fibronectin with beta-tricalcium phosphate (β -TCP) improve bone regeneration in rat calvarial bone defects (48). Thrombin was found to control osteoblast function and fracture healing in mice (49). Fibrinogen 3D scaffolds improve bone regeneration by increasing transforming growth factor-beta ($\text{TGF-}\beta$) (52). In osteoblast cell cultures, the inhibition of FXIII-A transglutaminase reduces fibronectin, collagen matrix assembly and mineralization (43). The complement system maintains cell proliferation, cell turnover, and enhances angiogenesis and tissue regeneration (53) (Table 2).

Leukocytes

PCs may contain leukocytes depending on the preparation protocol. The leukocyte count in whole blood ranges from 4.5 to $11.0 \times 10^9/\text{L}$. In PCs, leukocyte counts range from 0/L in platelet poor plasma (PPP) to $35.8 \times 10^9/\text{L}$ in L-PRP (57, 58). Leukocytes could enhance or impair tissue healing depending on their environment (58). Neutrophils are the primary white blood cells that migrate to the injured tissue and promote phagocytosis of dead tissue and microbes (59). Monocytes enhance host defense and promote arteriogenesis (60). Lymphocytes are not required at the early stages of wound tissue healing, but an innate cellular immune response is required for tissue repair (61). Some studies proposed that leukocytes stimulate the healing process in damaged tissue as they secrete GFs, and simultaneously suppress the growth of bacteria, thus reducing the infection (62–65). However, there is no evidence support the antimicrobial effects of leukocyte-rich PCs (66). Furthermore, other reports showed a positive correlation between the total number of leukocytes in PRP and increased levels of pro-inflammatory cytokines and reactive oxygen species (ROS) released by neutrophils in damaged tissue indicating that high concentration of leukocytes in PRP may inhibit the healing process, and thus the presence of leukocytes should be controlled (67).

Red Blood Cells (RBCs)

Erythrocytes are the most common cell type of blood cells that carry oxygen to all body tissues. PCs can be prepared with or without erythrocytes. However, many researchers do not pay attention to erythrocyte found in PC and their possible role on tissue regeneration and repair. In addition, there is a lack of clinical and *in vivo* studies assessing the effect of RBCs in tissue regeneration. Thus, the role of erythrocytes on tissue regeneration is unknown. Only an *in vitro* study done by Braun et al. (68) showed that RBC rich-PRP resulted in synoviocyte cell death and thus RBCs may have deleterious effect on cartridge regeneration (68).

Clinical Applications

PCs have been used alone or as an adjunctive treatment to enhance soft and hard tissue regeneration in several oral and maxillofacial interventions such as sinus augmentation, implant surgery, alveolar ridge augmentation, socket preservation, periodontal surgery, endodontic surgery, jaw cysts, oral ulcers, alveolar clefts, jaw osteonecrosis, Oroantral communication, and temporomandibular disorders. Underneath we discuss each

one of those interventions in details (Table 3). Figures 1–6 showing some clinical applications of PCs in oral and craniofacial tissue regeneration.

Sinus Augmentation

Sinus augmentation is a surgical procedure aims to restore the resorbed bone in the posterior maxilla caused by tooth loss and is indicated in cases with no enough bone height to accommodate dental implants. PRF has been used as sole filling material or combined with bone graft materials in sinus augmentation. The use of PRF as a sole filling material with simultaneous implant placement showed successful results, although there was no control group and these findings could be similar to the use of implant as a tent in which blood clot filled the gap around the dental implant. Furthermore, PRF reduced the healing time when combined with demineralized freeze-dried bone allograft. However, it does not affect the maturation of deproteinized bovine bone (69). In addition, PRF can be used as a membrane to cover the lateral window approach in sinus augmentation. The use of PRF or collagen as membranes in sinus augmentation with xenografts showed similar results in terms of percentages of new bone formation and the amount residual bone (70). The addition of PRP to anorganic bovine bone (ABB) increased the volume of newly formed bone and improved the osteoconductive properties of ABB, although it did not affect the implant success compared to ABB alone (71). However, in a systematic review, assessed the effect of PRP combined with bone grafts in sinus augmentation, showed no differences in clinical outcomes including implant success and complications (72). Also, the use of PCs as an adjunctive treatment for maxillary sinus elevation was not supported by other studies (73). Therefore, further standardized randomized clinical trials (RCTs) are required.

Alveolar Ridge Augmentation

Different augmentation techniques such as ridge augmentation, guided bone regeneration (GBR), and ridge splitting and expansion can be used to manage alveolar ridge resorption. Different bone grafts (autografts, allograft, xenografts, alloplasts) with or without biological materials such as PCs are used for alveolar ridge augmentation. In alveolar ridge augmentation with the titanium mesh (Ti-mesh), it was found that covering the mesh with PRP prevents the mesh exposure and bone resorption (74). PRP was found to significantly increase alveolar ridge width and the percentage of vital bone achieved with cancellous allograft (75). In addition, covering the autogenous bone blocks in anterior maxillary augmentation with PRF increased bone width and decreased bone resorption (110). On the other hand, limited evidence was found regarding the effect of PRGF on new bone formation when combined with other bone substitutes, but it also shown some benefits on soft tissue healing, pain, and swelling (76). However, due to limited studies, further research is required.

Periodontal Surgery

PCs have been used alone or as an adjunctive material in the treatment of intra-bony periodontal defects. Overall, PRP as an adjunctive material significantly enhanced periodontal

TABLE 3 | Clinical applications of platelet concentrates (PCs) in oral tissue regeneration.

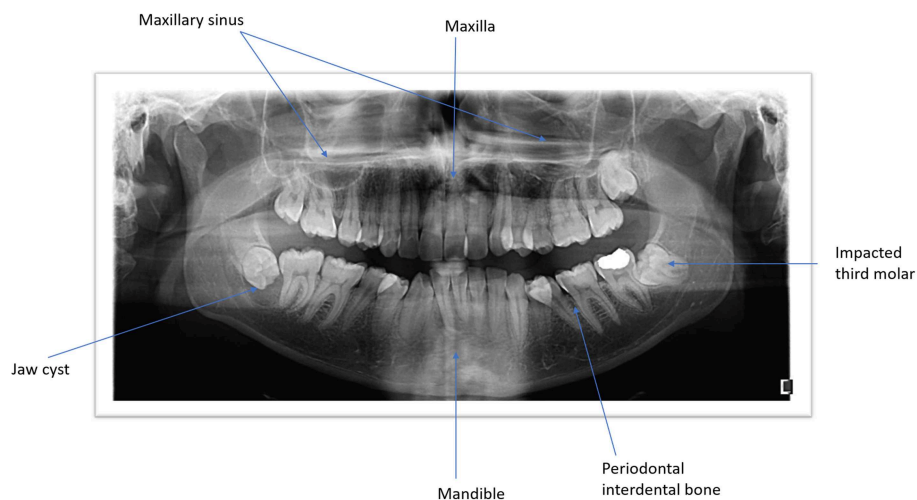
Material	Comparisons	Effect of PCs	References
Maxillary sinus augmentation			
PRF	PRF alone, PRF vs. allograft, PRF vs. xenograft,	PRF alone or combined with allografts accelerate bone healing, although it does not affect the maturation of xenograft	(69)
	PRF vs. collagen membrane	similar new bone formation was reported in both groups	(70)
PRP	PRP with xenograft vs. xenograft	Increased volume of newly formed bone in PRP/xenograft combination	(71)
	PRP with autogenous bone graft or bone substitute	PRP does not improve implant success	(72)
	PRP with bone graft vs. bone graft	PRP did not add beneficial difference to the percentage of newly formed bone or to the implant survival	(73)
Alveolar ridge resorption			
PRF	PRF with Ti-Mesh vs. Ti-Mesh	PRF prevents mesh exposure and bone resorption	(74)
	PRF/allograft vs. allograft	PRF increase alveolar ridge width, and the percentages of vital bone	(75)
	PRF/autogenous bone vs. autogenous bone	Adding PRF increases bone width and decreases bone resorption	(75)
PRP	PRGF alone or combined with bone grafts	Enhances soft tissue healing and reduces inflammatory complications	(76)
Periodontal bone defects			
PRP	PRP/ bone graft vs. bone graft	PRP as an adjunctive material enhances bone regeneration, reduces pocket depth and enhances clinical attachment level	(77)
	PRP/GTR vs. GTR	No beneficial effect was found when used with GTR	(78)
PRF	PRF alone vs. PRF/ open flap debridement (OFD)	PRF enhance bone regeneration when combined with OFD, whereas it did improve the outcomes with GTR	(78)
Gingival recession			
PRF	PRF vs. connective tissue graft	No evidence supports the use of PCs in the treatment of gingival recession	(79)
Alveolar socket			
L-PRF	L-PRF vs. blood clot	L-PRF enhanced the preservation of the alveolar width and reduced buccal bone resorption	(80)
L-PRF	L-PRF vs. PRP	L-PRF resulted in better preservation of alveolar height and width compared to PRP	(81)
PRF, PRP	PRF or PRP vs. empty socket	PCs reduce alveolar osteitis compared to empty socket	(82, 83)
PRP, PRF	PRF or PRP vs. empty socket	PCs showed inconsistent results in hard tissue regeneration	(4, 5)
PRF	PRF/bone graft/ bone graft or empty defect	PRF enhances alveolar ridge preservation	(84)
Implant surgery			
PRF	PRF/bone graft/ bone graft or empty defect	PRF improves the early phases of osseointegration	(84)
PRF	PRF vs. no graft	PRF enhances implant stability and reduces marginal bone loss	(80)
PRP	PRP around dental implant	Inconclusive results due to lack of studies	(85)
PRF	PRF vs. no graft	PRF does not improve bone healing	(86)
PRF	PRF vs. flap surgery (in peri-implantitis)	PRF improves clinical outcomes	(87)
Endodontic diseases			
PRF, PRP	PRF or PRP	PCs enhance bone regeneration, root development, and regaining of pulp vitality, although the level of evidence was weak	(88)
PRF, PRP	PRF or PRP	PCs enhance root development	(89)
Jaw cysts			
PRP	PRP/bone graft vs. bone graft	PRP accelerates bone healing	(90, 91)
PRP	PRP vs. empty defect	PRP did not improve bone healing	(92)
Cleft palate			
PRP	PRP vs. surgical closure alone	PCs enhance soft tissue closure of complete cleft palate and reduce the incidence of oronasal fistula	(93)
	PRGF with bone graft	Mixing the PRGF with bone grafts resulted in complete closure of 90.9% cases with recurrent cleft palate fistulas	(94)
Alveolar clefts			
PRP	PRP with iliac bone graft vs. bone graft	The combination of iliac graft and PRP reduce bone resorption compared to iliac graft	(95, 96)
PRF	PRF with iliac bone graft vs. bone graft	adding PRF to iliac bone grafts, did not enhance maxillary alveolar bone thickness, height, and density or the percentage of newly formed bone	(97, 98)

(Continued)

TABLE 3 | Continued

Material	Comparisons	Effect of PCs	References
BRONJ			
PRF	PRF with surgical debridement	PRF has been found to enhance soft tissue healing and reduce pain of surgically debrided BRONJ cases	(99)
PRP	PRP with laser	Laser assisted surgery and PRP application enhance tissue healing of BRONJ cases	(100)
L-PRF	L-PRF with BMP-2	The combination of BMP-2 and L-PRF accelerate healing compared to L-PRF alone	(101)
Osteoradionecrosis			
L-PRF	L-PRF	Application of PCs combined with surgical debridement improve tissue healing	(102)
PRP	PRP gel	PRP enhance bone regeneration of mandibular bone defect	(103)
Oroantral communication (OAC)			
PRF	PRF vs. buccal advancement flap	PRF clots provides successful results and reduces pain and swelling compared to buccal advancement flap.	(104)
PRF	PRF	PRF membrane could be used to manage OAC of size ≤ 5 mm	(105)
Oral ulcers			
PRP	PRP	Application of PCs in autoimmune derived ulcers accelerate tissue healing and reduce pain and discomfort during mastication	(106, 107)
PRF	PRF	PRF membrane could improve tissue healing after excision of oral mucosal lesions such as leukoplakia and lichen planus	(108)
TMDs PRP	PRP vs. Hyaluronic acid or saline injection	PCs injection reduces pain and improves mouth opening in patients with TMDs	(109)

PRF, platelet rich fibrin; PRP, platelet rich plasma; PRGF, plasma rich in growth factors; PCs, platelet concentrates; BRONJ, Bisphosphonate related osteonecrosis of the jaw; TMDs, Temporomandibular disorders; BMPs, bone morphogenic proteins.

**FIGURE 1 |** Panoramic view of both jaws illustrating the anatomic structures and the pathological conditions.

bone healing (77). PRP or PRF can reduce pocket depth and enhance clinical attachment level (77). Moreover, the use of PRP combined with bone grafts was found to enhance periodontal bone healing, whereas, no beneficial effect was found when used with guided tissue regeneration (GTR) (78). PRF was effective when used alone or combined with open flap debridement, although, there was a lack of evidence regarding its role when combined with bone grafts or GTR (78).

Regarding the efficacy of PCs in periodontal plastic surgery, studies showed that PRF membrane did not regenerate gingival recession, clinical attachment level, or the width of keratinized

mucosa compared to other biomaterials such as connective tissue grafts (111).

Socket Preservation

Platelet concentrates have been used to accelerate healing and regenerate bone in socket preservation procedures. L-PRF reduced the healing time and bone resorption when applied on the extracted tooth socket (80). Moreover, L-PRF was reported to enhance alveolar socket preservation in height and width compared to PRP (81). In addition, PCs reduce alveolar osteitis compared to empty socket after third molar extraction (82, 83).

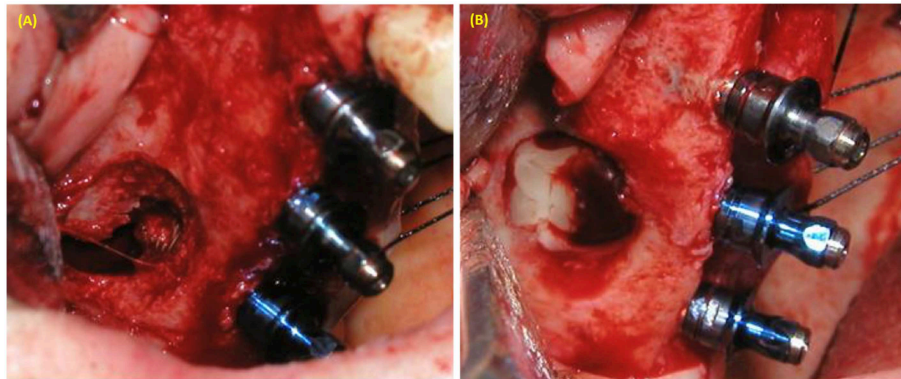


FIGURE 2 | Maxillary sinus augmentation using lateral window approach. **(A)** Perforation of sinus membrane during lateral window approach in sinus lifting with simultaneous implant placement. **(B)** PRP was used to cover the perforated sinus membrane.

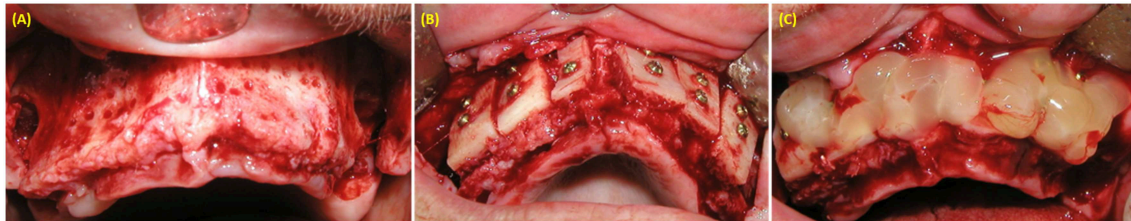


FIGURE 3 | Alveolar maxillary width reconstruction using allograft cortico-cancellous block. **(A)** exposure of alveolar ridge. **(B)** Fixation of bone graft with fixation screws. **(C)** PRP covering the bone graft material.

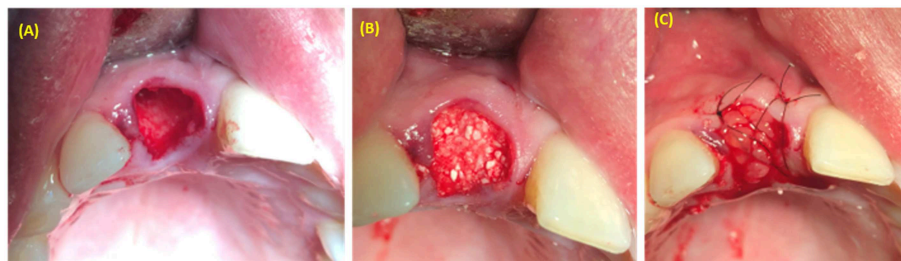


FIGURE 4 | Alveolar socket preservation using bone graft covered with PRP. **(A)** Extraction socket. **(B)** Bone graft applied inside the extraction socket. **(C)** PRP covered bone graft material.



FIGURE 5 | PRP use in treatment of alveolar osteitis (inflammation of alveolar socket). **(A)** Alveolar socket. **(B)** PRP applied in the extraction socket. **(C)** Figure 8 suture type used to stabilize the PRP in the alveolar socket.

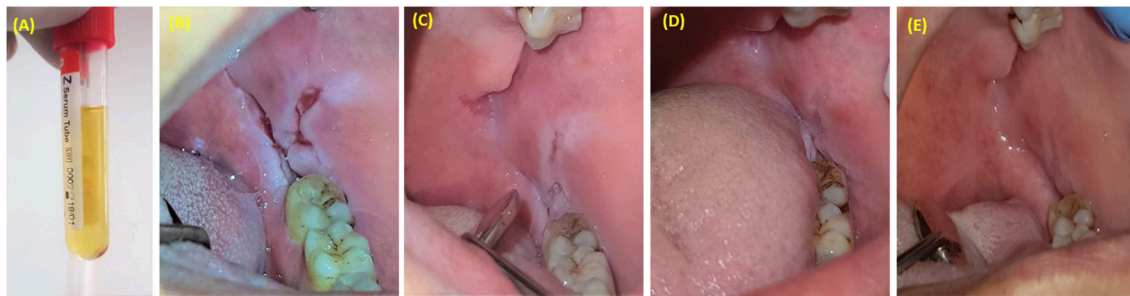


FIGURE 6 | PRP injection as an adjunctive treatment with corticosteroids for resistant oral pemphigus vulgaris. **(A)** 1.5 ml of PRP was injected once a week for 3 weeks. **(B)** Pemphigus vulgaris lesion located posterior to mandibular third molar. **(C)** One week after last injection. **(D)** Six weeks after last injection. **(E)** Six months after last injection.

However, PCs have a beneficial effect in accelerating wound healing and reducing postoperative symptoms; some studies showed conflicting results in hard tissue regeneration (4, 5). Overall, there was a moderate evidence to support the use of PCs in socket preservation (84).

Implant Surgery

Platelet concentrates have been used to enhance bone regeneration prior to implant placement or to treat bone defects in cases of peri-implantitis. Overall, there is moderate evidence to support the use of PCs in the early phases of osseointegration (84). Both PRF and PRP have been found to enhance bone regeneration and reduce marginal bone loss around dental implants (80, 112). However, the application of PRF during immediate implantation does not seem to improve implant stability or bone healing (86). In cases of peri-implantitis, PRF application after surgical debridement was found to reduce peri-implant pocket depth and to increase keratinized mucosa compared to conventional flap surgery only (87).

Regenerative Endodontics

PCs have been used in different root canal procedures including apexification, apexogenesis, pulpotomy, and endodontic apical surgery. PCs have been found to enhance peri apical bone regeneration, root development, and pulp vitality (88). In addition, a comprehensive systematic review of clinical evidence, showed that application of PCs are successful procedures in the treatment of immature teeth, although the level of evidence was weak (only 5 RCTs and 21 case reports were included), and thus further well-designed RCTs with longer follow-up are required (89).

Jaw Cysts

Jaw cysts are pathological cavities formed within the jaw bones or soft tissues, that are usually lined by epithelial layer. Radicular cysts are the most common inflammatory cysts that are usually located around the apexes of necrotic teeth. The standard treatment for inflammatory jaw cysts is enucleation (113). Fewer reports assessed PCs application in bone regeneration after cyst enucleation. Filling the cyst cavity with bone graft and PRP was found to accelerate bone healing compared to bone graft only (90). In addition in a pilot study consisted of 10 cystic lesions,

PRP was found to significantly accelerate bone healing after 6 months of follow-up (91). However, in another study, PRP did not show any significant acceleration in bone healing after 6 months of treatment compared to the control group (empty defect) (92). Thus, due to limited evidence and absence of RCTs, further standardized studies are required.

Cleft Lip and Palate

Cleft lip and palate are congenital defects in maxillary and nasal processes that resulted in cleft lip and/or palate. Surgical closure is the treatment of choice in such patients. PRP was found to enhance soft tissue closure of complete cleft palate and to reduce the incidence of oronasal fistula compared to surgical closure alone. Therefore, it could reduce the need for additional surgical procedures (93). In addition, mixing the PRGF with bone grafts resulted in complete closure of 90.9% cases with recurrent cleft palate fistulas (94). However, due to limited evidence, further standardized studies are required.

Alveolar Clefts Reconstruction

Alveolar clefts are congenital bone defects in the alveolar bone that affect 75% of cleft lip or cleft lip and palate patients (114). The combination of iliac graft and PRP reduce bone resorption compared to iliac graft alone in patients requiring alveolar cleft augmentation (95, 96). On the other hand, adding PRF to iliac bone grafts, did not benefit maxillary alveolar bone thickness, height, and density or the percentage of newly formed bone (97, 98). However, due to limited evidence, further studies are required.

Bisphosphonate Related Osteonecrosis of the Jaw (BRONJ)

BRONJ is commonly manifested by exposed necrotic bone that persists for 8 weeks or more in patients with metastasis or osteoporosis under antiresorptive medications such as alendronate. PRF has been found to enhance soft tissue healing and reduce pain of surgically debrided BRONJ cases (99). In addition, Erbium Chromium: Yttrium Scandium Gallium Garnet laser (Er,Cr:YSGG) assessed surgery combined with PRP application enhance healing of BRONJ cases (100). The

combination of bone morphogenetic protein 2 (BMP-2) and L-PRF in treating BRONJ cases was found to accelerate healing compared to L-PRF alone (101). However, in a systematic review summarizing the combination protocol (surgery and PCs), concluded that there is no sufficient data to support this protocol and thus further studies are required (115).

Osteoradionecrosis

Osteoradionecrosis of the jaws is defined as bone death caused by radiation therapy of head and neck cancer. PCs have been found to be effective in treating patient with osteoradionecrosis (103). The application of L-PRF combined with surgical debridement using piezosurgery in managing cases of osteoradionecrosis, resulted in complete healing of 67% of cases within 1-year follow-up (102). This promising application could reduce the need for bone resection in such cases, although due to the limited evidence, further research is required.

Oroantral Communication

Oroantral communication (OAC) is an abnormal communication between the oral cavity and the maxillary sinus that could occur due to pathological conditions or during extraction of maxillary posterior teeth. The treatment of OAC depends on the size of the communication and it is usually achieved by buccal advancement flaps. PRF clots enhance wound closure and reduce pain and swelling compared to buccal advancement flap (104). In addition, PRF membrane could be used to manage OAC of size ≤ 5 mm (105). However, further standardized studies are required to shed the light in such promised application.

Oral Ulcers

Pemphigus vulgaris is an autoimmune disease that affects the skin and mucous membranes. It manifests in oral cavity as painful erosive lesions (ulcers) that are usually treated with corticosteroids (116). In such cases, the repeated injections of PRP accelerate tissue healing and reduce pain and discomfort during mastication (106). Oral ulcers can also occur after bone marrow allogeneic transplants resulting in graft vs. host disease (GvHD). Application of PCs gel in those patients reduces pain, enhances mastication, and accelerates healing (107). In addition, PRF membrane could improve tissue healing after excision of oral mucosal lesions such as leukoplakia and lichen planus (108). PCs seem to be promising materials for managing oral ulcers, however, due to limited studies, further research is required.

Temporomandibular Disorders (TMD)

TMDs are diseases of multifactorial origin that affect the temporomandibular joint articular surfaces as well as the surrounding masticatory muscles (117). PRP injection has been used to reduce pain and to improve mouth opening in patients with TMDs. In a systematic review, PRP injection was found to reduce pain and improve jaw movements in 4 out of 6 studies compared to hyaluronic acid or saline injections (109).

DISCUSSION

PCs have been used extensively in oral and craniofacial interventions for hard tissue regeneration. Overall, PCs seem to have a beneficial role on bone regeneration in different clinical procedures, such as socket preservation, implant surgery, sinus augmentation, periodontal surgery, osteonecrosis, and alveolar ridge augmentation (71, 75, 78, 80, 90, 95, 96, 100, 112), although, there was some inconsistent findings. The regenerative potential of PCs is attributed to their contents of bioactive molecules, specifically GFs known to enhance osteogenesis, angiogenesis and tissue regeneration (28–30). However, due to limited studies, further research is required.

PCs seem to improve soft tissue healing in socket preservation (81), periodontal surgery (77), oral ulcers (106), oroantral communication (105), osteonecrosis (100), and alveolar ridge augmentation (74, 75). The improvement on soft tissue closure obtained by PCs could indirectly enhance bone regeneration in different surgical procedures.

In addition, PCs could reduce inflammatory complications such as pain and swelling and could improve mouth opening and masticatory function in patients with in temporomandibular disorders (TMDs) (109, 118), oral ulcers (106), jaw osteonecrosis, and extraction sockets (4). This beneficial role could be attributed to their content of GFs that have an essential role in inflammation, cell movement and metabolism (119). In addition, PCs may have immunomodulatory effects; PC can induce considerable changes in the level of proinflammatory mediators such as an increased level of lipoxin A4 (LXA₄) and thus suggests that PCs could prohibit cytokine secretion, reduce inflammation and promote tissue healing (120).

However, the effect of PCs on bone regeneration has been clinically inconsistent across the literature (4, 76). Differences in study designs and types of PCs used with variable concentration of platelets, growth factors, and leucocytes, as well as different application forms and techniques could explain these contradictory results (6). Also, the centrifuge characteristics and different centrifugation protocols could affect the cells, growth factors and fibrin architecture of PCs and thus any modification of the original protocols should be investigated separately in order to avoid confusion and inaccurate results (121). In addition, a recent study suggested the possible negative effect of platelet dense granule contents including serotonin in bone regeneration (23). Serotonin inhibits osteogenic activity and osteoblastic differentiation and proliferation (42, 122, 123). Therefore, PCs preparation protocols require standardization.

PRP and PRF are the main types of PCs that applied in different clinical procedures. PRP is the only PCs that can be injected inside the temporomandibular joint (TMJ) spaces in treating patients with TMDs (109, 118). PRF membrane is used for closure of oroantral communication (105). Both materials could be used for other clinical interventions. Their main challenges are the stability in the application site and the need for an optimized delivery system that allow gradual release of growth factors (124).

CONCLUSION

Platelet concentrate (PC) are biological blood-derived products that are rich in platelets and GFs. Overall, PCs seem to enhance bone and soft tissue healing, reduce pain and swelling in alveolar ridge augmentation, periodontal surgery, sinus augmentation, socket preservation, implant surgery, endodontic regeneration, BRONJ, osteoradionecrosis, closure of oroantral communication (OAC), oral ulcers, and temporomandibular disorders. On the other hand, no effect was reported in gingival recession and guided tissue regeneration (GTR) procedures. However, due to the limited available evidence and the heterogeneity among different studies, further research is required to shed light on these promising biological materials.

AUTHOR CONTRIBUTIONS

FA-H wrote the review and participated in design the review. MM participated in writing part of the clinical applications. HA-W revised the manuscript. JT revised the manuscript. ZB and FT designed and revised the manuscript.

REFERENCES

- Dohan Ehrenfest DM, Rasmusson L, Albrektsson T. Classification of platelet concentrates: from pure platelet-rich plasma (P-PRP) to leucocyte- and platelet-rich fibrin (L-PRF). *Trends Biotechnol.* (2009) 27:158–67. doi: 10.1016/j.tibtech.2008.11.009
- Dohan Ehrenfest DM, Bielecki T, Mishra A, Borzini P, Inchingolo F, Sammartino G, et al. In search of a consensus terminology in the field of platelet concentrates for surgical use: platelet-rich plasma (PRP), platelet-rich fibrin (PRF), fibrin gel polymerization and leukocytes. *Curr Pharm Biotechnol.* (2012) 13:1131–7. doi: 10.2174/138920112800624328
- Harrison P. The use of platelets in regenerative medicine and proposal for a new classification system: guidance from the SSC of the ISTH. *J Thromb Haemost.* (2018) 16:1895–900. doi: 10.1111/jth.14223
- Al-Hamed FS, Tawfik MA, Abdelfadil E, Al-Saleh MAQ. Efficacy of platelet-rich fibrin after mandibular third molar extraction: a systematic review and meta-analysis. *J Oral Maxillofac Surg.* (2017) 75:1124–35. doi: 10.1016/j.joms.2017.01.022
- Annunziata M, Guida L, Nastri L, Piccirillo A, Sommese L, Napoli C. The role of autologous platelet concentrates in alveolar socket preservation: a systematic review. *Transfusion Med Hemother.* (2018) 45:195–203. doi: 10.1159/000488061
- Del Corso M, Vervelle A, Simonpieri A, Jimbo R, Inchingolo F, Sammartino G, et al. Current knowledge and perspectives for the use of platelet-rich plasma (PRP) and platelet-rich fibrin (PRF) in oral and maxillofacial surgery part 1: periodontal and dentoalveolar surgery. *Curr Pharmaceut Biotechnol.* (2012) 13:1207–30. doi: 10.2174/138920112800624391
- Sela JJ, Bab IA. Healing of Bone Fracture: general concepts, principles of bone regeneration. *Principles Bone Regenerat.* (2012) 2012:1–8. doi: 10.1007/978-1-4614-2059-0_1
- Cho TJ, Gerstenfeld LC, Einhorn TA. Differential temporal expression of members of the transforming growth factor beta superfamily during murine fracture healing. *J Bone Miner Res.* (2002) 17:513–20. doi: 10.1359/jbmr.2002.17.3.513
- Gerstenfeld LC, Cullinane DM, Barnes GL, Graves DT, Einhorn TA. Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation. *J Cell Biochem.* (2003) 88:873–84. doi: 10.1002/jcb.10435

FUNDING

This work was supported by grants from NSERC RGPIN-2019-04340, Faculty of Dentistry, McGill University, International Team for Implantology (ITI) grant (ITI 1362-2018 and ITI 1320-2018), Dr. Florence Johnston grant, CRC 241487, FRQ-S/RSBO/UdeMtl 5213, CFI/IOF 31705, and MEDTEQ (FT). FA-H was supported by scholarships from Al Awn Foundation for Development, Yemen, Ph.D training award from Fonds de Recherche Québec - Santé (FRQS code: 257709), Alpha Omega Foundation of Canada grant and he received the Graduate Excellence Award from Faculty of Dentistry, McGill University. MM was supported by scholarships from the College of Dentistry, Jazan University through Saudi Arabian Cultural Bureau, Saudi Arabia.

ACKNOWLEDGMENTS

We would like to thank Dr. Abdel-Hameed Hijazi from Cairo University for sharing photos for pemphigus vulgaris case.

- Kon T, Cho TJ, Aizawa T, Yamazaki M, Nooh N, Graves D, et al. Expression of osteoprotegerin, receptor activator of NF-kappaB ligand (osteoprotegerin ligand) and related proinflammatory cytokines during fracture healing. *J Bone Miner Res.* (2001) 16:1004–14. doi: 10.1359/jbmr.2001.16.6.1004
- Granero-Molto F, Weis JA, Miga MI, Landis B, Myers TJ, O'Rear L, et al. Regenerative effects of transplanted mesenchymal stem cells in fracture healing. *Stem Cells.* (2009) 27:1887–98. doi: 10.1002/stem.103
- Lee KS, Kim HJ, Li QL, Chi XZ, Ueta C, Komori T, et al. Runx2 is a common target of transforming growth factor beta1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. *Mol Cell Biol.* (2000) 20:8783–92. doi: 10.1128/MCB.20.23.8783-8792.2000
- Dhurat R, Sukesh M. Principles and methods of preparation of platelet-rich plasma: a review and author's perspective. *J Cutaneous Aesthetic Surg.* (2014) 7:189–97. doi: 10.4103/0974-2077.150734
- Perez AG, Lana JF, Rodrigues AA, Luzo AC, Belangero WD, Santana MH. Relevant aspects of centrifugation step in the preparation of platelet-rich plasma. *ISRN Hematol.* (2014) 2014:176060. doi: 10.1155/2014/176060
- Slichter SJ, Harker LA. Preparation and storage of platelet concentrates. I. Factors influencing the harvest of viable platelets from whole blood. *Br J Haematol.* (1976) 34:395–402. doi: 10.1111/j.1365-2141.1976.tb03586.x
- Kahn RA, Cossette I, Friedman LI. Optimum centrifugation conditions for the preparation of platelet and plasma products. *Transfusion.* (1976) 16:162–5. doi: 10.1046/j.1537-2995.1976.16276155111.x
- Harrison S, Vavken P, Kevy S, Jacobson M, Zurakowski D, Murray MM. Platelet activation by collagen provides sustained release of anabolic cytokines. *Am J Sports Med.* (2011) 39:729–34. doi: 10.1177/0363546511401576
- Cavallo C, Roffi A, Grigolo B, Mariani E, Pratelli L, Merli G, et al. Platelet-rich plasma: the choice of activation method affects the release of bioactive molecules. *Biomed Res Int.* (2016) 2016:6591717. doi: 10.1155/2016/6591717
- Paterson KL, Nicholls M, Bennell KL, Bates D. Intra-articular injection of photo-activated platelet-rich plasma in patients with knee osteoarthritis: a double-blind, randomized controlled pilot study. *BMC Musculoskelet Disord.* (2016) 17:67. doi: 10.1186/s12891-016-0920-3
- Dohan DM, Choukroun J, Diss A, Dohan SL, Dohan AJJ, Mouhyi J, et al. Platelet-rich fibrin (PRF): a second-generation platelet concentrate. Part II:

- Platelet-related biologic features. *Oral Surg Oral Med Oral Pathol Oral Radiol Endodontology*. (2006) 101:e45–e50. doi: 10.1016/j.tripleo.2005.07.009
21. Dohan DM, Choukroun J, Diss A, Dohan SL, Dohan AJJ, Mouhyi J, et al. Platelet-rich fibrin (PRF): a second-generation platelet concentrate. Part I: Technological concepts and evolution. *Oral Surg Oral Med Oral Pathol Oral Radiol Endodontology*. (2006) 101:e37–e44. doi: 10.1016/j.tripleo.2005.07.008
 22. Gassling V, Douglas T, Warnke PH, Acil Y, Wiltfang J, Becker ST. Platelet-rich fibrin membranes as scaffolds for periosteal tissue engineering. *Clin Oral Implants Res*. (2010) 21:543–9. doi: 10.1111/j.1600-0501.2009.01900.x
 23. Badran Z, Abdallah MN, Torres J, Tamimi F. Platelet concentrates for bone regeneration: current evidence and future challenges. *Platelets*. (2018) 29:105–12. doi: 10.1080/09537104.2017.1327656
 24. Thon JN, Italiano JE. Platelet formation. *Semin Hematol*. (2010) 47:220–6. doi: 10.1053/j.seminhematol.2010.03.005
 25. White JG. Platelet structure. In: Michelson AD, editor. *Platelets*. San Diego, CA: Elsevier/Academic Press (2013). p. 117–44. doi: 10.1016/B978-0-12-387837-3.00007-9
 26. White JG. Platelet secretion. In: Michelson AD, editor. *Platelets*. San Diego, CA: Elsevier/Academic Press (2013). p. 343–66. doi: 10.1016/B978-0-12-387837-3.00018-3
 27. Gremmel T, Frelinger AL, III, Michelson AD. Platelet Physiology. Seminars in thrombosis and hemostasis. *Semin Thromb Hemost*. (2016) 42:191–204. doi: 10.1055/s-0035-1564835
 28. Yakar S, Rosen CJ, Beamer WG, Ackert-Bicknell CL, Wu Y, Liu JL, et al. Circulating levels of IGF-1 directly regulate bone growth and density. *J Clin Invest*. (2002) 110:771–81. doi: 10.1172/JCI15463
 29. Wu M, Chen G, Li YP. TGF-beta and BMP signaling in osteoblast, skeletal development, and bone formation, homeostasis and disease. *Bone Res*. (2016) 4:16009. doi: 10.1038/boneres.2016.9
 30. Shakir S, MacIsaac ZM, Naran S, Smith DM, Bykowski MR, Cray JJ, et al. Transforming growth factor beta 1 augments calvarial defect healing and promotes suture regeneration. *Tissue Eng Part A*. (2015) 21:939–47. doi: 10.1089/ten.tea.2014.0189
 31. Masuki H, Okudera T, Watanebe T, Suzuki M, Nishiyama K, Okudera H, et al. Growth factor and pro-inflammatory cytokine contents in platelet-rich plasma (PRP), plasma rich in growth factors (PRGF), advanced platelet-rich fibrin (A-PRF), and concentrated growth factors (CGF). *Int J Implant Dentistry*. (2016) 2:19. doi: 10.1186/s40729-016-0052-4
 32. Ornitz DM, Marie PJ. Fibroblast growth factor signaling in skeletal development and disease. *Genes Dev*. (2015) 29:1463–86. doi: 10.1101/gad.266551.115
 33. Zhou WL, Li LL, Qiu XR, An Q, Li MH. Effects of combining insulin-like growth factor 1 and platelet-derived growth factor on osteogenesis around dental implants. *Chin J Dent Res*. (2017) 20:105–9. doi: 10.3290/j.cjdr.a38275
 34. Ferrara N, Gerber HP. The role of vascular endothelial growth factor in angiogenesis. *Acta Haematol*. (2001) 106:148–56. doi: 10.1159/000046610
 35. Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nat Med*. (1999) 5:623–8. doi: 10.1038/9467
 36. Kilian O, Hartmann S, Dongowski N, Karnati S, Baumgart-Vogt E, Hartel FV, et al. BDNF and its TrkB receptor in human fracture healing. *Ann Anatomy*. (2014) 196:286–95. doi: 10.1016/j.aanat.2014.06.001
 37. Lee YM, Fujikado N, Manaka H, Yasuda H, Iwakura Y. IL-1 plays an important role in the bone metabolism under physiological conditions. *Int Immunol*. (2010) 22:805–16. doi: 10.1093/intimm/dxq431
 38. Franchimont N, Wertz S, Malaise M. Interleukin-6: an osteotropic factor influencing bone formation? *Bone*. (2005) 37:601–6. doi: 10.1016/j.bone.2005.06.002
 39. Yang A, Lu Y, Xing J, Li Z, Yin X, Dou C, et al. IL-8 enhances therapeutic effects of BMSCs on bone regeneration via CXCR2-mediated PI3k/Akt signaling pathway. *Cell Physiol Biochem*. (2018) 48:361–70. doi: 10.1159/000491742
 40. Adams CS, Mansfield K, Perlot RL, Shapiro IM. Matrix regulation of skeletal cell apoptosis. Role of calcium and phosphate ions. *J Biol Chem*. (2001) 276:20316–22. doi: 10.1074/jbc.M006492200
 41. Cutarelli A, Marini M, Tancredi V, D'Arcangelo G, Murdocca M, Frank C, et al. Adenosine Triphosphate stimulates differentiation and mineralization in human osteoblast-like Saos-2 cells. *Dev Growth Differ*. (2016) 58:400–8. doi: 10.1111/dgd.12288
 42. Nam SS, Lee JC, Kim HJ, Park JW, Lee JM, Suh JY, et al. Serotonin inhibits osteoblast differentiation and bone regeneration in rats. *J Periodontol*. (2016) 87:461–9. doi: 10.1902/jop.2015.150302
 43. Cui C, Kaartinen MT. Serotonin (5-HT) inhibits Factor XIII-A-mediated plasma fibronectin matrix assembly and crosslinking in osteoblast cultures via direct competition with transamidation. *Bone*. (2015) 72:43–52. doi: 10.1016/j.bone.2014.11.008
 44. Hoac B, Kiffer-Moreira T, Millan JL, McKee MD. Polyphosphates inhibit extracellular matrix mineralization in MC3T3-E1 osteoblast cultures. *Bone*. (2013) 53:478–86. doi: 10.1016/j.bone.2013.01.020
 45. Muller WE, Wang X, Diehl-Seifert B, Kropf K, Schlossmacher U, Lieberwirth I, et al. Inorganic polymeric phosphate/polyphosphate as an inducer of alkaline phosphatase and a modulator of intracellular Ca²⁺ level in osteoblasts (SaOS-2 cells) *in vitro*. *Acta Biomater*. (2011) 7:2661–71. doi: 10.1016/j.actbio.2011.03.007
 46. Skaliczki G, Schandl K, Weszl M, Major T, Kovacs M, Skaliczki J, et al. Serum albumin enhances bone healing in a nonunion femoral defect model in rats: a computer tomography micromorphometry study. *Int Orthopaedics*. (2013) 37:741–5. doi: 10.1007/s00264-012-1770-8
 47. Lenselink EA. Role of fibronectin in normal wound healing. *Int Wound J*. (2015) 12:313–6. doi: 10.1111/iwj.12109
 48. Escoda-Francoli J, Sanchez-Garces MA, Gimeno-Sandig A, Munoz-Guzon F, Barbany-Cairo JR, Badiella-Busquets L, et al. Guided bone regeneration using beta-tricalcium phosphate with and without fibronectin—An experimental study in rats. *Clin Oral Implants Res*. (2018) 29:1038–49. doi: 10.1111/clr.13370
 49. Sato N, Ichikawa J, Wako M, Ohba T, Saito M, Sato H, et al. Thrombin induced by the extrinsic pathway and PAR-1 regulated inflammation at the site of fracture repair. *Bone*. (2016) 83:23–34. doi: 10.1016/j.bone.2015.10.005
 50. Aronovich A, Nur Y, Shezen E, Rosen C, Zlotnikov Klinsky Y, Milman I, et al. A novel role for factor VIII and thrombin/PAR1 in regulating hematopoiesis and its interplay with the bone structure. *Blood*. (2013) 122:2562–71. doi: 10.1182/blood-2012-08-447458
 51. Kawao N, Tamura Y, Okumoto K, Yano M, Okada K, Matsuo O, et al. Plasminogen plays a crucial role in bone repair. *J Bone Miner Res*. (2013) 28:1561–74. doi: 10.1002/jbmr.1921
 52. Vasconcelos DM, Goncalves RM, Almeida CR, Pereira IO, Oliveira MI, Neves N, et al. Fibrinogen scaffolds with immunomodulatory properties promote *in vivo* bone regeneration. *Biomaterials*. (2016) 111:163–78. doi: 10.1016/j.biomaterials.2016.10.004
 53. Rutkowski MJ, Sughrue ME, Kane AJ, Ahn BJ, Fang S, Parsa AT. The complement cascade as a mediator of tissue growth and regeneration. *Inflamm Res*. (2010) 59:897–905. doi: 10.1007/s00011-010-0220-6
 54. Napoli N, Chandran M, Pierroz DD, Abrahamsen B, Schwartz AV, Ferrari SL. Mechanisms of diabetes mellitus-induced bone fragility. *Nat Rev Endocrinol*. (2017) 13:208–19. doi: 10.1038/nrendo.2016.153
 55. Aravamudhan A, Ramos DM, Nip J, Subramanian A, James R, Harmon MD, et al. Osteoinductive small molecules: growth factor alternatives for bone tissue engineering. *Curr Pharmaceut Des*. (2013) 19:3420–8. doi: 10.2174/1381612811319190008
 56. Jonnalagadda D, Izu LT, Whiteheart SW. Platelet secretion is kinetically heterogeneous in an agonist-responsive manner. *Blood*. (2012) 120:5209–16. doi: 10.1182/blood-2012-07-445080
 57. Fitzpatrick J, Bulsara MK, McCrory PR, Richardson MD, Zheng MH. Analysis of platelet-rich plasma extraction: variations in platelet and blood components between 4 common commercial kits. *Orthopaedic J Sports Med*. (2017) 5:2325967116675272. doi: 10.1177/2325967116675272
 58. King W, Toler K, Woodell-May J. Role of white blood cells in blood- and bone marrow-based autologous therapies. *Biomed Res Int*. (2018) 2018:6510842. doi: 10.1155/2018/6510842
 59. Kolaczowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nature reviews. Immunology*. (2013) 13:159–75. doi: 10.1038/nri3399
 60. Bergmann CE, Hoefer IE, Meder B, Roth H, van Royen N, Breit SM, et al. Arteriogenesis depends on circulating monocytes and macrophage

- accumulation and is severely depressed in op/op mice. *J Leukocyte Biol.* (2006) 80:59–65. doi: 10.1189/jlb.0206087
61. Schaffer M, Barbul A. Lymphocyte function in wound healing and following injury. *Br J Surg.* (1998) 85:444–60. doi: 10.1046/j.1365-2168.1998.00734.x
 62. Badade PS, Mahale SA, Panjwani AA, Vaidya PD, Warang AD. Antimicrobial effect of platelet-rich plasma and platelet-rich fibrin. *Indian J Dent Res.* (2016) 27:300–4. doi: 10.4103/0970-9290.186231
 63. Kasten P, Vogel J, Luginbuhl R, Niemeyer P, Weiss S, Schneider S, et al. Influence of platelet-rich plasma on osteogenic differentiation of mesenchymal stem cells and ectopic bone formation in calcium phosphate ceramics. *Cells Tissues Organs.* (2006) 183:68–79. doi: 10.1159/000095511
 64. Pavlovic V, Ciric M, Jovanovic V, Stojanovic P. Platelet rich plasma: a short overview of certain bioactive components. *Open Med.* (2016) 11:242–7. doi: 10.1515/med-2016-0048
 65. Barrick B, Campbell EJ, Owen CA. Leukocyte proteinases in wound healing: roles in physiologic and pathologic processes. *Wound Repair Regen.* (1999) 7:410–22. doi: 10.1046/j.1524-475X.1999.00410.x
 66. D'Asta F, Halstead F, Harrison P, Zecchi Orlandini S, Moiemmen N, Lord J. The contribution of leucocytes to the antimicrobial activity of platelet-rich plasma preparations: a systematic review. *Platelets.* (2018) 29:9–20. doi: 10.1080/09537104.2017.1317731
 67. Anitua E, Zalduendo M, Troya M, Padilla S, Orive G. Leukocyte inclusion within a platelet rich plasma-derived fibrin scaffold stimulates a more pro-inflammatory environment and alters fibrin properties. *PLoS ONE.* (2015) 10:e0121713. doi: 10.1371/journal.pone.0121713
 68. Braun HJ, Kim HJ, Chu CR, Dragoo JL. The effect of platelet-rich plasma formulations and blood products on human synoviocytes: implications for intra-articular injury and therapy. *Am J Sports Med.* (2014) 42:1204–10. doi: 10.1177/0363546514525593
 69. Ali S, Bakry SA, Abd-Elhakam H. Platelet-rich fibrin in maxillary sinus augmentation: a systematic review. *J Oral Implantol.* (2015) 41:746–53. doi: 10.1563/AAID-JOI-D-14-00167
 70. Gassling V, Purcz N, Braesen JH, Will M, Gierloff M, Behrens E, et al. Comparison of two different absorbable membranes for the coverage of lateral osteotomy sites in maxillary sinus augmentation: a preliminary study. *J Cranio-Maxillo-Facial Surg.* (2013) 41:76–82. doi: 10.1016/j.jcms.2012.10.015
 71. Torres J, Tamimi F, Martinez PP, Alkhraisat MH, Linares R, Hernandez G, et al. Effect of platelet-rich plasma on sinus lifting: a randomized-controlled clinical trial. *J Clin Periodontol.* (2009) 36:677–87. doi: 10.1111/j.1600-051X.2009.01437.x
 72. Esposito M, Grusovin MG, Rees J, Karasoulos D, Felice P, Alissa R, et al. Effectiveness of sinus lift procedures for dental implant rehabilitation: a Cochrane systematic review. *Eur J Oral Implantol.* (2010) 3:7–26.
 73. Pocaterra A, Caruso S, Bernardi S, Scagnoli L, Continenza MA, Gatto R. Effectiveness of platelet-rich plasma as an adjunctive material to bone graft: a systematic review and meta-analysis of randomized controlled clinical trials. *Int J Oral Maxillofac Surg.* (2016) 45:1027–34. doi: 10.1016/j.ijom.2016.02.012
 74. Torres J, Tamimi F, Alkhraisat MH, Manchon A, Linares R, Prados-Frutos JC, et al. Platelet-rich plasma may prevent titanium-mesh exposure in alveolar ridge augmentation with anorganic bovine bone. *J Clin Periodontol.* (2010) 37:943–51. doi: 10.1111/j.1600-051X.2010.01615.x
 75. Eskin MA, Greenwell H, Hill M, Morton D, Vidal R, Shumway B, et al. Platelet-rich plasma-assisted guided bone regeneration for ridge augmentation: a randomized, controlled clinical trial. *J Periodontol.* (2014) 85:661–8. doi: 10.1902/jop.2013.130260
 76. Dragonas P, Schiavo JH, Avila-Ortiz G, Palaologou A, Katsaros T. Plasma rich in growth factors (PRGF) in intraoral bone grafting procedures: a systematic review. *J Cranio-Maxillo-Facial Surg.* (2019) 47:443–53. doi: 10.1016/j.jcms.2019.01.012
 77. Zhou S, Sun C, Huang S, Wu X, Zhao Y, Pan C, et al. Efficacy of adjunctive bioactive materials in the treatment of periodontal intrabony defects: a systematic review and meta-analysis. *Biomed Res Int.* (2018) 2018:8670832. doi: 10.1155/2018/8670832
 78. Panda S, Doraiswamy J, Malaipappan S, Varghese SS, Del Fabbro M. Additive effect of autologous platelet concentrates in treatment of intrabony defects: a systematic review and meta-analysis. *J Invest Clin Dentistry.* (2016) 7:13–26. doi: 10.1111/jicd.12117
 79. Schliephake H. Clinical efficacy of growth factors to enhance tissue repair in oral and maxillofacial reconstruction: a systematic review. *Clin Implant Dent Relat Res.* (2015) 17:247–73. doi: 10.1111/cid.12114
 80. Castro AB, Meschi N, Temmerman A, Pinto N, Lambrechts P, Teughels W, et al. Regenerative potential of leucocyte-and platelet-rich fibrin. Part B: sinus floor elevation, alveolar ridge preservation and implant therapy. A systematic review. *J Clin Periodontol.* (2017) 44:225–34. doi: 10.1111/jcpe.12658
 81. Niu W, Wang P, Ge S, Ji P. Effects of platelet concentrates used in alveolar ridge preservation: a systematic review. *Implant Dent.* (2018) 27:498–506. doi: 10.1097/ID.0000000000000797
 82. Unsul H, Erbasar HGN. Evaluation of the effect of platelet-rich fibrin on the alveolar osteitis incidence and periodontal probing depth after extracting partially erupted mandibular third molars extraction. *Niger J Clin Pract.* (2018) 21:201–5. doi: 10.4103/njcp.njcp_1_17
 83. Prataap N, Sunil PM, Sudeep CB, Ninan VS, Tom A, Arjun MR. Platelet-rich plasma and incidence of alveolar osteitis in high-risk patients undergoing extractions of mandibular molars: a case-control study. *J Pharm Bioallied Sci.* (2017) 9:S173–9. doi: 10.4103/jpbs.JPBS_151_17
 84. Strauss FJ, Stahli A, Gruber R. The use of platelet-rich fibrin to enhance the outcomes of implant therapy: a systematic review. *Clin Oral Implants Res.* 29 (Suppl. 1) (2018) 18:6–19. doi: 10.1111/clr.13275
 85. Kotsovilis S, Markou N, Pepelassi E, Nikolidakis D. The adjunctive use of platelet-rich plasma in the therapy of periodontal intraosseous defects: a systematic review. *J Periodontol Res.* (2010) 45:428–43. doi: 10.1111/j.1600-0765.2009.01236.x
 86. Diana C, Mohanty S, Chaudhary Z, Kumari S, Dabas J, Bodh R. Does platelet-rich fibrin have a role in osseointegration of immediate implants? A randomized, single-blind, controlled clinical trial. *Int J Oral Maxillofac Surg.* (2018) 47:1178–88. doi: 10.1016/j.ijom.2018.01.001
 87. Hamzacebi B, Oduncuoglu B, Alaaddinoglu EE. Treatment of peri-implant bone defects with platelet-rich fibrin. *Int J Periodontics Restorat Dentistry.* (2015) 35:415–22. doi: 10.11607/prd.1861
 88. Meschi N, Castro AB, Vandamme K, Quirynen M, Lambrechts P. The impact of autologous platelet concentrates on endodontic healing: a systematic review. *Platelets.* (2016) 27:613–33. doi: 10.1080/09537104.2016.1226497
 89. Metterska J, Fagogeni I, Nowicka A. Efficacy of autologous platelet concentrates in regenerative endodontic treatment: a systematic review of human studies. *J Endodontics.* (2019) 45:20–30.e1. doi: 10.1016/j.joen.2018.09.003
 90. Nagaveni NB, Praveen RB, Umashankar KV, Pranav B, Sreedevi R, Radhika NB. Efficacy of platelet-rich-plasma (PRP) in bone regeneration after cyst enucleation in pediatric patients—a clinical study. *J Clin Pediatr Dentistry.* (2010) 35:81–7. doi: 10.17796/jcpd.35.1.q69168v5268234k9
 91. Meshram VS, Lambade PN, Meshram PV, Kadu A, Tiwari MS. The autologous platelet rich fibrin: a novel approach in osseous regeneration after cystic enucleation: a pilot study. *Indian J Dent Res.* (2015) 26:560–4. doi: 10.4103/0970-9290.176915
 92. Ramanathan A, Cariappa KM. Effect of platelet-rich plasma on bone regeneration after removal of cysts and benign tumours of the jaws. *Oral Maxillofac Surg.* (2014) 18:445–52. doi: 10.1007/s10006-013-0435-0
 93. El-Anwar MW, Nofal AA, Khalifa M, Quriba AS. Use of autologous platelet-rich plasma in complete cleft palate repair. *Laryngoscope.* (2016) 126:1524–8. doi: 10.1002/lary.25868
 94. Gonzalez-Sanchez JG, Jimenez-Barragan K. [Closure of recurrent cleft palate fistulas with plasma rich in growth factors]. *Acta Otorrinolaringol Esp.* (2011) 62:448–53. doi: 10.1016/j.otoeng.2011.06.001
 95. Marukawa E, Oshina H, Iino G, Morita K, Omura K. Reduction of bone resorption by the application of platelet-rich plasma (PRP) in bone grafting of the alveolar cleft. *J Cranio-Maxillofac Surg.* (2011) 39:278–83. doi: 10.1016/j.jcms.2010.04.017
 96. Gupta C, Mehrotra D, Mohammad S, Khanna V, Kumar Singh G, Singh G, et al. Alveolar bone graft with platelet rich plasma in cleft alveolus. *J Oral Biol Craniofac Res.* (2013) 3:3–8. doi: 10.1016/j.jobcr.2013.02.002

97. Omidkhoda M, Jahnabin A, Khoshandam F, Eslami F, Hosseini Zarch SH, Tavakol Afshari J, et al. Efficacy of platelet-rich fibrin combined with autogenous bone graft in the quality and quantity of maxillary alveolar cleft reconstruction. *Iran J Otorhinolaryngol.* (2018) 30:329–34. doi: 10.4103/njcp.njcp_1_17
98. Saruhan N, Ertas U. Evaluating of platelet-rich fibrin in the treatment of alveolar cleft with iliac bone graft by means of volumetric analysis. *J Craniofac Surg.* (2018) 29:322–6. doi: 10.1097/SCS.00000000000004125
99. Fernando de Almeida Barros Mourao C, Calasans-Maia MD, Del Fabbro M, Le Drapper Vieira F, Coutinho de Mello Machado R, Capella R, et al. The use of Platelet-rich Fibrin in the management of medication-related osteonecrosis of the jaw: a case series. *J Stomatol Oral Maxillofac Surg.* (in press). doi: 10.1016/j.joms.2019.02.011
100. Mauceri R, Panzarella V, Maniscalco L, Bedogni A, Licata ME, Albanese A, et al. Conservative surgical treatment of bisphosphonate-related osteonecrosis of the jaw with Er,Cr:YSGG laser and platelet-rich plasma: a longitudinal study. *Biomed Res Int.* (2018) 2018:3982540. doi: 10.1155/2018/3982540
101. Park JH, Kim JW, Kim SJ. Does the addition of bone morphogenetic protein 2 to platelet-rich fibrin improve healing after treatment for medication-related osteonecrosis of the jaw? *J Oral Maxillofac Surg.* (2017) 75:1176–84. doi: 10.1016/j.joms.2016.12.005
102. Bilimoria R, Young H, Patel D, Kwok J. The role of piezoelectric surgery and platelet-rich fibrin in treatment of ORN and MRONJ: a clinical case series. *Oral Surg.* (2018) 11:136–43. doi: 10.1111/ors.12318
103. Scala M, Gipponi M, Mereu P, Strada P, Corvo R, Muraglia A, et al. Regeneration of mandibular osteoradionecrosis defect with platelet rich plasma gel. *In vivo.* (2010) 24:889–93.
104. Bilginaylar K. Comparison of the clinical outcomes of buccal advancement flap versus platelet-rich fibrin application for the immediate closure of acute oroantral communications. *J Craniofac Surg.* (2019) 30:e45–9. doi: 10.1097/SCS.00000000000004958
105. Demetoglu U, Ocak H, Bilge S. Closure of oroantral communication with plasma-rich fibrin membrane. *J Craniofac Surg.* (2018) 29:e367–70. doi: 10.1097/SCS.00000000000004360
106. EL-Komy MHM, Hassan AS, Abdel Raheem HM, Doss SS, EL-Kaliouby M, Saleh NA, et al. Platelet-rich plasma for resistant oral erosions of pemphigus vulgaris: a pilot study. *Wound Repair Regen.* (2015) 23:953–5. doi: 10.1111/wrr.12363
107. Picardi A, Ferraro AS, Miranda M, Meconi F, Lanti A, Adorno G, et al. Therapeutic efficiency of platelet gel for the treatment of oral ulcers related to chronic graft versus host disease after allogeneic haematopoietic stem cell transplantation. *Oral Implantol.* (2017) 10:398–405. doi: 10.11138/orl/2017.10.4.398
108. Pathak H, Mohanty S, Urs AB, Dabas J. Treatment of oral mucosal lesions by scalpel excision and platelet-rich fibrin membrane grafting: a review of 26 sites. *J Oral Maxillofac Surg.* (2015) 73:1865–74. doi: 10.1016/j.joms.2015.03.041
109. Bousnaki M, Bakopoulou A, Koidis P. Platelet-rich plasma for the therapeutic management of temporomandibular joint disorders: a systematic review. *Int J Oral Maxillofac Surg.* (2018) 47:188–98. doi: 10.1016/j.jiom.2017.09.014
110. Moussa M, El-Dahab OA, El Nahass H. Anterior maxilla augmentation using palatal bone block with platelet-rich fibrin: a controlled trial. *Int J Oral Maxillofac Implants.* (2016) 31:708–15. doi: 10.11607/jomi.3926
111. Moraschini V, Barboza Edos S. Use of platelet-rich fibrin membrane in the treatment of gingival recession: a systematic review and meta-analysis. *J Periodontol.* (2016) 87:281–90. doi: 10.1902/jop.2015.150420
112. Stahli A, Strauss FJ, Gruber R. The use of platelet-rich plasma to enhance the outcomes of implant therapy: a systematic review. *Clin Oral Implants Res.* 29 (Suppl. 1) (2018) 18:20–36. doi: 10.1111/clr.13296
113. Hill CM, Renton T. Oral surgery II: Part 3. Cysts of the mouth and jaws and their management. *Br Dent J.* (2017) 223:573–84. doi: 10.1038/sj.bdj.2017.916
114. Guo J, Li C, Zhang Q, Wu G, Deacon SA, Chen J, et al. Secondary bone grafting for alveolar cleft in children with cleft lip or cleft lip and palate. *Cochrane Database Syst Rev.* (2011) 2011:CD008050. doi: 10.1002/14651858.CD008050.pub2
115. Lopez-Jornet P, Sanchez Perez A, Amaral Mendes R, Tobias A. Medication-related osteonecrosis of the jaw: is autologous platelet concentrate application effective for prevention and treatment? A systematic review. *J Cranio-Maxillo-Facial Surg.* (2016) 44:1067–72. doi: 10.1016/j.jcms.2016.05.004
116. Kala N, Manjeu J, Dominic N, Babu S. Oral pemphigus without skin lesions treated with pulse steroid therapy. *J Indian Soc Periodontol.* (2018) 22:551–4. doi: 10.4103/jisp.jisp_345_18
117. Ahmad M, Schiffman EL. Temporomandibular joint disorders and orofacial pain. *Dent Clin N Am.* (2016) 60:105–24. doi: 10.1016/j.cden.2015.08.004
118. Fernandez-Ferro M, Fernandez-Sanroman J, Blanco-Carrion A, Costas-Lopez A, Lopez-Betancourt A, Arenaz-Bua J, et al. Comparison of intra-articular injection of plasma rich in growth factors versus hyaluronic acid following arthroscopy in the treatment of temporomandibular dysfunction: a randomised prospective study. *J Cranio-Maxillo-Facial Surg.* (2017) 45:449–54. doi: 10.1016/j.jcms.2017.01.010
119. Andia I, Maffulli N. Platelet-rich plasma for managing pain and inflammation in osteoarthritis. *Nat Rev Rheumatol.* (2013) 9:721–30. doi: 10.1038/nrrheum.2013.141
120. El-Sharkawy H, Kantarci A, Dedy J, Hasturk H, Liu H, Alshahat M, et al. Platelet-rich plasma: growth factors and pro- and anti-inflammatory properties. *J Periodontol.* (2007) 78:661–9. doi: 10.1902/jop.2007.060302
121. Dohan Ehrenfest DM, Pinto NR, Pereda A, Jimenez P, Corso MD, Kang BS, et al. The impact of the centrifuge characteristics and centrifugation protocols on the cells, growth factors, fibrin architecture of a leukocyte- and platelet-rich fibrin (L-PRF) clot and membrane. *Platelets.* (2017) 1–14. doi: 10.1080/09537104.2017.1293812
122. Bradaschia-Correa V, Josephson AM, Mehta D, Mizrahi M, Neibart SS, Liu C, et al. The selective serotonin reuptake inhibitor fluoxetine directly inhibits osteoblast differentiation and mineralization during fracture healing in mice. *J Bone Miner Res.* (2017) 32:821–33. doi: 10.1002/jbmr.3045
123. Wu X, Al-Abedalla K, Rastikerdar E, Abi Nader S, Daniel NG, Nicolau B, et al. Selective serotonin reuptake inhibitors and the risk of osseointegrated implant failure: a cohort study. *J Dent Res.* (2014) 93:1054–61. doi: 10.1177/0022034514549378
124. Yung YL, Fu SC, Cheuk YC, Qin L, Ong MT, Chan KM, et al. Optimisation of platelet concentrates therapy: composition, localisation, duration of action. *Asia-Pacific J Sports Med Arthroscopy Rehabil Technol.* (2017) 7:27–36. doi: 10.1016/j.asmart.2016.11.003

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Al-Hamed, Mahri, Al-Waeli, Torres, Badran and Tamimi. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Platelets and Complement Cross-Talk in Early Atherogenesis

Hugh Kim^{1,2,3*†} and Edward M. Conway^{1,4*†}

¹ Centre for Blood Research, Life Sciences Institute, University of British Columbia, Vancouver, BC, Canada, ² Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada, ³ Faculty of Dentistry, University of British Columbia, Vancouver, BC, Canada, ⁴ Faculty of Medicine, University of British Columbia, Vancouver, BC, Canada

Atherosclerosis remains a ubiquitous and serious threat to human health. The initial formation of the atherosclerotic lesion (atheroma) is driven by pro-inflammatory signaling involving monocytes and vascular endothelial cells; later stages of the disease involve rupture of well-established atherosclerotic plaques, thrombosis, and blood vessel occlusion. While the central role of platelets in thrombosis is undisputed, platelets exhibit pro-inflammatory activities, and contribute to early-stage atheroma formation. Platelets also engage components of the complement system, an essential element of innate immunity that contributes to vascular inflammation. Here we provide an overview of the complex interplay between platelets and the complement system, with a focus on how the crosstalk between them may impact on the initiation of atheroma formation.

OPEN ACCESS

Edited by:

Paul Jurasz,
University of Alberta, Canada

Reviewed by:

Teresa Padro,
Sant Pau Institute for Biomedical
Research, Spain
Christoph Eugen Hagemeyer,
Monash University, Australia

*Correspondence:

Hugh Kim
hughkim@dentistry.ubc.ca
Edward M. Conway
ed.conway@ubc.ca

[†]These authors have contributed
equally to this work

Specialty section:

This article was submitted to
Atherosclerosis and Vascular
Medicine,
a section of the journal
Frontiers in Cardiovascular Medicine

Received: 08 June 2019

Accepted: 22 August 2019

Published: 06 September 2019

Citation:

Kim H and Conway EM (2019)
Platelets and Complement Cross-Talk
in Early Atherogenesis.
Front. Cardiovasc. Med. 6:131.
doi: 10.3389/fcvm.2019.00131

Keywords: platelets, complement, atheroma, inflammation, atherosclerosis, endothelium, innate immunity, vascular

INTRODUCTION

Cardio-cerebrovascular diseases are commonly caused by atherosclerosis, an inflammatory vasculopathy characterized by the formation of atheromatous plaques along the arterial wall. If the disease progresses, blood flow becomes limited, with resultant tissue injury, and organ dysfunction. Several pro-inflammatory events lead to plaque formation [reviewed in (1–3)]. Early in the process, endothelial activation promotes binding of monocytes to the vascular wall. These cells subsequently migrate into the subendothelial space, where they differentiate into macrophages. They then phagocytose oxidized low-density lipoproteins (oxLDL) which are deposited within the vascular wall, resulting in their transformation into lipid-laden foam cells. In parallel, vascular smooth muscle cells synthesize a fibrous connective tissue cap that surrounds the central lipid-macrophage core. Long-standing pro-coagulant and pro-inflammatory atherosclerotic plaques are prone to rupture, resulting in life-threatening thrombosis and ischemia, as is observed during myocardial infarction, and stroke. Platelets play a central role in thrombus formation secondary to plaque rupture. However, platelets are also highly immune-competent cells that, with activation of the complement system, are believed to orchestrate the initial signaling events during vascular inflammation that are critical for atheroma formation. In this review, we focus on the participation of platelet-complement crosstalk in early atherogenesis. We begin with a broad overview of the concepts of platelet activation and how this may trigger atheroma formation via interactions with leukocytes and endothelial cells [readers are referred to detailed reviews on this topic (4–6)]. This is followed by a description of the complement cascade and its regulation. We finally focus on the inter-relationship between platelets and the complement system, highlighting several of the complex links, how they may impact on atherogenesis, and on the potential clinical utility of recently uncovered pathways.

PLATELET SIGNALING PROMOTES ATHEROGENESIS

Platelet Activation and Secretion

Platelets are 2–4 μm diameter cells that circulate in resting discoid forms at a concentration of 150,000–450,000 cells/ μl of whole blood, and become activated upon stimulation of their cell surface receptors by corresponding ligands (4). The role of platelets in thrombosis following plaque rupture in late-stage atherosclerosis is well-known. However, platelets also store >30 cytokines and growth factors in granules—alpha (α)-granules, dense granules, and lysosomes (7). Platelet activation is typically accompanied by rapid shape change, with fusion of the granule membranes with the platelet's plasma membrane, and the inner membranous network, to form the so-called open canalicular system (7, 8). Platelet secretion that occurs with this activation, is characterized by rapid translocation of P-selectin from the α -granules to the plasma membrane and extracellular release of soluble cytokines, chemokines, growth factors, and complement components (9). Activated platelets also uniquely express multiple receptors on their surface, and release platelet microparticles (PMPs), which are <1 μm diameter vesicular bodies containing a variety of cytokines, including for example, interleukin-1 β (IL-1 β) (10). Importantly, platelet-derived cytokines from activated platelets are believed to contribute to vascular inflammation at the early stages of atheroma formation (11). Strong evidence exists that platelets also promote atherogenesis by acting as lipid-carrying structures (12), by signaling to vascular endothelium, and by recruiting leukocytes to the nascent atheroma (13).

Platelet-Endothelial Cell Signaling

Under physiologic conditions, the endothelial cell layer is separated from circulating platelets and leukocytes by a proteoglycan-rich layer termed the glycocalyx (14). Endothelial cells also produce prostacyclin (15), and nitric oxide (16) which serve to maintain platelets in their quiescent states. However, activation of the endothelial cell layer promotes atheroma formation. Activated endothelial cells express platelet-adhesive molecules including for example, E-selectin (17) and von Willebrand factor (VWF) (18), and release platelet agonists such as thromboxane (19). These molecules serve to recruit, activate, and tether platelets to the vessel wall. The activated platelets in turn, express P-selectin (20), and release multiple cytokines and chemokines that further induce endothelial cell activation (21), with the result being more recruitment and activation of platelets at the site of the vascular lesion. Unless dampened by natural or pharmacologic interventions, the process becomes self-sustaining with progressive vascular damage, and atherogenesis.

Platelet-Derived Chemokines Recruit and Retain Monocytes at Sites of Vascular Inflammation

In addition to promoting endothelial cell activation, platelet-derived chemokines recruit monocytes, thereby propagating atheroma development. CXCL4/PF4 (platelet factor four), a major constituent of platelet α -granules (22–25), is one

of several well-characterized monocyte chemoattractants. CCL5/RANTES (regulated on activation normal T-cell expressed and secreted) is also a platelet-derived chemokine with monocyte chemoattractant properties (26, 27).

The pathophysiologic relevance of these and other platelet-released factors in atheroma formation is supported by several observations. For example, *in vitro* analyses revealed that monocytes preferentially adhere to endothelial cells pre-incubated with PF4 and/or RANTES (28, 29), while *in vivo* studies showed that the size of experimentally-induced atherosclerotic lesions were significantly reduced in PF4-null mice (30). Evidence also exists from clinical studies to support the contribution of PF4 and RANTES in atherosclerosis. In analyses of 132 carotid atheromatous plaques, PF4 presence directly correlated with lesion severity, in terms of histological grading of the lesions, and the history of significant clinical events such as myocardial infarction (31). RANTES plasma levels in patients hospitalized with acute coronary syndrome also correlated with progressive disease (32). That the chemotactic properties of PF4 and RANTES promote vascular disease severity underlines the importance of understanding how these and other similarly biologically active factors are released by platelets. Increasing evidence supports the notion that the complement system plays an important role.

THE COMPLEMENT SYSTEM

Activation via 3 Pathways

The complement system is a tightly regulated blood borne proteolytic system, a key component of innate immunity, that responds rapidly to clear damaged host cells and invading pathogens, to limit tissue destruction and to effect healing. This system is intimately involved in platelet function and the pathogenesis and progression of atherosclerosis. Thus, a brief review is provided [more extensive reviews can be found in (33–36)].

Comprising over 30 soluble and membrane bound proteins, complement activation is triggered by exposure to damage-associated molecular patterns, initiated via the lectin (LP), classical (CP), or alternative (AP) pathways (**Figure 1**). The CP is triggered by C1q recognition of antibodies or other targets (e.g., C-reactive protein, apoptotic cells) bound to antigens or microbial surfaces. C1q circulates in complex with zymogen forms of serine proteases C1r and C1s. Exposure of C1q to its target results in activation of C1r and C1s (37), followed by C1s-mediated cleavage of C4 into C4a (an anaphylatoxin) and the opsonin C4b. C2 complexes with immobilized C4b, and is also cleaved by C1s into C2b and C2a. The resultant C4b2a complex is the CP C3 convertase which cleaves C3 into C3b, liberating the anaphylatoxin C3a. C3b binds to the surface of nearby cells/microbes for downstream complement activation.

Similar to the CP, in the LP, mannose binding lectin (MBL), ficolins, and/or collectin-11 circulate in complex with MBL-associated zymogens of serine proteases, MASP1/MASP3, and MASP2 (38). These complexes bind to sugars on microorganisms or damaged cells, whereupon MASP1 autoactivates

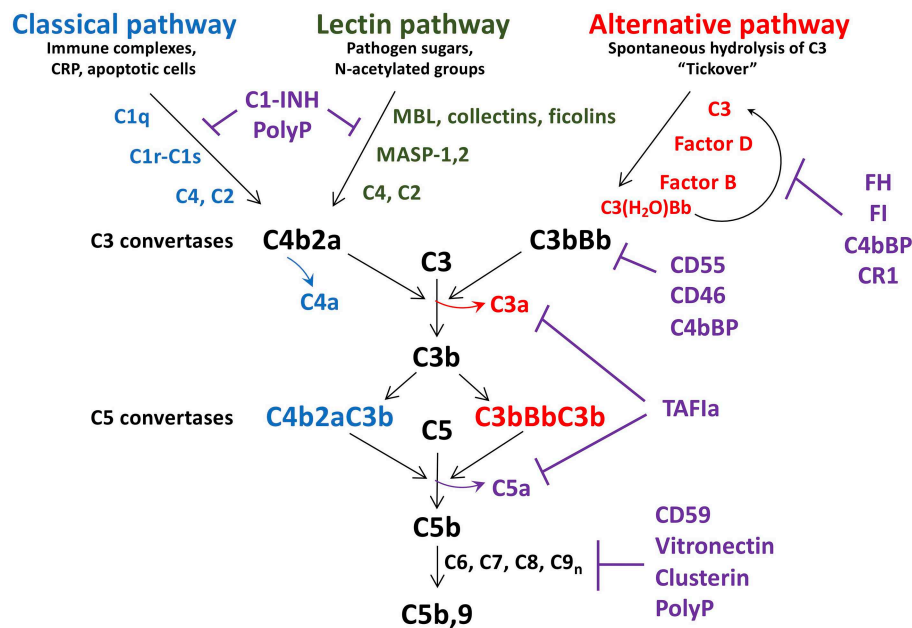


FIGURE 1 | Schematic of complement activation and regulation. Complement activation occurs via the classical, lectin, or alternative pathways, triggered by exposure of C1q, MBL, collectins and ficolins, to danger signals. The alternative pathway is constitutively active, due to spontaneous hydrolysis of C3 to C3(H₂O). All pathways converge to form C3 convertases, with release of C4a, and C3a. As C3b is further generated, C5 convertases C4bBbC3b, and C3bBbC3b are formed, resulting in release of the potent anaphylatoxin C5a, in concert with C5b. C5b is the initial factor required for assembly of the C5b-9 membrane attack complex which induces lysis/damage to the cellular target. Tight regulation is achieved at multiple levels by soluble and membrane associated factors (C1-INH, FH, FI, CD55, CD46, polyphosphate (polyP), CD59, clusterin, and vitronectin). C1-INH, C1 esterase inhibitor; MBL, mannose binding lectin; CRP, C-reactive protein; MASP, MBL associated serine protease; FH, factor H; FI, factor I; TAFIa, activated thrombin activatable fibrinolysis inhibitor.

and cleaves C2, as well as MASP2, which then cleave C2 and C4, yielding C4b2a, the LP C3 convertase (39).

In contrast to the CP/LP, the AP is constitutively active via a “tick-over” mechanism in which small amounts of circulating C3 spontaneously hydrolyze into C3(H₂O) (40). This yields a binding site for factor B (FB) which is cleaved into Ba and Bb by factor D. Bb binds to C3(H₂O) to form a fluid-phase C3 convertase, which can cleave C3 to generate C3a and C3b. Relevant to a relationship between platelets and complement, surface contact may also trigger hydrolysis of C3 and thus activation of the AP (41). This is achieved via the release of properdin by inflammatory leukocytes, which binds to activated platelets and recruits C3(H₂O) to promote formation of cell-bound C3(H₂O)Bb (42). This pathway supports the notion that platelets cooperate with activated leukocytes to trigger complement activation via the AP, amplifying generation of C3b, and formation of a stable C3bBb AP C3 convertase.

As noted above, the three complement pathways converge with the formation of their respective C3 convertases, and generation of C3a, and C3b (Figure 1). If activation is sufficient, excess C3b binds to these convertases, to generate C5 convertases, which cleave C5 into C5b, and C5a. C5a is the most potent anaphylatoxin, with a range of pro-inflammatory and pro-coagulant properties (see below). C5b binds to C6, and assembles with C7, C8, and multiple C9 molecules, yielding C5b-9, the so-called membrane attack complex (MAC), which has pore-forming, lytic properties designed to destroy invading organisms and damaged/foreign cells (see below) (43).

Regulation of Complement

Complement activation is tightly regulated at multiple levels to prevent host cell damage and to allow healing to proceed. This is achieved via the coordinated actions of several membrane anchored and fluid-phase regulators, some of which will be discussed [(35); Figure 1]. C1-esterase inhibitor (C1-INH) is a serine protease inhibitor that dampens the CP and LP by neutralizing C1r, C1s, MASP-1, and MASP-2, each interaction variably potentiated by heparan sulfate and polyphosphate (37, 44, 45). It is synthesized by hepatocytes, but also by fibroblasts, endothelial cells, monocytes, megakaryocytes, and platelets. C1-INH also interferes with several pro-coagulant and pro-inflammatory enzymes, including factors XIa, XIIa, and kallikrein. Factor H (FH) is the major fluid-phase negative regulator of the AP (46). Synthesized by hepatocytes, but also by endothelial cells, platelets, and monocytes (47, 48), FH is a cofactor for factor I (FI) mediated inactivation of C3b, a decay accelerating factor of the AP C3 convertase, and a competitor to FB binding to C3b. A platelet-released kinase (49) can reduce FH binding to C3b, thereby enhancing the inflammatory response (50). FH also colocalizes with VWF in Weibel-Palade bodies and variably modulates ADAMTS13-mediated proteolysis of ultra large VWF (ULVWF) multimers, thereby impacting on platelet-vessel wall interactions (51–54), and thus atherogenesis. Negative regulators of the terminal pathway of complement activation include CD59, which binds to C8 and C9 and prevents C9 polymerization (55), clusterin and vitronectin, which reduce membrane integration of C5b-9 (56, 57), and polyphosphate,

which destabilizes the C5b,6 complex, reducing C5b-9 insertion into the membrane (58).

PLATELET-COMPLEMENT CROSS-TALK SYNERGIZES IN ATHEROMA FORMATION

Considerable evidence supports the notion that complement and platelets act in concert to orchestrate the early cellular and molecular events that promote atheroma formation [reviewed (59, 60)]. Importantly, there exist reciprocal signaling pathways between platelets and the complement system (61) that impact on the vascular endothelium, and potentiate or attenuate each other's pro-atherogenic properties.

Pro-Atherogenic Properties of Complement: Targeting the Vascular Endothelium

The complement system is a major contributor to inflammation and thrombosis, critical features underlying atheroma formation, and progression of atherosclerosis. Indeed, many complement components are found in atheromatous lesions where they participate in initiating and sustaining inflammation (62, 63). Endothelial cells express receptors for several complement components, most notably C1q (64), C3a (65), and C5a (66). The anaphylatoxins C3a and C5a bind to their cognate G protein-coupled receptors (C3aR for C3a; C5aR1, and C5L2 for C5a) and trigger pro-inflammatory and pro-thrombotic activities. Both C3a and C5a induce endothelial cell expression of pro-inflammatory IL-8, IL-1, and RANTES. They upregulate expression of key leukocyte adhesion molecules, VCAM-1, ELAM-1, ICAM-1, and P-selectin (67, 68). They also induce a pro-thrombotic phenotype through C5a-mediated tissue factor (TF) expression on neutrophils (69) and endothelial cells, and VWF secretion from endothelial cells (67). Indeed, C3a and C5a are pivotal in recruiting and activating monocytes, neutrophils and macrophages, promoting endothelial permeability (70), and providing a nidus for clot formation, all of which are required for the initiation, and expansion of an atherosclerotic plaque. The terminal pathway complexes, C5b-7, C5b-8, and C5b-9 also participate by augmenting VCAM-1 expression by endothelial cells (71), inducing cellular release of pro-inflammatory mediators, such as IL1- α , that cause leukocyte recruitment (72), and promote functional expression of TF. This occurs partly via activation of MAPKs leading to its transcriptional upregulation (73), and by inducing its activation via oxidation of cell surface protein disulfide isomerase (PDI) (43, 74).

Complement-Platelet Crosstalk

Like endothelial cells, platelets express receptors for C1q (75), C3a (76), C4 (77), and C5a (59). They also release complement components upon activation, including C1q, C3, C4, and C5b-9 (61). These platelet-derived complement factors/complexes may promote atherogenesis in several ways. Firstly, secreted platelet-derived complement components can activate other platelets via autocrine and paracrine signaling. Secondly, as

recognized in the preceding section, complement proteins from activated platelets propagate vascular inflammation by further activating the endothelium and/or recruiting leukocytes to nascent atheroma. Thirdly, and as further discussed, various complement components retained at the platelet surface can serve as substrates for continued complement activation, resulting in a tightly regulated, positive feedback loop of complement, and platelet activation (78). Several of the following described complement-platelet interactions that may impact on atherogenesis are depicted schematically in **Figure 2**.

The direct correlation of expression levels of receptors for C3a and C5a with platelet activation in patients with coronary artery disease supports their potential pathophysiologic relevance (59). Via their cognate receptors, C3a and C5a trigger platelet activation and aggregation (79), inducing exposure of P-selectin and the receptor for C1q (C1qR). P-selectin is a receptor for C3b, providing a site for assembly of the AP C3 convertase, and ultimately, if not checked by negative regulators, for formation of the C5b-9 MAC (80). P-selectin is also a ligand for leukocyte-expressed P-selectin glycoprotein ligand-1 (PSGL-1), which together are strongly implicated in promoting atherogenesis by enhancing recruitment of leukocytes to sites of inflammation (81).

C1q binding to C1qR cooperates with chondroitin sulfate A (CSA) that is released from α -granules of activated platelets to support C1q-mediated complement activation via the CP (82, 83). The C1q:C1qR interaction also induces P-selectin release and platelet activation, adhesion, and aggregation (84). Interestingly, C1q plays dual and apparently opposing roles in the inflammatory response, as it also dampens platelet-neutrophil aggregate formation (85) by interfering with P-selectin-PSGL-1 interactions. This is consistent with platelets possessing multiple negative regulatory mechanisms to keep inflammation in check, and to also provide protection to the platelet from complement mediated destruction. Indeed, the anaphylatoxins C3a and C5a, acting via their cognate receptors, also trigger platelet release of FH and C4b binding protein (C4bBP), both of which are negative regulators of the AP of complement (**Figure 2**), the latter which also negatively regulates toll like receptor (TLR)1/2 mediated pro-inflammatory cytokine production (86, 87).

Evidence from human studies supports the notion that the C3a/C3aR axis participates in atheroma formation. In a cross-sectional analysis of >500 individuals, plasma levels of C3a strongly and positively correlated with carotid artery intima-media thickness (88). In patients with coronary artery disease, there was also a strong positive correlation of C3aR expression on activated platelets with that of GpIIb/IIIa, with experimental evidence that the C3a/C3aR axis regulates platelet function via activation of the small GTPase Rap1b (59, 76). In that manner, the C3a receptor (C3aR) directly impacts on hemostasis, since mice lacking C3aR exhibit prolonged bleeding times, with reduced ADP-triggered platelet aggregation. They are also protected against C3a-triggered thrombosis, with reduced severity of experimental stroke and myocardial ischemia (76).

The two structurally homologous but distinct C5a receptors, C5aR1 (CD88), and C5aR2 (C5L2), have been reported to be highly expressed in atherosclerotic lesions (6). Blockade or gene

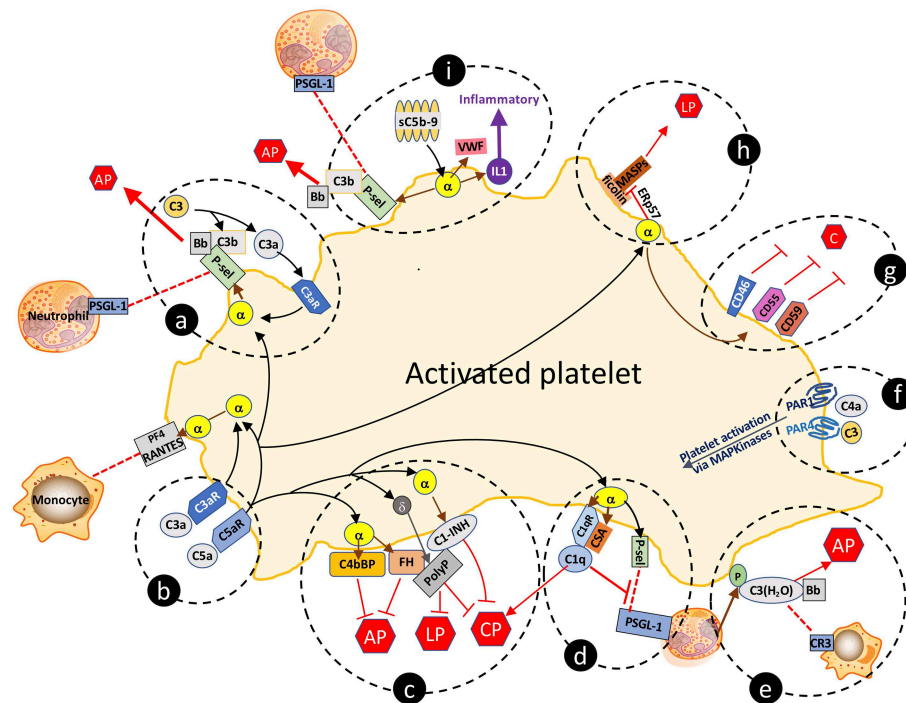


FIGURE 2 | Complement-platelet interactions that can facilitate an inflammatory response that favors atheroma formation. Selected interactions between complement and platelets, as described in the manuscript, are highlighted. **(a,b)** C3a and C5a bind to their cognate receptors to trigger release of factors from α -granules. P-selectin localizes to the platelet surface and is a receptor for leukocyte expressed PSGL-1 and for C3b, the latter which allows for initiation of the AP and amplification of C3a-triggered platelet activation. **(c)** Complement activation on the platelet surface is dampened by α -granule release of cofactors C4bBP, FH and C1-INH, and δ -granule release of the anti-complement, prothrombotic polyphosphate. Polyphosphate binds to FH and C1-INH, and downregulates complement activation via the CP and the terminal pathway. **(d)** By binding to C1qR, C1q also triggers α -granule release of P-selectin and chondroitin sulfate (CSA), the latter which enhances the C1q-C1qR interaction, initiating the CP but negatively regulates leukocyte recruitment by interfering with P-selectin-PSGL-1 interactions. **(e)** Neutrophil-released properdin (P) stabilizes the convertases and is also a receptor for C3(H₂O) which complexes with Bb to form the AP C3-convertase, and is a ligand for leukocyte-expressed CR3, thereby facilitating leukocyte cell migration to the site of inflammation. **(f)** C4a and C3 can activate platelets via distinct interactions with PAR1 and PAR4. **(g)** Activated platelets are also protected against complement-mediated destruction by granule release of negative regulators of complement, cell surface expressed CD46, CD55, and CD59. **(h)** Sublytic C5b-9 (sC5b-9) triggers platelet activation with release of VWF, P-selectin and inflammatory cytokines (e.g., IL1), the latter which further promotes inflammation. **(i)** Ficolins on the surface of activated platelets are receptors for MASPs which can trigger the LP. Release of the isomerase ERp57 modifies the ficolin to limit its functional capacity to trigger complement. C, complement activation pathways; AP, alternative pathway; LP, lectin pathway; CP, classical pathway; PF4, platelet factor 4; C3aR, C3a receptor; C5aR, C5a receptor; C1qR, C1q receptor; P-sel, P-selectin; PSGL-1, P-selectin glycoprotein ligand-1; sC5b-9, sublytic C5b-9; VWF, von Willebrand factor; α , α -granule; δ , δ -granule; ERp57, endoplasmic reticulum protein 57; PAR, protease activated receptor; P, properdin; CR3, complement receptor 3; PolyP, polyphosphate; IL1, interleukin 1.

inactivation in mice of either of these, results in protection against diet-induced atherosclerosis (89), consistent with what is observed when C5a is blocked (90). Most intriguing, blockade of both C5aR1 and C5aR2 had added benefit in reducing neointimal plaque size and inflammation in a wire injury model. Since the C5a receptors are widely expressed by many cell types, the specific role of the receptors on platelets was not determined. Nonetheless, the findings are impressive, and imply that a multi-modal approach to tackle atherosclerosis will likely yield improved outcomes.

C4a, generally accepted to be the weakest of the three anaphylatoxins, has unique properties in terms of crosstalk with other innate immune pathways to induce platelet activation. Although a specific receptor for C4a has remained elusive, recent studies indicate that C4a binds to PAR1 and PAR4 as an untethered agonist, triggering activation of downstream

pro-inflammatory MAPKines, thereby participating in platelet activation via alternative routes (91). The pathophysiologic relevance of this pathway in atherogenesis has not yet been explored.

C3 itself also plays an important role in platelet function. C3-deficient mice exhibit delayed hemostasis, based on tail bleeding times and are protected against atheroma formation in the atherosclerosis-prone low density lipoprotein receptor-null (*Ldlr*^{-/-}) mice (92). Aggregation of the C3-deficient platelets was significantly dampened in response to the PAR-4 agonist peptide (93), a defect that was rescued by the addition of exogenous plasma C3 [(93), Figure 2]. Consistent with these findings, C3-null platelets stimulated with convulxin (an agonist for glycoprotein VI, GPVI), respond with reduced surface exposure of P-selectin, von Willebrand factor (VWF), and annexin V (94), effects that dampen both inflammation

and hemostasis/thrombosis. As mentioned previously, C3 is readily hydrolyzed to C3(H₂O) which may bind to the activated platelet surface in the presence of leukocyte derived properdin, promoting formation of a platelet surface-bound C3(H₂O)Bb convertase (42). This would thus enable complement activation to proceed via the AP. When associated with the platelet, C3(H₂O) may also serve as a ligand for leukocyte cell surface receptor CD11b/CD18 [also referred to as complement receptor 3 (CR3) or integrin $\alpha_M\beta_2$], facilitating formation of platelet-leukocyte interactions and recruitment of activated macrophages to atheroma. Again, the physiologic relevance of each of these independent interactions remain unclear, but taken together support the *in vivo* evidence of a role for C3/C3(H₂O) in atherosclerosis (92).

In contrast to the strong evidence that C3, C3a/C3aR, and C5a/C5aR are important contributors to platelet activation and atherogenesis, the role of C5 in modulating platelet function in health and vascular disease is more controversial. This is in spite of most studies concluding that C5 participates in promoting tissue factor mediated fibrin clot formation *in vivo*. In murine models, histones trigger aggregation of platelets from wild-type mice but not from C5-deficient mice (95), suggesting that C5 is essential for normal platelet function. These findings however, seem in conflict with the work of others, in which platelets from C5-deficient mice responded normally to agonist induced platelet release of P-selectin, vWF, and annexin V, and furthermore, did not exhibit any hemostatic or vessel wall platelet deposition defects *in vivo* (94). Moreover, the development of atherosclerosis in *ApoE*(^{-/-}) mice was not affected by genetic deletion of C5 (96). Such apparent discrepancies in the interpretation of the role of C5 in platelet function and vascular disease highlight the need for further study.

The lectin pathway (LP) also participates in platelet activation and function (97). Ficolins (but not MBL), MASP-1, and MASP-2, were detected as complexes on the surface of activated platelets, indicating that the initiating factors can assemble to trigger cleavage of C4 and C2 (Figure 2). Moreover, ficolins and MBL were present in the plaques of atherosclerotic lesions derived from patients undergoing carotid endarterectomy (98). This pathway appears to be dampened on the cell surface by the release from activated platelets of C1-INH (Figure 2). Recent data indicate that the LP can also be attenuated by the release from activated platelets of the thiol isomerase ERp57, which interferes with ficolin recognition via disruption of its multimerization (99). While not proven, these pathways may reasonably impact on the inflammatory response to injury that leads to atheroma formation. Further *in vivo* studies will be required for validation and to ascertain whether there are targetable steps for treatment design.

The concept of platelet-released enzymes, as described above for ERp57, was reported >20 years ago (49), but is now a re-emerging area of interest. Protein kinases released from the α -granules of activated platelets in concert with ATP and divalent cations (Ca²⁺) from dense granules, have been shown to phosphorylate plasma proteins, including coagulation factors XI, Va, and protein S, thereby modifying their functions. In the context of complement, C3 phosphorylation occurs by this

method, resulting in the C3b cleavage fragment being more resistant to factor I-mediated inactivation to iC3b. Degradation of the kinase-modified C3 also generates C3d that binds more avidly to complement receptor 1 (CR1/CD35), thereby enhancing opsonin activity (50) and clearance of immune complexes by phagocytosis. Genetic variations of the CR1 gene have been linked to the risk of incident coronary artery disease and inflammation by unknown mechanisms. Although entirely speculative, it is possible that the kinase-mediated modification of C3 from activated platelets may contribute.

The terminal pathway of complement also participates in platelet activation and atherogenesis. Sublytic concentrations of C5b-9 bind to the platelet surface and induce activation and α -granule secretion (79, 100). C5b-9 also induces changes in the orientation of the phospholipid membranes of platelets that favor binding of factor Va, prothrombinase assembly, and generation of thrombin (74, 101, 102). Similar to C3a and C5a, C5b-9 triggers platelet secretion of VWF, P-selectin and pro-inflammatory cytokines (e.g., IL1) (Figure 2), the expression of adhesion molecules on platelets (103, 104), and the release of platelet microparticles (PMPs), any of which may modulate vascular responses to thrombo-inflammatory stimuli.

Self-Preservation of the Activated Platelet by Complement Regulators

The profound changes in the structure and the expression pattern of proteins and glycolipids on the surface of the activated platelet, would be expected to trigger a host innate immune response that would destroy the cell, rendering it unable to complete its prothrombotic/prohemostatic function in the setting of injury and bleeding. Yet, in spite of the platelet being activated by several complement factors (e.g., C3a, C5a, C5b-9), and providing sites for assembly of the convertases (e.g., P-selectin, CSA, C1qR), complete formation and integration of a lytic C5b-9 MAC, is normally held in check, preserving the integrity of the prothrombotic platelet. This is achieved through the action of numerous negative regulators of complement, stored in the platelet and released upon activation. Thus, platelet α -granules contain C1-INH, FH, CD55, CD59, CD46, and clusterin, while polyphosphate is housed in dense granules (105). All can accumulate on the activated platelet surface and prevent generation of the C5b-9 MAC via the CP, the LP and/or the terminal pathway (Figure 2). Indeed, we have shown that polyphosphate directly interacts with C1-INH, augmenting the serine protease inhibitor's anticomplement activity, while retaining the prothrombotic properties of polyphosphate (106), and thus, presumably, the prothrombotic function of the platelet (45, 58). Although the physiologic relevance of the platelet pool of these negative regulators in atherogenesis, has not been specifically validated, global blockade via gene inactivation or pharmacologic interventions have established their importance. Thus, for example, lack of CD55 or CD59 in atherosclerosis-susceptible *ApoE*^{-/-} mice, resulted in worse disease, while CD59 administration reduced the severity of experimental atherosclerosis by abrogating MAC formation (107, 108). Similarly, atheroma formation

in *Ldlr*^{-/-} mice, was attenuated by administration of C1-INH (109).

These complement regulatory factors and a soluble form of C5b-9 also are found in/on PMPs that are released in the setting of platelet activation. The function of these PMPs is not clear, as they variably contain other complement activating factors that are found in and on platelets (60, 78, 110, 111). The PMPs also contain/express variable amounts of tissue factor, factor V, other coagulation-related factors, growth factors, cytokines, lipids, ions, and microRNAs [reviewed in (112)]. Given the complex nature of PMPs and their variability in composition under different pathological conditions, it has been challenging to discern their primary roles in different disease states and stages. Nonetheless, speculation abounds, including the notion that PMPs serve to clear sublytic C5b-9 away from the platelet (60), thereby providing local protection to the platelet.

CONCLUSION

In this brief review, we highlight the complexity of the cross-talk pathways between platelets and the complement system, particularly as they pertain to early stages of atherosclerosis. As is evident, there are multiple apparently opposing factors and pathways that may promote or prevent inflammation and atheroma formation. In the delicate balance that maintains immune and vascular homeostasis in the face of multiple stresses, this is as expected. When this balance is unfavorably tipped due to genetic, epigenetic and/or environmental factors, inflammation, and atherogenesis may proceed. By understanding which factors are tipping that balance and how they function, more effective preventative and therapeutic strategies may be designed.

Notably, and in spite of promising data that complement activation directly correlates with atheroma formation and atherosclerosis, and that interfering with the complement cascade may be beneficial, at least in preclinical models, anti-complement therapies have not entered the clinic. This may be because the mouse model does not fully recapitulate the human condition. It may reflect the current high cost of the very few anti-complement drugs that are available for clinical use (e.g., eculizumab). Or it may be that appropriate trials have yet to be performed. From the complement cascade, it is evident that there are multiple potential steps at which interventions might be envisaged, that could potentially dampen feed-forward loops in/on the platelet that drive inflammation and atherogenesis. Indeed, strategic initiatives by industry and academia that aim to target the complement system to treat a range of vascular and inflammatory diseases abound. A full discussion of drugs at various stages of development is beyond the scope of this brief

report. However, several of the opportunities and challenges are well-addressed in recent reviews (113–117). From the relatively simple scheme that we offer in **Figure 2**, treatments could include, for example, agents that interfere with C1q interactions with C1qR and/or CSA to dampen activation of the CP and reduce monocyte adhesion to endothelial cells. Antibodies against C1s are being used successfully to suppress CP-triggered cold-agglutinin disease (118) and might reasonably have efficacy in preventing atheroma formation. Cp40 is a cyclic peptide and analog of compstatin that inhibits C3-mediated activation of endothelial cells to reduce leukocyte adhesion (119). Rapamycin upregulates expression of CD55 by inducing protein kinase C α , AMP-activated kinase, and CREB-dependent pathways, thereby dampening allograft vasculopathy, a benefit that synergizes with statin therapy (120). With experimental evidence of a role for the LP in atherogenesis, MASP-2 inhibitors that are currently being evaluated for hereditary angioedema and other disorders (121), could also be considered to intervene in the platelet-complement crosstalk driving atherogenesis.

Indeed, the near future will likely see many complement-targeted therapies enter the clinic for various innate immune/inflammatory disorders. The challenges will be how to select which ones are best for intervening in atheroma formation, whether multiple pathways should be targeted, how to select when to administer, how long, how much, and how to monitor (122). No matter which anti-complement interventions are used, effective treatments will undoubtedly require maneuvers to mitigate against the critical dietary, environmental and epi/genetic triggers that drive the disease (123).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

HK and EC were supported by grants from the Canadian Institutes of Health Research (CIHR) and the Canada Foundations for Innovation (CFI) and are members of the Life Sciences Institute (LSI). HK holds awards as a CIHR Clinician-Scientist and a Michael Smith Foundation for Health Research (MSFHR) Scholar. EC also received funding from the Natural Sciences and Engineering Research Council of Canada (NSERC), the Heart and Stroke Foundation of Canada, and CanVECTOR. He holds a Tier 1 Canada Research Chair in Endothelial Cell Biology and is an Adjunct Scientist with the Canadian Blood Services.

REFERENCES

- Raggi P, Genest J, Giles JT, Rayner KJ, Dwivedi G, Beanlands RS, et al. Role of inflammation in the pathogenesis of atherosclerosis and therapeutic interventions. *Atherosclerosis*. (2018) 276:98–108. doi: 10.1016/j.atherosclerosis.2018.07.014
- Libby P. Current concepts of the pathogenesis of the acute coronary syndromes. *Circulation*. (2001) 104:365–72. doi: 10.1161/01.CIR.104.3.365
- Woollard KJ. Immunological aspects of atherosclerosis. *Clin Sci*. (2013) 125:221–35. doi: 10.1042/CS20120576
- van der Meijden PEJ, Heemskerk WMJ. Platelet biology and functions: new concepts and clinical perspectives. *Nat Rev Cardiol*. (2019) 16:166–79. doi: 10.1038/s41569-018-0110-0

5. Marchio P, Guerra-Ojeda S, Vila JM, Aldasoro M, Victor VM, Mauricio MD. Targeting early atherosclerosis: a focus on oxidative stress and inflammation. *Oxid Med Cell Longev*. (2019) 2019:8563845. doi: 10.1155/2019/8563845
6. Hovland A, Jonasson L, Garred P, Yndestad A, Aukrust P, Lappégard KT, et al. The complement system and toll-like receptors as integrated players in the pathophysiology of atherosclerosis. *Atherosclerosis*. (2015) 241:480–94. doi: 10.1016/j.atherosclerosis.2015.05.038
7. Heijnen H, van der Sluijs P. Platelet secretory behaviour: as diverse as the granules... or not? *J Thromb Haemost*. (2015) 13:2141–51. doi: 10.1111/jth.13147
8. Selvadurai MV, Hamilton JR. Structure and function of the open canalicular system - the platelet's specialized internal membrane network. *Platelets*. (2018) 29:319–25. doi: 10.1080/09537104.2018.1431388
9. Joshi S, Whiteheart SW. The nuts and bolts of the platelet release reaction. *Platelets*. (2017) 28:129–37. doi: 10.1080/09537104.2016.1240768
10. Boilard E, Nigrovic PA, Larabee K, Watts GF, Coblyn JS, Weinblatt ME, et al. Platelets amplify inflammation in arthritis via collagen-dependent microparticle production. *Science*. (2010) 327:580–3. doi: 10.1126/science.1181928
11. Bakogiannis C, Sachse M, Stamatelopoulou K, Stellos K. Platelet-derived chemokines in inflammation and atherosclerosis. *Cytokine*. (2017) 122:154157. doi: 10.1016/j.cyt.2017.09.013
12. Magwenzi S, Woodward C, Wraith KS, Aburima A, Raslan Z, Jones H, et al. Oxidized LDL activates blood platelets through CD36/NOX2-mediated inhibition of the cGMP/protein kinase G signaling cascade. *Blood*. (2015) 125:2693–703. doi: 10.1182/blood-2014-05-574491
13. Gleissner CA, von Hundelshausen P, Ley K. Platelet chemokines in vascular disease. *Arterioscler Thromb Vasc Biol*. (2008) 28:1920–7. doi: 10.1161/ATVBAHA.108.169417
14. Machin DR, Phuong TT, Donato AJ. The role of the endothelial glycocalyx in advanced age and cardiovascular disease. *Curr Opin Pharmacol*. (2019) 45:66–71. doi: 10.1016/j.coph.2019.04.011
15. Stitham J, Midgett C, Martin KA, Hwa J. Prostacyclin: an inflammatory paradox. *Front Pharmacol*. (2011) 2:24. doi: 10.3389/fphar.2011.00024
16. van Hinsbergh VW. Endothelium—role in regulation of coagulation and inflammation. *Semin Immunopathol*. (2012) 34:93–106. doi: 10.1007/s00281-011-0285-5
17. Silva M, Videira PA, Sackstein R. E-selectin ligands in the human mononuclear phagocyte system: implications for infection, inflammation, and immunotherapy. *Front Immunol*. (2017) 8:1878. doi: 10.3389/fimmu.2017.01878
18. Holthenrich A, Gerke V. Regulation of von-willebrand factor secretion from endothelial cells by the annexin A2-S100A10 complex. *Int J Mol Sci*. (2018) 19:1752. doi: 10.3390/ijms19061752
19. Capra V, Back M, Angiolillo DJ, Cattaneo M, Sakariassen KS. Impact of vascular thromboxane prostanoid receptor activation on hemostasis, thrombosis, oxidative stress, and inflammation. *J Thromb Haemost*. (2014) 12:126–37. doi: 10.1111/jth.12472
20. Larsen E, Celi A, Gilbert GE, Furie BC, Erban JK, Bonfanti R, et al. PADGEM protein: a receptor that mediates the interaction of activated platelets with neutrophils and monocytes. *Cell*. (1989) 59:305–12. doi: 10.1016/0092-8674(89)90292-4
21. Wu F, Liu L, Zhou H. Endothelial cell activation in central nervous system inflammation. *J Leukoc Biol*. (2017) 101:1119–32. doi: 10.1189/jlb.3RU0816-352RR
22. Walz DA, Wu VY, de Lamo R, Dene H, McCoy LE. Primary structure of human platelet factor 4. *Thromb Res*. (1977) 11:893–8. doi: 10.1016/0049-3848(77)90117-7
23. Deuel TF, Senior RM, Chang D, Griffin GL, Heinrikson RL, Kaiser ET. Platelet factor 4 is chemotactic for neutrophils and monocytes. *Proc Natl Acad Sci USA*. (1981) 78:4584–7. doi: 10.1073/pnas.78.7.4584
24. Osterman DG, Griffin GL, Senior RM, Kaiser ET, Deuel TF. The carboxyl-terminal tridecapeptide of platelet factor 4 is a potent chemotactic agent for monocytes. *Biochem Biophys Res Commun*. (1982) 107:130–5. doi: 10.1016/0006-291X(82)91679-5
25. CX CL4, Kausar F, Day A, Osborne M, Hussain K, Mueller A, et al. CXCL4/Platelet factor 4 is an agonist of CCR1 and drives human monocyte migration. *Sci Rep*. (2018) 8:9466. doi: 10.1038/s41598-018-27710-9
26. Schall TJ, Bacon K, Toy KJ, Goeddel DV. Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. *Nature*. (1990) 347:669–71. doi: 10.1038/347669a0
27. Zernecke A, Shagdarsuren E, Weber C. Chemokines in atherosclerosis: an update. *Arterioscler Thromb Vasc Biol*. (2008) 28:1897–908. doi: 10.1161/ATVBAHA.107.161174
28. Baltus T, von Hundelshausen P, Mause SF, Buhre W, Rossaint R, Weber C. Differential and additive effects of platelet-derived chemokines on monocyte arrest on inflamed endothelium under flow conditions. *J Leukoc Biol*. (2005) 78:435–41. doi: 10.1189/jlb.0305141
29. von Hundelshausen P, Koenen RR, Sack M, Mause SF, Adriaens W, Proudfoot AE, et al. Heterophilic interactions of platelet factor 4 and RANTES promote monocyte arrest on endothelium. *Blood*. (2005) 105:924–30. doi: 10.1182/blood-2004-06-2475
30. Sachais BS, Turrentine T, Dawicki McKenna JM, Rux AH, Rader D, Kowalska MA. Elimination of platelet factor 4 (PF4) from platelets reduces atherosclerosis in C57Bl/6 and apoE^{-/-} mice. *Thromb Haemost*. (2007) 98:1108–13. doi: 10.1160/TH07-04-0271
31. Pitsilos S, Hunt J, Mohler ER, Prabhakar AM, Poncz M, Dawicki J, et al. Platelet factor 4 localization in carotid atherosclerotic plaques: correlation with clinical parameters. *Thromb Haemost*. (2003) 90:1112–20. doi: 10.1160/TH03-02-0069
32. Blanchet X, Cesarek K, Brandt J, Herwald H, Teupser D, Kuchenhoff H, et al. Inflammatory role and prognostic value of platelet chemokines in acute coronary syndrome. *Thromb Haemost*. (2014) 112:1277–87. doi: 10.1160/th14-02-0139
33. Baines AC, Brodsky RA. Complementopathies. *Blood Rev*. (2017) 31:213–23. doi: 10.1016/j.blre.2017.02.003
34. Reddy YN, Siedlecki AM, Francis JM. Breaking down the complement system: a review and update on novel therapies. *Curr Opin Nephrol Hypertens*. (2017) 26:123–8. doi: 10.1097/MNH.0000000000000305
35. Merle NS, Church SE, Fremiaux-Bacchi V, Roumenina LT. Complement system part I - molecular mechanisms of activation and regulation. *Front Immunol*. (2015) 6:262. doi: 10.3389/fimmu.2015.00262
36. Conway EM. Complement-coagulation connections. *Blood Coagul Fibrinolysis*. (2018) 29:243–51. doi: 10.1097/MBC.0000000000000720
37. Wijeyewickrema LC, Yongqing T, Tran TP, Thompson PE, Viljoen JE, Coetzer TH, et al. Molecular determinants of the substrate specificity of the complement-initiating protease, C1r. *J Biol Chem*. (2013) 288:15571–80. doi: 10.1074/jbc.M113.451757
38. Degen SE, Jensen L, Hansen AG, Duman D, Tekin M, Jensenius JC, et al. Mannan-binding lectin-associated serine protease (MASP)-1 is crucial for lectin pathway activation in human serum, whereas neither MASP-1 nor MASP-3 is required for alternative pathway function. *J Immunol*. (2012) 189:3957–69. doi: 10.4049/jimmunol.1201736
39. Dobo J, Schroeder V, Jenny L, Cervenak L, Zavodszky P, Gal P. Multiple roles of complement MASP-1 at the interface of innate immune response and coagulation. *Mol Immunol*. (2014) 61:69–78. doi: 10.1016/j.molimm.2014.05.013
40. Lachmann PJ, Hughes-Jones NC. Initiation of complement activation. *Spring Sem Immunopathol*. (1984) 7:143–62. doi: 10.1007/BF01893018
41. Nilsson B, Ekdahl KN. The tick-over theory revisited: is C3a contact-activated protein? *Immunobiology*. (2012) 217:1106–10. doi: 10.1016/j.imbio.2012.07.008
42. Saggi G, Cortes C, Emch HN, Ramirez G, Worth RG, Ferreira VP. Identification of a novel mode of complement activation on stimulated platelets mediated by properdin and C3(H2O). *J Immunol*. (2013) 190:6457–67. doi: 10.4049/jimmunol.1300610
43. Langer F, Spath B, Fischer C, Stolz M, Ayuk FA, Kroger N, et al. Rapid activation of monocyte tissue factor by antithymocyte globulin is dependent on complement and protein disulfide isomerase. *Blood*. (2013) 121:2324–35. doi: 10.1182/blood-2012-10-460493
44. Pike RN, Wijeyewickrema LC. The molecular switches controlling the interaction between complement proteases of the classical and lectin pathways and their substrates. *Curr Opin Struct Biol*. (2013) 23:820–7. doi: 10.1016/j.sbi.2013.07.016
45. Wijeyewickrema LC, Lameignere E, Hor L, Duncan RC, Shiba T, Travers RJ, et al. Polyphosphate is a novel cofactor for regulation

- of complement by a serpin, C1 inhibitor. *Blood*. (2016) 128:1766–76. doi: 10.1182/blood-2016-02-699561
46. Licht C, Pluthero FG, Li L, Christensen H, Habbig S, Hoppe B, et al. Platelet-associated complement factor H in healthy persons and patients with atypical HUS. *Blood*. (2009) 114:4538–45. doi: 10.1182/blood-2009-03-205096
 47. Friese MA, Hellwege J, Jokiranta TS, Meri S, Peter HH, Eibel H, et al. FHL-1/reconectin and factor H: two human complement regulators which are encoded by the same gene are differently expressed and regulated. *Mol. Immunol.* (1999) 36:809–18. doi: 10.1016/S0161-5890(99)00101-7
 48. Alexander JJ, Quigg RJ. The simple design of complement factor H: looks can be deceiving. *Mol Immunol.* (2007) 44:123–32. doi: 10.1016/j.molimm.2006.07.287
 49. Ekdahl KN, Nilsson B. Phosphorylation of complement component C3 and C3 fragments by a human platelet protein kinase. Inhibition of factor I-mediated cleavage of C3b. *J Immunol.* (1995) 154:6502–10.
 50. Nilsson-Ekdahl K, Nilsson B. Phosphorylation of C3 by a casein kinase released from activated human platelets increases opsonization of immune complexes and binding to complement receptor type 1. *Euro J Immunol.* (2001) 31:1047–54. doi: 10.1002/1521-4141(200104)31:4<1047::AID-IMMU1047>3.3.CO;2-P
 51. Feng S, Liang X, Cruz MA, Vu H, Zhou Z, Pemmaraju N, et al. The interaction between factor H and von willebrand factor. *PLoS ONE*. (2013) 8:e73715. doi: 10.1371/journal.pone.0073715
 52. Rayes J, Roumenina LT, Dimitrov JD, Repesse Y, Ing M, Christophe O, et al. The interaction between factor H and VWF increases factor H cofactor activity and regulates VWF prothrombotic status. *Blood*. (2014) 123:121–5. doi: 10.1182/blood-2013-04-495853
 53. Nolasco L, Nolasco J, Feng S, Afshar-Kharghan V, Moake J. Human complement factor H is a reductase for large soluble von willebrand factor multimers—brief report. *Arterioscler Thromb Vasc Biol.* (2013) 33:2524–8. doi: 10.1161/ATVBAHA.113.302280
 54. Turner N, Nolasco L, Nolasco J, Sartain S, Moake J. Thrombotic microangiopathies and the linkage between von Willebrand factor and the alternative complement pathway. *Sem Thromb Hemost.* (2014) 40:544–50. doi: 10.1055/s-0034-1383547
 55. Ninomiya H, Sims PJ. The human complement regulatory protein CD59 binds to the alpha-chain of C8 and to the “b” domain of C9. *J Biol Chem.* (1992) 267:13675–80.
 56. Falgarone G, Chiochia G. Chapter 8: clusterin: a multifacet protein at the crossroad of inflammation and autoimmunity. *Adv Cancer Res.* (2009) 104:139–70. doi: 10.1016/S0065-230X(09)04008-1
 57. Podack ER, Kolb WP, Muller-Eberhard HJ. The SC5b-7 complex: formation, isolation, properties, and subunit composition. *J Immunol.* (1977) 119:2024–9.
 58. Wat J, Foley JH, Krisinger MJ, Ocariza LM, Lei V, Wasney G, et al. Polyphosphate suppresses complement via the terminal pathway. *Blood*. (2014) 123:768–76. doi: 10.1182/blood-2013-07-515726
 59. Patzelt J, Mueller KA, Breuning S, Karathanos A, Schleicher R, Seizer P, et al. Expression of anaphylatoxin receptors on platelets in patients with coronary heart disease. *Atherosclerosis*. (2015) 238:289–95. doi: 10.1016/j.atherosclerosis.2014.12.002
 60. Eriksson O, Mohlin C, Nilsson B, Ekdahl KN. The human platelet as an innate immune cell: Interactions between activated platelets and the complement system. *Front Immunol.* (2019) 10:1590. doi: 10.3389/fimmu.2019.01590
 61. Speth C, Rambach G, Wurzner R, Lass-Flörl C, Kozarcanin H, Hamad OA, et al. Complement and platelets: mutual interference in the immune network. *Mol Immunol.* (2015) 67:108–18. doi: 10.1016/j.molimm.2015.03.244
 62. Howes JM, Richardson VR, Smith KA, Schroeder V, Somani R, Shore A, et al. Complement C3 is a novel plasma clot component with anti-fibrinolytic properties. *Diab Vasc Dis Res.* (2012) 9:216–25. doi: 10.1177/1479164111432788
 63. Niculescu F, Rus H. The role of complement activation in atherosclerosis. *Immunol Res.* (2004) 30:73–80. doi: 10.1385/IR:30:1:073
 64. Peerschke EI, Malhotra R, Ghebrehiwet B, Reid KB, Willis AC, Sim RB. Isolation of a human endothelial cell C1q receptor (C1qR). *J Leukoc Biol.* (1993) 53:179–84. doi: 10.1002/jlb.53.2.179
 65. Monsinjon T, Gasque P, Chan P, Ischenko A, Brady JJ, Fontaine MC. Regulation by complement C3a and C5a anaphylatoxins of cytokine production in human umbilical vein endothelial cells. *FASEB J.* (2003) 17:1003–14. doi: 10.1096/fj.02-0737com
 66. Laudes JJ, Chu JC, Huber-Lang M, Guo RF, Riedemann NC, Sarma JV. Expression and function of C5a receptor in mouse microvascular endothelial cells. *J Immunol.* (2002) 169:5962–70. doi: 10.4049/jimmunol.169.10.5962
 67. Foreman KE, Vaporciyan AA, Bonish BK, Jones ML, Johnson KJ, Glosky MM, et al. C5a-induced expression of P-selectin in endothelial cells. *J Clin Invest.* (1994) 94:1147–55. doi: 10.1172/JCI117430
 68. Lozada C, Levin RI, Huie M, Hirschhorn R, Naime D, Whitlow M, et al. Identification of C1q as the heat-labile serum cofactor required for immune complexes to stimulate endothelial expression of the adhesion molecules E-selectin and intercellular and vascular cell adhesion molecules 1. *Proc Natl Acad Sci USA.* (1995) 92:8378–82. doi: 10.1073/pnas.92.18.8378
 69. Ritis K, Doumas M, Mastellos D, Micheli A, Gaglioli S, Magotti P, et al. A novel C5a receptor-tissue factor cross-talk in neutrophils links innate immunity to coagulation pathways. *J Immunol.* (2006) 177:4794–802. doi: 10.4049/jimmunol.177.7.4794
 70. Schraufstatter IU, Trieu K, Sikora L, Sriramarao P, DiScipio R. Complement c3a and c5a induce different signal transduction cascades in endothelial cells. *J Immunol.* (2002) 169:2102–10. doi: 10.4049/jimmunol.169.4.2102
 71. Tedesco F, Pausa M, Nardon E, Introna M, Mantovani A, Dobrina A. The cytolytically inactive terminal complement complex activates endothelial cells to express adhesion molecules and tissue factor procoagulant activity. *J Exp Med.* (1997) 185:1619–27. doi: 10.1084/jem.185.9.1619
 72. Seifert PS, Hugo F, Hansson GK, Bhakdi S. Prelesional complement activation in experimental atherosclerosis. Terminal C5b-9 complement deposition coincides with cholesterol accumulation in the aortic intima of hypercholesterolemic rabbits. *Lab Invest.* (1989) 60:747–54.
 73. Saadi S, Platt JL. Role of complement in xenotransplantation. *Clin Exp Pharmacol Physiol.* (1999) 26:1016–9. doi: 10.1046/j.1440-1681.1999.03184.x
 74. Wiedmer T, Esmon CT, Sims PJ. Complement proteins C5b-9 stimulate procoagulant activity through platelet prothrombinase. *Blood*. (1986) 68:875–80.
 75. Peerschke EI, Murphy TK, Ghebrehiwet B. Activation-dependent surface expression of gC1qR/p33 on human blood platelets. *Thromb Haemost.* (2003) 89:331–9. doi: 10.1055/s-0037-1613450
 76. Sauter RJ, Sauter M, Reis ES, Emschermann FN, Nording H, Ebenhoch S, et al. Functional relevance of the anaphylatoxin receptor C3aR for platelet function and arterial thrombus formation marks an intersection point between innate immunity and thrombosis. *Circulation.* (2018) 138:1720–35. doi: 10.1161/CIRCULATIONAHA.118.034600
 77. Vik DP, Fearon DT. Cellular distribution of complement receptor type 4 (CR4): expression on human platelets. *J Immunol.* (1987) 138:254–8.
 78. Peerschke EI, Yin W, Ghebrehiwet B. Complement activation on platelets: implications for vascular inflammation and thrombosis. *Mol Immunol.* (2010) 47:2170–5. doi: 10.1016/j.molimm.2010.05.009
 79. Martel C, Cointe S, Maurice P, Matar S, Ghitescu M, Theroux P, et al. Requirements for membrane attack complex formation and anaphylatoxins binding to collagen-activated platelets. *PLoS ONE*. (2011) 6:e18812. doi: 10.1371/journal.pone.0018812
 80. Del Conde I, Cruz MA, Zhang H, Lopez JA, Afshar-Kharghan V. Platelet activation leads to activation and propagation of the complement system. *J Exp Med.* (2005) 201:871–9. doi: 10.1084/jem.20041497
 81. Ye Z, Zhong L, Zhu S, Wang Y, Zheng J, Wang S, et al. The P-selectin and PSGL-1 axis accelerates atherosclerosis via activation of dendritic cells by the TLR4 signaling pathway. *Cell Death Dis.* (2019) 10:507. doi: 10.1038/s41419-019-1736-5
 82. Okayama M, Oguri K, Fujiwara Y, Nakanishi H, Yonekura H, Kondo T, et al. Purification and characterization of human platelet proteoglycan. *Biochem J.* (1986) 233:73–81. doi: 10.1042/bj2330073
 83. Hamad OA, Ekdahl KN, Nilsson PH, Andersson J, Magotti P, Lambris JD, et al. Complement activation triggered by chondroitin sulfate released by thrombin receptor-activated platelets. *J Thromb Haemost.* (2008) 6:1413–21. doi: 10.1111/j.1538-7836.2008.03034.x
 84. Nayak A, Ferluga J, Tsolaki AG, Kishore U. The non-classical functions of the classical complement pathway recognition subcomponent

- C1q. *Immunol Lett.* (2010) 131:139–50. doi: 10.1016/j.imlet.2010.03.012
85. Skoglund C, Wettero J, Tengvall P, Bengtsson T. C1q induces a rapid up-regulation of P-selectin and modulates collagen- and collagen-related peptide-triggered activation in human platelets. *Immunobiology.* (2010) 215:987–95. doi: 10.1016/j.imbio.2009.11.004
 86. Hamad OA, Nilsson PH, Lasasoa M, Ricklin D, Lambris JD, Nilsson B, et al. Contribution of chondroitin sulfate A to the binding of complement proteins to activated platelets. *PLoS ONE.* (2010) 5:e12889. doi: 10.1371/journal.pone.0012889
 87. Morita N, Yamai I, Takahashi K, Kusumoto Y, Shibata T, Kobayashi T, et al. C4b binding protein negatively regulates TLR1/2 response. *Innate Immun.* (2017) 23:11–9. doi: 10.1177/1753425916672312
 88. Hertle E, van Greevenbroek MM, Arts IC, van der Kallen CJ, Geijselaers SL, Feskens EJ, et al. Distinct associations of complement C3a and its precursor C3 with atherosclerosis and cardiovascular disease. The CODAM study. *Thromb Haemost.* (2014) 111:1102–11. doi: 10.1160/TH13-10-0831
 89. Selle J, Asare Y, Kohnke J, Alampour-Rajabi S, Shagdarsuren G, Klos A, et al. Atheroprotective role of C5ar2 deficiency in apolipoprotein E-deficient mice. *Thromb Haemost.* (2015) 114:848–58. doi: 10.1160/TH14-12-1075
 90. Manthey HD, Thomas AC, Shiels IA, Zerneck A, Woodruff TM, Rolfe B, et al. Complement C5a inhibition reduces atherosclerosis in ApoE^{-/-} mice. *FASEB J.* (2011) 25:2447–55. doi: 10.1096/fj.10-174284
 91. Wang H, Ricklin D, Lambris JD. Complement-activation fragment C4a mediates effector functions by binding as untethered agonist to protease-activated receptors 1 and 4. *Proc Natl Acad Sci USA.* (2017) 114:10948–53. doi: 10.1073/pnas.1707364114
 92. Buono C, Come CE, Witztum JL, Maguire GF, Connelly PW, Carroll M, et al. Influence of C3 deficiency on atherosclerosis. *Circulation.* (2002) 105:3025–31. doi: 10.1161/01.CIR.0000019584.04929.83
 93. Gushiken FC, Hyojeong H, Pradhan S, Langlois KW, Alrehani N, Cruz MA, et al. The catalytic subunit of protein phosphatase 1 gamma regulates thrombin-induced murine platelet alpha(IIB)beta(3) function. *PLoS ONE.* (2009) 4:e8304. doi: 10.1371/journal.pone.0008304
 94. Subramaniam S, Jurk K, Hobohm L, Jackel S, Saffarzadeh M, Schwierczek K, et al. Distinct contributions of complement factors to platelet activation and fibrin formation in venous thrombus development. *Blood.* (2017) 129:2291–302. doi: 10.1182/blood-2016-11-749879
 95. Mizuno T, Yoshioka K, Mizuno M, Shimizu M, Nagano F, Okuda T, et al. Complement component 5 promotes lethal thrombosis. *Sci Rep.* (2017) 7:42714. doi: 10.1038/srep42714
 96. Patel S, Thelander EM, Hernandez M, Montenegro J, Hassing H, Burton C, et al. ApoE^{-/-} mice develop atherosclerosis in the absence of complement component C5. *Biochem Biophys Res Commun.* (2001) 286:164–70. doi: 10.1006/bbrc.2001.5276
 97. Kozarcanin H, Lood C, Munthe-Fog L, Sandholm K, Hamad OA, Bengtsson AA, et al. The lectin complement pathway serine proteases (MASPs) represent a possible crossroad between the coagulation and complement systems in thromboinflammation. *J Thromb Haemost.* (2016) 14:531–45. doi: 10.1111/jth.13208
 98. Fumagalli S, Perego C, Zangari R, De Blasio D, Oggioni M, De Nigris F, et al. Lectin pathway of complement activation is associated with vulnerability of atherosclerotic plaques. *Front Immunol.* (2017) 8:288. doi: 10.3389/fimmu.2017.00288
 99. Eriksson O, Chiu J, Hogg PJ, Atkinson JP, Liszewski MK, Flaumenhaft R, et al. Thiol isomerase ERp57 targets and modulates the lectin pathway of complement activation. *J Biol Chem.* (2019) 294:4878–88. doi: 10.1074/jbc.RA118.006792
 100. Rinder CS, Rinder HM, Smith MJ, Tracey JB, Fitch J, Li L, et al. Selective blockade of membrane attack complex formation during simulated extracorporeal circulation inhibits platelet but not leukocyte activation. *J Thorac Cardiovasc Surg.* (1999) 118:460–6. doi: 10.1016/S0022-5223(99)70183-2
 101. Hamilton KK, Hattori R, Esmon CT, Sims PJ. Complement proteins C5b-9 induce vesiculation of the endothelial plasma membrane and expose catalytic surface for assembly of the prothrombinase enzyme complex. *J Biol Chem.* (1990) 265:3809–14.
 102. Sims PJ, Faioni EM, Wiedmer T, Shattil SJ. Complement proteins C5b-9 cause release of membrane vesicles from the platelet surface that are enriched in the membrane receptor for coagulation factor Va and express prothrombinase activity. *J Biol Chem.* (1988) 263:18205–12.
 103. Hattori R, Hamilton KK, McEver RP, Sims PJ. Complement proteins C5b-9 induce secretion of high molecular weight multimers of endothelial von willebrand factor and translocation of granule membrane protein GMP-140 to the cell surface. *J Biol Chem.* (1989) 264:9053–60.
 104. Ando B, Wiedmer T, Hamilton KK, Sims PJ. Complement proteins C5b-9 initiate secretion of platelet storage granules without increased binding of fibrinogen or von willebrand factor to newly expressed cell surface GPIIb-IIIa. *J Biol Chem.* (1988) 263:11907–14.
 105. Farkas I, Baranyi L, Ishikawa Y, Okada N, Bohata C, Budai D, et al. CD59 blocks not only the insertion of C9 into MAC but inhibits ion channel formation by homologous C5b-8 as well as C5b-9. *J Physiol.* (2002) 539:537–45. doi: 10.1113/jphysiol.2001.013381
 106. Smith SA, Morrissey JH. Polyphosphate: a new player in the field of hemostasis. *Curr Opin Hematol.* (2014) 21:388–94. doi: 10.1097/MOH.0000000000000069
 107. Wu G, Hu W, Shahsafaei A, Song W, Dobarro M, Sukhova GK, et al. Complement regulator CD59 protects against atherosclerosis by restricting the formation of complement membrane attack complex. *Circulat Res.* (2009) 104:550–8. doi: 10.1161/CIRCRESAHA.108.191361
 108. Liu F, Sahoo R, Ge X, Wu L, Ghosh P, Qin X, et al. Deficiency of the complement regulatory protein CD59 accelerates the development of diabetes-induced atherosclerosis in mice. *J Diabetes Complicat.* (2017) 31:311–7. doi: 10.1016/j.jdiacomp.2016.08.021
 109. Shagdarsuren E, Bidzhekov K, Djalali-Talab Y, Liehn EA, Hristov M, Matthijsen RA, et al. C1-esterase inhibitor protects against neointima formation after arterial injury in atherosclerosis-prone mice. *Circulation.* (2008) 117:70–8. doi: 10.1161/CIRCULATIONAHA.107.715649
 110. Yin W, Ghebrehwet B, Peerschke EI. Expression of complement components and inhibitors on platelet microparticles. *Platelets.* (2008) 19:225–33. doi: 10.1080/09537100701777311
 111. Chen F, Liao Z, Peng D, Han L. Role of platelet microparticles in blood diseases: future clinical perspectives. *Ann Clin Lab Sci.* (2019) 49:161–170.
 112. Horstman LL, McCauley RF, Jy W, Ahn YS. Tissue factor-negative cell-derived microparticles play a distinctive role in hemostasis: a viewpoint review. *Sem Thromb Hemost.* (2019) 45:509–13. doi: 10.1055/s-0039-1688570
 113. Harris CL, Pouw RB, Kavanagh D, Sun R, Ricklin D. Developments in anti-complement therapy; from disease to clinical trial. *Mol Immunol.* (2018) 102:89–119. doi: 10.1016/j.molimm.2018.06.008
 114. Ricklin D, Lambris JD. Progress and trends in complement therapeutics. *Adv Exp Med Biol.* (2013) 735:1–22. doi: 10.1007/978-1-4614-4118-2_1
 115. Ricklin D, Lambris JD. Complement in immune and inflammatory disorders: therapeutic interventions. *J Immunol.* (2013) 190:3839–47. doi: 10.4049/jimmunol.1203200
 116. Zhou H, Hara H, Cooper KCD. The complex functioning of the complement system in xenotransplantation. *Xenotransplantation.* (2019) 26:e12517. doi: 10.1111/xen.12517
 117. Vlaicu SI, Tatomir A, Rus V, Mekala AP, Mircea PA, Niculescu F, et al. The role of complement activation in atherogenesis: the first 40 years. *Immunol Res.* (2016) 64:1–13. doi: 10.1007/s12026-015-8669-6
 118. Jager U, D'Sa S, Schorrenhofer C, Bartko J, Derhaschnig U, Sillaber C, et al. Inhibition of complement C1s improves severe hemolytic anemia in cold agglutinin disease: a first-in-human trial. *Blood.* (2019) 133:893–901. doi: 10.1182/blood-2018-06-856930
 119. Reis ES, DeAngelis RA, Chen H, Resuello RR, Ricklin D, Lambris JD. Therapeutic C3 inhibitor Cp40 abrogates complement activation induced by modern hemodialysis filters. *Immunobiology.* (2015) 220:476–82. doi: 10.1016/j.imbio.2014.10.026
 120. Hamdulay SS, Wang B, Calay D, Kiprianos AP, Cole J, Dumont O, et al. Synergistic therapeutic vascular cytoprotection against complement-mediated injury induced via a PKCalpha-, AMPK-, and CREB-dependent pathway. *J Immunol.* (2014) 192:4316–27. doi: 10.4049/jimmunol.1301702

121. Szakacs D, Kocsis A, Szasz R, Gal P, Pal G. Novel MASP-2 inhibitors developed via directed evolution of human TFPI1 are potent lectin pathway inhibitors. *J Biol Chem.* (2019) 294:8227–37. doi: 10.1074/jbc.RA119.008315
122. Prohaszka Z, Kirschfink M, Frazer-Abel A. Complement analysis in the era of targeted therapeutics. *Mol Immunol.* (2018) 102:84–8. doi: 10.1016/j.molimm.2018.06.001
123. Torzewski M, Bhakdi S. Complement and atherosclerosis—united to the point of no return? *Clin Biochem.* (2013) 46:20–5. doi: 10.1016/j.clinbiochem.2012.09.012

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Kim and Conway. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Platelets Are at the Nexus of Vascular Diseases

Héloïse Lebas, Katia Yahiaoui, Raphaël Martos and Yacine Boulaftali*

Laboratory of Vascular Translational Science, U1148 Institut National de la Santé et de la Recherche Médicale (INSERM), Sorbonne Paris Cite, Univ Paris Diderot, Paris, France

Platelets are important actors of cardiovascular diseases (CVD). Current antiplatelet drugs that inhibit platelet aggregation have been shown to be effective in CVD treatment. However, the management of bleeding complications is still an issue in vascular diseases. While platelets can act individually, they interact with vascular cells and leukocytes at sites of vascular injury and inflammation. The main goal remains to better understand platelet mechanisms in thrombo-inflammatory diseases and provide new lines of safe treatments. Beyond their role in hemostasis and thrombosis, recent studies have reported the role of several aspects of platelet functions in CVD progression. In this review, we will provide a comprehensive overview of platelet mechanisms involved in several vascular diseases.

Keywords: vascular diseases, leukocytes, inflammation, platelets activation, thrombosis

OPEN ACCESS

Edited by:

Marie Lordkipanidzé,
Université de Montréal, Canada

Reviewed by:

Owen McCarty,
Oregon Health & Science University,
United States
Cécile Oury,
University of Liège, Belgium

*Correspondence:

Yacine Boulaftali
yacine.boulaftali@inserm.fr

Specialty section:

This article was submitted to
Atherosclerosis and Vascular
Medicine,
a section of the journal
Frontiers in Cardiovascular Medicine

Received: 20 June 2019

Accepted: 23 August 2019

Published: 11 September 2019

Citation:

Lebas H, Yahiaoui K, Martos R and
Boulaftali Y (2019) Platelets Are at the
Nexus of Vascular Diseases.
Front. Cardiovasc. Med. 6:132.
doi: 10.3389/fcvm.2019.00132

Since their first description by Osler (1) and later by Bizzozzero (2), platelets have gained a lot of attention in many biological processes. Among the earliest evidence that platelets are crucial for human hemostasis, is based on platelet transfusions in thrombocytopenic patients that can restore hemostatic function. Platelets play a critical role in hemostasis by maintaining the integrity of blood vessels. They provide the first line of defense following injury, forming thrombi that patch-up damaged endothelial tissue and thereby play an indispensable role in hemostasis. However, dysregulated platelet activation can lead to thrombosis, myocardial infarction and stroke. Platelets are also involved in the development of atherosclerosis in coronary or carotid arteries, which is commonly the trigger for thrombosis. Plaque rupture is a common cause of arterial thrombosis and leads to the exposure of thrombogenic components to the flowing blood. The current dogma is that arterial thrombi are composed of aggregated platelets and venous thrombi are enriched in fibrin. However, this view has been challenged with landmark works by several groups on the role of platelets in venous thrombosis (3, 4). The molecular mechanism of thrombus formation has been extensively reviewed in detail elsewhere (5). Here we will provide a brief description of the role of platelets in clot formation and discuss their implication in vascular diseases.

PLATELET MECHANISMS IN ARTERIAL THROMBOSIS AND VASCULAR INFLAMMATION

At sites of vascular injury, the subendothelial extracellular matrix (ECM) is exposed to the blood, to which platelets promptly adhere in order to limit hemorrhage and promote tissue healing. This matrix contains several adhesive macromolecules such as collagen, von Willebrand factor (vWF), laminin, fibronectin and thrombospondin, all of which serve as ligands for different platelet surface receptors. Among these subendothelial substrates, the thrombogenic fibrillar collagens type I and III are by far the most potent mediators of platelet adhesion due to their strong platelet activating potential and affinity for vWF. Transient adhesion of platelets (tethering) depends largely on

vWF and its receptor, the GPIb-V-IX complex. Platelets express multiple surface receptors that directly or indirectly interact with collagen, among which integrin $\alpha 2\beta 1$ (GPIa/IIa) and the Ig-like receptor glycoprotein VI (GPVI) are the most important ones. Integrin $\alpha 2\beta 1$ predominantly mediates adhesion, whereas GPVI is the collagen-activated receptor in platelets. GPVI is a transmembrane protein of 62 kDa and belongs to the family of immunoreceptor tyrosine-based activation motif (ITAM) receptors. GPVI binds to the Fc receptor γ chain (FcR γ) which triggers the signaling cascade. Activation of platelets by GPVI–collagen interactions leads to the activation of phospholipase C and the subsequent mobilization of the second messengers calcium (Ca^{2+}) and diacylglycerol (DAG). DAG is critical for protein kinase C (PKC) activation, a key event in platelet granule release and integrin activation (6). Ca^{2+} regulates various adhesive platelet responses such as integrin activation and the release of ADP and thromboxane A2 (TxA2) that can activate the G protein-coupled receptors (GPCRs), ADP purinergic receptors P2Y1 (7) and P2Y12 (8) and the thromboxane receptors (TP) TP α and TP β . These second wave mediators allow the recruitment of circulating platelets which reinforce thrombus formation on collagen surfaces (9). In the clinic, pharmacological inhibition of TxA2 generation and/or the P2Y12 receptor are effective strategies to reduce thrombus formation at sites of vascular injury (10). Importantly, GPVI plays a central role in collagen-induced exposure of procoagulant phospholipids at the platelet surface, allowing efficient thrombin generation (11) and platelet activation. Of note, human platelets express the thrombin receptors, PAR1 and PAR4, whereas mouse platelets express a PAR3/PAR4 complex (12).

The specificity of GPVI is not restricted to collagen. Laminin as well as fibronectin, present in the basement membrane, have been shown to support platelet adhesion and spreading through $\alpha 6\beta 1$ and GPVI (13, 14). The exclusive expression of GPVI on platelets makes it an attractive target. A recent placebo-controlled phase 1 study evaluated the safety and tolerability of a humanized Fab anti GPVI (Act017) in healthy donors. This study reported no bleeding events or increased of the bleeding time suggesting a promising effect of targeting GPVI in thrombotic diseases (15). The hemITAM receptor, C-type lectin 2 (CLEC2), may also contribute to platelet activation and

thrombus formation in the deeper layers of the ECM. The known ligand for CLEC-2 is podoplanin which is expressed by type-1 alveolar cells, fibrotic reticular cells, lymphatic endothelial cells (EC) but not by vascular ECs. While the role of CLEC-2 in arterial thrombosis is still not clear (16–18), a recent *in vitro* study shows that podoplanin-expressing perivascular mesenchymal stromal cells are able to protrude through ECs and activate platelets in a CLEC-2 dependent manner (19). However, further *in vivo* studies are needed to support this observation in the context of inflammation or vascular injury.

Platelets are also known to play an important role in inflammation by recruiting leukocytes. This crosstalk has been well-studied and contributes to the increased leukocyte infiltration in tissue. The molecular mechanism of this crosstalk has been experimentally documented in different organs and in various inflammatory situations. Experimental studies show that thrombocytopenic animals have a significant reduction in leukocyte numbers in inflamed organs (20–22). Similarly, mice deficient for the main platelet adhesion receptors (P-selectin, GPIb α , GPVI, $\beta 3$ integrin) show reduced tissue inflammation (23–25). Importantly, the direct interaction between platelets and leukocytes not only occurs locally but also in circulation. Increased levels of neutrophil/platelet and/or monocyte/platelet aggregates have been reported in patients with various inflammatory diseases (26–28). Platelet activation and secretion have been shown to recruit leukocytes, upregulate adhesion molecules by EC and destabilize EC junctions. Platelets can release a variety of chemokines (e.g., platelet factor 4, IL1 β , PAF, RANTES) that can up regulate endothelial adhesion molecules (ICAM, $\alpha v\beta 3$) (29), as well as the release of Weibel palade content and open endothelial junctions (30). Among the pro-permeable platelet factors, the GPVI-dependent serotonin release has been shown to contribute to the inflammation in the joints of arthritic mice (31). Furthermore, the release of soluble factors by platelets is also central to stimulate leukocytes. For example, platelet-released adenine nucleotides or platelet factor 4 and PDGF can favor superoxide anion generation by neutrophils (32). Conversely, activated neutrophils through leukocyte-released substances, such as platelet-activating factor (PAF), elastase, and cathepsin G, may induce platelet aggregation and secretion (33). The overall effect of the platelet-leukocytes interaction on tissue integrity can be either beneficial or deleterious depending of the inflammatory situation. This dichotomous aspect of the platelet-leukocyte interaction is further documented by the formation of neutrophil extracellular traps (NETs) that entrap bacteria (34) but also cause significant damage to the surrounding tissue (35). Based on the intertwined interaction between platelet and leukocyte in pathological situations, platelet/leukocyte aggregates (PLA) are rather important regulators of disease than just a read-out for platelet activation. The use of platelet inhibitors in patients with cardiovascular disease (e.g., clopidogrel, aspirin, eptifibatide) has been shown to reduce leukocyte recruitment, cytokine release and subsequently improve disease outcome. For instance, clopidogrel pretreatment in addition of ASA therapy was shown to reduce the C-Reactive protein (CRP) level, an inflammatory marker, in patients with percutaneous

Abbreviations: AAA, abdominal aortic aneurysm; ACS, acute coronary syndrome; BAPN, beta 3-aminopropionitrile fumaralt salt; CalDAG-GEFI, calcium and diacylglycerol-regulated guanine nucleotide exchange factor I; CLEC2, C-type lectin 2; COX-1, cyclooxygenase-1; CVD, cardiovascular diseases; DAG, diacylglycerol; DAPT, dual antiplatelet treatment; EC, endothelial cells; ECM, extracellular matrix; FcR γ , Fc receptor γ chain; GPCRs, G protein-coupled receptors; GPIb α , glycoprotein Ib α ; GPVI, glycoprotein VI; ILT, intra-luminal thrombus; IR, ischemia-reperfusion; ITAM, immunoreceptor tyrosine-based activation motif receptors; MI, myocardial infarction; MMP, matrix-degrading proteinases; NETs, neutrophil extracellular traps; oxLDL, oxidized low density lipoproteins; PAF, platelet-activating factor; PAR, protease-activated receptor; PDGF, platelet derived growth factor; Pg, *Porphyromonas gingivalis*; PKC, protein kinase C; PLA, platelet-leukocyte aggregates; PLD1, phospholipase D1; rt-PA, recombinant tissue plasminogen activator; ROS, reactive oxygen species; STEMI, ST-segment elevation myocardial infarction; tMCAO, transient middle cerebral artery occlusion model; TP, TxA2 receptor; TxA2, thromboxane A2; vWF, von Willebrand factor.

coronary intervention (36). Apart from antiplatelet therapy, anti-inflammatory agent such as colchicine has been used in various settings of cardiovascular disease (e.g., myocardial infarction) by inhibiting interleukin-1 production by neutrophils (37, 38). Considering the anti-inflammatory properties of antiplatelet therapy, one could speculate that antiplatelet therapy could be considered as either a complementary or a second line of treatment to inflammatory medications (e.g., colchicine) in vascular disease.

ROLE OF PLATELETS IN EARLY AND LATE STAGES OF ATHEROSCLEROSIS

Atherosclerosis is a chronic inflammatory vascular disease involving ECs, vascular smooth muscle cells and mononuclear cells. Atherosclerosis classification as an inflammatory disease is based on the finding that immune competent cells and pro-inflammatory cytokines are abundant in atherosclerotic lesions. It is characterized by the formation of an atheromatous plaque mainly composed of pro-inflammatory oxidized low density lipoproteins (oxLDL) and foam cells accumulation in the intima of medium or large arteries, in high-shear stress areas. It results in vessel occlusion inducing CVD onset (39). The role of platelets in early atherosclerosis have been proposed by pioneer studies. Russel Ross postulated in his “*response to injury theory*” that “*lesions of atherosclerosis result from injury to the artery wall*” and result in “*subtle arterial endothelial cells desquamation*” (40). Of the many possible injuries, mechanical stresses may occur at particular anatomic sites and lead to the detachment of ECs from the artery wall and subsequently platelet adherence (41). Recent studies also reported endothelial breaches in the intima of human coronary arteries as well as in ApoE mice at sites of flow perturbation leading to the infiltration of red blood cells and leukocytes (42). Almost 30 years after Russel Ross hypothesis, the Massberg group in a landmark paper showed that in high-fat diet fed ApoE mice, platelet adhesion to the endothelium precedes the development of atherosclerotic lesions and leukocyte recruitment in atherosclerotic plaque supporting a major role of platelets in atherogenesis (43). Mechanistic studies showed that prolonged blockade of platelet adhesion in atherosclerosis animal model reduces leukocyte recruitment in arterial wall and results in fewer lesion formation (43). To decipher the involvement of platelets in this disease, several genetically modified mice lacking diverse platelet receptors were used in animal models of atherosclerosis.

Platelet glycoprotein Ib α (GPIb α), the ligand-binding subunit of the GPIb-V-IX receptor complex is known to interact with several proteins like vWF, P-selectin, Mac-1 and α -thrombin (44–47). Injections of anti-GPIb α antibodies in 10 weeks old ApoE $^{-/-}$ mice reduced both platelet transient and firm adhesion to the vascular surface of the common carotid. Genetic depletion of the GPIb α subunit leads to severe thrombocytopenia and reduced atherosclerosis progression with smaller lesion area (48). This reported protective effect could be a consequence of thrombocytopenia since mice with extracytoplasmic GPIb α domain genetic deletion (IL4R/GPIb α mice) develop milder thrombocytopenia and are not protected against atherosclerosis

(48). It indicates that GPIb α binding site for vWF, P-selectin, Mac-1 and α -thrombin might be dispensable for atherosclerosis development. This is quite surprising since vWF genetic depletion is protective in an animal model of atherosclerosis (49). Similarly, a role for Mac1/GPIb α interaction has been shown in leukocyte recruitment at sites of vascular inflammation (50, 51). The subunit GPIb β has been also investigated in atherosclerosis by using GPIb $\beta^{-/-}$ /ApoE $^{-/-}$ mice fed a chow diet for 30 weeks. Despite the moderate thrombocytopenia of those mice, GPIb β was found dispensable in atheroprotection (52).

Overall, these studies suggest that redundant mechanisms in platelet recruitment occur at site of developing atherosclerosis. The integrin α IIB β 3 can also mediate platelet adhesion via vWF binding, especially in modest shear stress condition as in large arteries. GPIIb genetic depletion results in reduced platelet adhesion at sites of vascular injury, decreased inflammatory processes and fewer atherosclerotic plaque formation (53). Integrin α IIB β 3 activation can be induced by GPIb-V-IX receptor, but also by GPVI receptor (54). Several GPVI inhibition strategies led to reduced platelet adhesion and attenuated atherosclerosis in ApoE $^{-/-}$ mice (55).

Aside from platelet adhesion, platelet activation plays a significant role in atherogenesis. The presence of activated platelets was reported in the blood obtained from patients with unstable atherosclerotic disease (56). Increased platelet reactivity has been suggested as a potential mechanism contributing to the accelerated atherosclerosis seen in diabetic patients, via capillary microembolization and acute arterial thrombosis (57). Likewise, circulating activated platelets are involved in the formation of atherosclerotic lesions in ApoE $^{-/-}$ mice (58). Those activated platelets interact with the atherosclerotic endothelium, leading to the delivery of pro-inflammatory chemokines (e.g., CCL5 and CXCL4) promoting adhesion molecule expression (58). PLA formation is required for neutrophil recruitment to inflamed tissues as animal models studies revealed that platelets activate neutrophils for an efficient adhesion to vascular endothelium via integrin up-regulation (59). Platelets can also bind to the inflamed endothelium, enhancing leukocytes adhesion to the vessel wall (60–62). Indeed, deletion of P-selectin, a marker of granule secretion, in platelets and/or ECs leads to significantly impaired early atherosclerotic lesion development in mice (63, 64). In addition, several studies addressing the contribution of platelet receptors have been conducted. The role of the ADP receptor P2Y₁₂ has been extensively studied throughout the years. It has been shown that P2Y₁₂ genetic depletion is protective in ApoE $^{-/-}$ mice and mediates a reduced lesion area, an increased fibrous content at the plaque site and less inflammatory cells infiltration (65). However, the role of P2Y₁₂ expressed by vascular smooth muscle cells cannot be excluded (66). Mice deficient for P2Y₁₂ specifically in hematopoietic cells were generated, and a reduced atherosclerotic lesion formation was also reported (67). In contrast, treatment of ApoE $^{-/-}$ mice with the P2Y₁₂ inhibitor clopidogrel bisulfate was associated with inconsistent results. Clopidogrel administration induced delayed atherogenesis, a reduced lesion size, slower progression of atherosclerotic lesion (68–70). However, another study reported that clopidogrel-treated mice

have the same atherosclerotic burden as control mice (70). Moreover, clopidogrel administration in mice with established atherosclerotic lesions show no longer beneficial effect (69). A more recent P2Y₁₂ inhibitor, ticagrelor, has been tested in atherosclerosis models. Several studies conclude to a beneficial effect of ticagrelor administration, reporting a reduced lesion area and slower atherosclerotic lesion progression (71, 72). Nevertheless, one study related no effect on atherosclerotic lesion size in ticagrelor-treated mice, but showed an increased fibrous cap area along with a diminished ratio necrotic core/lesion area, indicating plaque stabilization process (73). Pharmacological inhibition is more likely to give variable results than a genetic approach. The differences in the experimental conditions and the inhibitor dose may be responsible for the discrepant results. The impact of the platelet thrombin receptor, PAR₄, has been also investigated in atherosclerosis. Indeed, transfusion of thrombin-activated platelets into mice increases plaque formation, suggesting that thrombin-induced platelet activation might contribute to platelet-dependent atherosclerosis (58). However, PAR₄ deletion is not protective in ApoE^{-/-} mice (74) suggesting other platelet activators than thrombin are involved.

Upon activation, platelets release soluble factors (e.g., PF₄, CD40L, RANTES, and TXA₂) enhancing their activation and leukocyte recruitment. Disruption of this amplification process leads to diminished atherosclerotic lesion formation. Indeed, PF₄ or CD40L genetic deletion protects ApoE^{-/-} mice from atherosclerosis (75, 76). Inhibition of RANTES or its receptors alters the progression of an established atherosclerotic lesion (77, 78). Biological response modulators such as CD40L and its receptor CD40 have been shown to exacerbate atherosclerosis progression by promoting leucocyte recruitment via molecule adhesion expression in vascular ECs (79).

Platelet TXA₂ generation is the product of cyclooxygenase-1 (COX-1) activation and contributes to the platelet activation amplification loop. The TXA₂ receptor (TP) antagonist administration induces a slight reduction of atherogenesis (80), and TP deficient ApoE^{-/-} mice showed delayed lesion development and reduced atherogenesis compared to control (81). Disruption of COX-1 expression in ApoE^{-/-} mice induces a decrease in atherosclerotic lesion formation, attesting TXA₂ deleterious role in this pathology (82). Acetylsalicylic acid, also known as aspirin, is one of the most widespread antiplatelet treatment and displays also anti-inflammatory properties. This irreversible COX-1 inhibitor blocks the formation of TXA₂ in platelet, producing an inhibitory effect on platelet aggregation. Most animal studies reported a beneficial effect of low-dose aspirin administration in ApoE^{-/-} mice. Atherogenesis and lesion progression is reduced in aspirin-treated mice compared to control (83–85). Low-dose aspirin also delayed the progression of established and advanced vascular atherosclerotic lesions (86). However, some studies reported no effect of aspirin in ApoE^{-/-} mice (70, 80), and one described a deleterious long-term effect on atherosclerotic lesion progression (87). Overall, these studies suggest a functional hierarchy and redundancy between the different receptors in the role of platelets in atheroprotection.

Downstream of the platelet receptors, the signaling molecule GTPase Rap1 is a critical node in platelet response. The calcium and diacylglycerol-regulated guanine nucleotide exchange factor I (CalDAG-GEFI; RasGRP2) has been identified as the major calcium sensor in platelets regulating the Rap1 activation (88). Studies led by the Bergmeier group uncovered key roles of CalDAG-GEFI in platelet responses: integrin activation, platelet adhesion and secretion, TXA₂ generation (89, 90). In an animal model of atherosclerosis, mice lacking CalDAG-GEFI specifically in hematopoietic cells have smaller lesions, reduced atherogenesis and decreased inflammation in areas of plaque development compared to control mice (67).

Even though the stenosis induced by atherosclerosis can restrict blood flow and thus induces CVD, the main mechanism implied in those diseases seems to be atherothrombosis. Indeed, following plaque rupture, prothrombotic materials (collagen and tissue factor) are exposed to the blood coagulation system leading to thrombus formation, decreased blood flow and CVD onset (39). Human postmortem studies showed that thrombi that form on disrupted plaques (e.g., asymptomatic coronary disease) appear small and non-occlusive (91). Animal models that can recapitulate spontaneous rupture of atherosclerotic lesions are very rare. To circumvent this issue, two experimental animal models have been developed to study platelet mechanism in thrombosis-induced plaque rupture. A model of ultrasound-induced plaque injury and a model of acute plaque rupture using a suture needle have been developed to test antiplatelet drugs in mice (92, 93). Ultrasound treatment resulted in a fissure at the shoulder region of the plaque leading to plaque material exposure (collagen) and unstable thrombus formation. Unlike the ultrasound model, the needle model is characterized by a frank rupture with larger and stable thrombi. At the site of plaque rupture, smaller thrombi were observed after P2Y₁₂ or thrombin or integrin α IIb β 3 inhibition in both models (92–94). The role of GPVI seems to be more pronounced in the ultrasound model presumably due to a higher amount of thrombin generated in the needle model (93). The role of the different platelet molecules in mouse atheroprotection has been summarized in **Table 1**.

The protective role of these drugs is difficult to assess in human clinical trials since atherosclerosis by itself is almost always asymptomatic. Thereby, their efficacy is studied in CVD with an atherosclerotic origin such as myocardial infarction (MI) and stroke.

PLATELETS CONTRIBUTE TO THROMBO-INFLAMMATION DURING STROKE

According to the World Health Organization, an estimated 7 million people died from stroke worldwide in 2016. Stroke represents the second most common cause of death and the third most common cause of disability (95). Strokes have mainly an ischemic origin (70%) and occur when an artery that supplies blood to the brain is blocked by a blood clot (96). Hemorrhagic stroke accounts for 15% of all strokes but they are responsible for about 40% of all stroke deaths (National Stroke Association).

TABLE 1 | A comprehensive analysis of platelet mechanisms in atheroprotection in mice (↓ decrease, ↑ increase, = no effect on plaque development).

Molecule targeted	Animal model	Plaque development effect	References
PGI ₂	ApoE ^{-/-} IP ^{-/-} (chow diet)	↑	(81)
GPIIb α	ApoE ^{-/-} + Fab anti-GPIIb α (HFD up to 18 weeks)	↓	(43)
	Ldlr ^{-/-} GPIIb α ^{-/-} (HFD for 16 weeks)	↓	(48)
	Ldlr ^{-/-} hIL4R/GPIIb α (HFD for 16 weeks)	=	(48)
vWF	Ldlr ^{-/-} vWF ^{-/-} (HFD up to 22 weeks)	↓	(49)
α IIb β 3	ApoE ^{-/-} GPIIb ^{-/-} (HFD for 8 at 12 weeks)	↓	(53)
GPVI	ApoE ^{-/-} + Fab anti-GPVI (HFD for 12 weeks)	↓	(55)
PAR4	ApoE ^{-/-} PAR4 ^{-/-} (HFD for 5 or 10 weeks)	=	(74)
CD40L	ApoE ^{-/-} CD154 ^{-/-} (chow diet)	↓	(75)
CD40	ApoE ^{-/-} CD40 ^{-/-} (HFD for 4 weeks)	↓	(79)
PF4	ApoE ^{-/-} PF4 ^{-/-} (HFD for 10 weeks)	↓	(76)
RANTES	Ldlr ^{-/-} + RANTES inhibitor (HFD up to 22 weeks)	↓	(77, 78)
TXA ₂	ApoE ^{-/-} + S18886 or aspirin (chow diet)	↓	(80)
	ApoE ^{-/-} COX-1 ^{-/-} (HFD for 8 weeks)	↓	(82)
	Ldlr ^{-/-} + aspirin (chow diet or HFD up to 26 weeks)	↓	(83–85)
	ApoE ^{-/-} + aspirin (chow diet or HFD up to 12 weeks)	=	(70, 80)
	ApoE ^{-/-} + aspirin long treatment (HFD up to 16 weeks)	↑	(87)
CalDAG-GEFI	CalDAG-GEFI ^{-/-} in hematopoietic cells (HFD for 12 weeks)	↓	(67)
P2Y ₁₂	ApoE ^{-/-} P2Y ₁₂ ^{-/-} (HFD up to 20 weeks)	↓	(65, 67)
	ApoE ^{-/-} + Clopidogrel (chow diet or HFD between 8 at 12 weeks)	↓	(68, 70)
	ApoE ^{-/-} + Clopidogrel (HFD for 6 months)	=	(69)
	ApoE ^{-/-} + Ticagrelor (HFD for 20 weeks)	↓	(71, 72)
	ApoE ^{-/-} + Ticagrelor (HFD for 12 weeks)	=	(73)

An ischemic stroke can occur in two ways: embolic stroke caused by thromboembolism of cardiac origin or thrombotic stroke with *in-situ* blood clot. Atherosclerosis in major intracranial arteries leads to changes ranging from minor wall thickening to luminal stenosis, and is one of the most common causes of

stroke worldwide (97). The middle cerebral arteries are the most common lesion site, followed by the basilar artery, the internal carotid arteries, and the intracranial vertebral arteries (98). Intracranial atherosclerotic disease may occur concomitantly with systemic atherosclerosis.

The current treatments for acute ischemic stroke are the use of a thrombolytic agent as recombinant tissue plasminogen activator (t-PA) (99) and mechanical thrombectomy (100). Nevertheless, in some patients, the recanalization by thrombolysis is not efficient and the persisting thrombus leads to severe brain damage. Recent clinical findings show that clots retrieved from stroke patients have a thick compact outer shell enriched in NETs and fibrin which might contribute to reperfusion resistance (101, 102). Following thrombolysis, the overall recanalization rate is 46% (103). However, reocclusion after initial recanalization occurs in 14–34% of patients and is associated with clinical deterioration and poor outcome (104–106). Reocclusion has been attributed to increased platelet aggregation caused by the local thrombus and endothelial injury. Thus, the start of antiplatelet therapy early after thrombolysis might reduce the risk of reocclusion and thereby improve functional outcome.

Animal models were used to decipher platelets involvement in stroke. The most common stroke animal model used is the transient middle cerebral artery occlusion (tMCAO) in mice and rats. Thrombocytopenic mice were submitted to transient occlusion of the middle cerebral artery, and 24 h after ischemia/reperfusion, infarct area was determined (107). Platelet depletion did not significantly affect the lesion area, but thrombocytopenic mice presented multiple hemorrhagic foci in the lesion whereas none were observed in mice with normal platelet count. Nevertheless, platelet adhesion and activation have been investigated in several stroke studies in mice. Blockade or genetic deficiency of GPIIb α improves stroke outcome without hemorrhagic transformation after tMCAO (108). Similarly, vWF deficiency is also associated with smaller infarct volumes and no bleeding was observed after tMCAO in those mice (109). Interestingly, the role of the GPIIb α -vWF axis in hemostasis can be decoupled from the one in brain injury highlighting a proinflammatory role of GPIIb α . The contribution of platelet activation receptors has been investigated during ischemia-reperfusion injury after tMCAO. Inhibition or genetic deficiency of GPVI, the collagen and fibrin receptor, has been shown to reduce the infarct volume and to improve stroke outcome (108, 110). Supporting the beneficial role of blocking the collagen receptor during a stroke, a recent study showed that GPVI inhibition plus intravenous infusion of rt-PA is safe in term of bleeding and has a better outcome than rt-PA alone (111). GPVI seems an attractive target in stroke since (i) it's only expressed on platelets, (ii) patients with a GPVI deficiency have no or mild bleeding phenotype (112), (iii) GPVI inhibition leads to a significant reduction of thrombus formation (113, 114), (iv) a novel humanized Fab anti GPVI (ACT017) in healthy donors didn't show bleeding complications (15). Overall, targeting GPVI in thrombosis can be a novel approach and compared to the current antiplatelet drugs, GPVI inhibition does not compromise hemostasis. Nevertheless, other larger studies

need to be conducted in patients with CVD to validate the use of anti-GPVI antibodies. Notably, the protective effect of GPIIb α and GPVI inhibition can also be observed in aged mice presenting comorbid factors such as atherosclerosis, diabetes or hypertension (115). Indeed, platelets from patients with comorbid factors are in a hyperactivated state (116). Enhanced platelet intracellular calcium responses to LDL cholesterol have been observed in diabetic patients with and without hypertension (117). Similarly, platelets from diabetic patients have been reported to have reduced sensitivity to prostacyclin (118) and hyperaggregate in response to platelet agonists (119). Other changes in platelets from diabetic patients include an increased expression of some platelet receptors GPIIb α and α IIb β IIIa (120) and an alteration of platelet membrane fluidity (121). Therefore, studies including comorbid factors should be performed to further assess the validity of future antiplatelet drugs in the context of CVD.

Other platelet receptors are involved in platelet activation during the ischemia-reperfusion mouse model. The thrombin PAR4 receptor is expressed not only in platelets but also in the central nervous system (122). In a transient stroke mouse model, its systemic deletion reduces the brain infarct volume and attenuates cognitive function deficit (123). Intravital microscopy studies showed fewer platelet/EC interactions in PAR4^{-/-} mice compared to control mice. Thus, PAR4 deficiency seems to be neuroprotective in transient middle cerebral artery occlusion, partially through the attenuation of cerebral microvascular inflammation. In addition, mice deficient for G α_{12} , G protein downstream of the ADP receptor P2Y₁₂, were subjected to tMCAO, then functional outcome and infarct size were assessed 24 h later (124). G α_{12} deficiency leads to a reduced lesion area and better functional outcome than control mice. Apart from platelet adhesion and activation, platelet granule secretion contributes to stroke. Mice lacking platelet α -granules (Nbeal2^{-/-}) and mice lacking platelet dense granules (Unc13d^{-/-}) showed a higher mortality rate due to intracranial hemorrhage. Nevertheless, the surviving animals developed significantly smaller brain infarctions and had a better outcome compared to WT mice (125). Platelet aggregation mediated by the integrin α IIb β 3 seems dispensable at sites of ischemia/reperfusion injury. Blockade of α IIb β 3 integrin increases intracranial hemorrhage risk in tMCAO mice (108, 126) and among the surviving mice, the treatment did not show any improvement (115) suggesting that platelet aggregation is dispensable for brain injury but still important for securing hemostasis in tMCAO.

Overall, these data suggest a deleterious role of platelet adhesion, activation and secretion in the stroke pathophysiology. On the opposite, platelet aggregation, which plays a crucial role in thrombus formation, is not required for stroke progression. Thus, the non-classical role of platelets, through their pro-inflammatory properties, may prevail in stroke disease. A comprehensive table summarizes platelet mechanisms studied in tMCAO models (Table 2A and Figure 1).

In humans, several antiplatelet drugs have been tested in stroke outcome. Among them, numerous studies have investigated the benefits and risks of aspirin for primary prevention in population at risk, during the acute management of cardiovascular events and in secondary prevention among

patients with CVD. In elderly populations, the risk of CVD is higher suggesting an increased benefit of aspirin administration in primary prevention for cardiovascular events. However, increased bleeding risk has also been observed in this population (147). According to a meta-analysis of aspirin primary prevention studies, reported by the Antithrombotic Trialists' Collaboration, aspirin did not reduce the risk of stroke. In this meta-analysis, aspirin non-significantly reduced the risk of ischemic stroke but increased non-significantly the risk of hemorrhagic stroke (148). The JPPP (Japanese Primary Prevention Project) clinical trial was designed to assess whether primary prevention would reduce the risk of non-fatal stroke in elderly Japanese patients (127). It appears that aspirin seems to reduce the non-fatal ischemic stroke risk, but it tends to increase the risk of hemorrhagic stroke. Recently, the ARRIVE (Aspirin to Reduce Risk of Initial Vascular Events) clinical trial investigated the benefits and risks of enteric-coated aspirin used in primary prevention of cardiovascular events, in patients with an average cardiovascular risk (128). Aspirin did not lower the risk of major cardiovascular events nor stroke incidence. Moreover, rates of gastrointestinal bleeding events and some other minor bleeding events were higher in the aspirin treatment group with no difference in the incidence of fatal events. The results of the ASPREE (Aspirin in Reducing Events in the Elderly) clinical trial investigating the role of aspirin in primary prevention of CVD were published recently (129). They confirm that the use of low-dose aspirin as a primary prevention strategy in older adults results in a significantly higher risk of major hemorrhage and did not trigger a significantly lower risk of CVD than placebo.

Low-dose aspirin efficacy has been widely established in secondary prevention trials, in which the benefits of reducing ischemic stroke rates have outweighed the risk of hemorrhage (149, 150). A meta-analysis including eight clinical trials involving more than 40,000 participants concluded that antiplatelet therapy with aspirin started within 48 h of the onset of ischemic stroke reduced the risk of early recurrent ischemic stroke without a major risk of early hemorrhagic complications (151). Moreover, long-term outcomes were also improved. The ARTIS (Antiplatelet therapy in combination with rt-PA Thrombolysis in Ischemic Stroke) clinical trial compared the effects of early intravenous aspirin addition to thrombolysis with standard treatment without aspirin (130). Patients with acute ischemic stroke treated with rt-PA thrombolysis were randomly assigned to intravenous aspirin within 90 min after the start of thrombolysis treatment or to no additional treatment. In addition, in both groups, oral antiplatelet therapy was started 24 h after thrombolysis treatment. This trial concluded that early administration of intravenous aspirin does not improve outcome at 3 months and increases the risk of intracranial hemorrhage without evidence of a beneficial effect on early neurological deterioration (131).

Current guidelines for the early management of patients with acute ischemic stroke, from the American Heart Association and the American Stroke Association, recommend starting aspirin administration 24 h after thrombolysis (152). However, the overall effect of aspirin in acute ischemic stroke is weak and better acute therapies are therefore necessary.

TABLE 2 | (A) A comprehensive analysis of platelet mechanisms in tMCAO mouse model and **(B)** in stroke patients (↓ decrease, ↑ increase, = no effect on infarct area or bleeding risk).

A					
Molecule targeted		Animal model	Effect on infarct area	References	
Platelets		Thombocytopenia	=	(107)	
α granules		Nbeal2 ^{-/-}	↓	(125)	
Dense granules		Unc13d ^{-/-}	↓	(125)	
PAR4		PAR4 ^{-/-}	↓	(123)	
Gαi2		Gαi2 ^{fl/fl} /PF4-Cre	↓	(124)	
GP1bα		Antibody anti-GP1bα	↓	(108, 126)	
GPVI		Antibody anti-GPVI	↓	(108)	
αIIbβ3		Antibody anti-GP1Ib/IIIa	↓	(108)	
B					
Molecule targeted		Clinical trial	Effect	Bleeding	References
TXA ₂	JPPP		↓	↑	(127)
	Aspirin				
	ARRIVE		=	↑	(128)
	Aspirin				
	ASPREE		=	↑	(129)
	Aspirin				
	ARTIS		=	↑	(130, 131)
	Early aspirin addition to thrombolysis				
	P2Y12	CAPRIE	=	↑	(132)
	Clopidogrel vs. aspirin - Ischemic stroke patients				
	CHARISMA		=	↑	(133)
	Clopidogrel + aspirin vs. aspirin alone				
	MATCH		=	↑	(134)
	Clopidogrel + aspirin vs. aspirin alone				
	CHANCE		↓	=	(135, 136)
	Clopidogrel + aspirin vs. aspirin alone				
	COMPRESS		=	↑ Tendency	(137)
	Clopidogrel + aspirin vs. aspirin alone				
	POINT		↓	↑	(138, 139)
	Clopidogrel + aspirin vs. aspirin alone				
	SOCRATES		=	=	(140, 141)
	Ticagrelor vs. aspirin				
	PRINCE		↓ Tendency	?	(142, 143)
	Ticagrelor vs. clopidogrel (+ aspirin)				
	TARDIS		=	↑	(144)
	Aspirin, clopidogrel and dipyridamole vs. guideline treatment				
	αIIbβ3	abESTT-II	=	↑	(145)
	Abciximab				
	SaTIS		=	=	(146)
	Tirofiban vs. placebo				

Over the years, several other antiplatelet agents were developed and then tested in clinical trials. A comprehensive table summarizes the different clinical trials that assessed the efficacy of antiplatelet agents in stroke outcome (**Table 2B**).

PLATELETS CONTRIBUTE TO MYOCARDIAL INJURY

Acute coronary syndrome (ACS) occurs when the blood flow is decreased or stopped in coronary arteries, leading to tissue

damage ranging from ischemia to infarction. This defect of blood supply is mainly due to atherosclerotic plaque growth and rupture, followed by a thrombus formation in coronary artery (153). ACS is commonly divided into myocardial infarction (MI) and unstable angina, considered to be an imminent precursor of MI. Biomarkers of cardiac tissue damage such as troponin and creatine-kinase myocardial band (CK-MB) are used to classify ACS into unstable angina or MI, the latter one presenting such blood biomarkers. A MI can have several consequences such as heart failure, an irregular heartbeat or a cardiac arrest.

According to the World Health Organization, in 2012 an estimated 7.5 million people died from MI worldwide. Survivors of MI are at increased risk of recurrent infarctions and have an annual death rate of 5%, representing a 6-time increase compared to people without coronary heart disease. MI can also be classified into ST-segment elevation myocardial infarction (STEMI) and non-STEMI (NSTEMI) according to the patient electrocardiogram. STEMI caused by a complete coronary vessel occlusion, and NSTEMI due to a partial artery occlusion, represent respectively 30 and 70% of all MI (154). Since unstable angina has similar pathophysiology to NSTEMI, they are referred together as non-ST-segment elevation ACS (NSTEMI-ACS) and are grouped for care management decisions. Current guidelines recommend an immediate treatment of ACS, but due to different pathophysiology between STEMI and NSTEMI-ACS, separate guidelines were edited. Guidelines for the management of NSTEMI-ACS recommend a pharmacological treatment of ischemia (via decreasing myocardial oxygen demand or increasing myocardial oxygen supply) (155). Guidelines for the treatment of STEMI recommend an immediate recanalization of coronary arteries via reperfusion therapies such as primary percutaneous coronary intervention and/or fibrinolysis strategy (156). Even though recanalization is necessary to provide oxygen and nutrients to the ischemic area, reperfusion by itself also exacerbates myocardial damage (157). This pathologic process is named ischemia-reperfusion (IR) injury. For long-term therapies, both guidelines agree to strongly recommend the use of antiplatelet agents.

Indeed, the atherosclerotic origin of MI led to numerous studies deciphering the platelet involvement in MI. A lower platelet count or no significant difference of the platelet count was observed between MI patients and stable angina or healthy donors (158, 159). However, the mean platelet volume reflecting platelet activation was higher in MI patients compared to stable coronary artery disease patients at the time of acute event (158). Increased levels of P-Selectin and CD63-exposing platelet microparticles have been found in MI patients (160). Plasma levels of vWF and serotonin are increased in patients with coronary artery syndrome (161, 162) highlighting the role of platelet activation in myocardial injury. Moreover, platelet-leucocyte aggregates are an early marker of acute MI and are also associated with myocardial no-reflow in STEMI patients (163, 164). Ventricular wall rupture is a fatal complication of acute MI and platelets seem to be involved in this phenomenon since an intramural thrombus was observed within the infarcted myocardium (165). Platelets potential involvement in this process was confirmed by their depletion which reduced the rate of myocardial wall rupture from 46 to 0% (166). Several studies investigated platelet mechanisms involved in MI and in myocardial (IR) injury.

Platelet activation is commonly observed in numerous pathologies, including MI (158). This phenomenon is mediated by specific platelet receptors that are involved in adhesion and activation. Inhibition of GPIIb/IIIa—involved in platelet tethering—via a Fab anti-GPIIb/IIIa did not change the infarct volume per the area at risk (INF/AAR) (167). In accordance with the results of GPIIb/IIIa inhibition, depletion of the phospholipase D1 (PLD1), enzyme involved in GPIIb/IIIa dependent α IIb β 3 activation,

did not protect mice from myocardial IR injury (167). These findings suggest that GPIIb/IIIa platelet receptor is dispensable in MI pathophysiology. On the contrary, mice deficient for the Fc receptor γ chain (FcR γ) coupled to GPVI were protected from myocardial IR injury with smaller infarct size and reduced leucocyte recruitment in the injured area (168). This was confirmed by pharmacological inhibition of the collagen receptor GPVI via a Fab anti-GPVI or soluble GPVI-Fc (Revacept) in a mouse model of the left anterior descending artery ligation with reperfusion. Inhibition of GPVI led to a reduced infarct size (167, 169). These findings suggest that a therapeutic strategy targeting GPVI could be a valuable approach in MI. This could be relevant in humans since it was recently described that patients with STEMI have an alteration of GPVI platelet signaling (170). Indeed, platelets from STEMI patients have an increased aggregation response compared to stable coronary artery disease patients. This could be due to the increased number of GPVI receptors observed in ACS patients (171). Therefore, studies focused on GPVI inhibition seem promising. Currently, a humanized Fab targeting GPVI without increasing the bleeding risk in healthy controls is developed and characterized (15) but their findings warrant further investigations in CVD patients under current antiplatelet drugs.

The contribution of other receptors in MI was studied such as CLEC-2, PAR receptors or P2Y₁₂ in mice. Pharmacological inhibition of CLEC-2 via Fab administration did not decrease the INF/AAR in mice (167). The thrombin receptors, PAR1 and PAR4 receptors are expressed at the surface of platelets, but also by cardiomyocytes (172, 173). It has been showed that PAR1 deficiency did not affect the infarct size after myocardial IR injury (174). However, interestingly, PAR1^{-/-} mice had reduced cardiac remodeling and decreased impairment of left ventricle function compared to control mice. In contrast to previous findings, PAR1 antagonist (SCH 79797) administration was shown to reduce infarct size after myocardial IR injury in rats (175). This discrepancy could be explained by off-target effects of SCH 79797 or a PAR4 compensation. Genetic depletion of PAR4 led to the development of larger infarcts and more myocardial apoptosis compared to control mice (176). However, another study attributes a cardioprotective effect of PAR4 deletion after myocardial IR injury as INF/AAR was decreased in PAR4^{-/-} mice compared to control mice (177). The administration of PAR4 antagonists confirmed the previous findings. Indeed, PAR4 inhibition in rats decreased infarct size after myocardial IR injury (178). The P2Y₁₂ receptor, which signaling is mediated by the G protein G α_{i2} , is involved in platelet activation. G α_{i2} ^{-/-} deficient mice have a reduced INF/AAR ratio suggesting a protective effect of platelet activation inhibition (124). However, this G protein may interact not only with P2Y₁₂, but also with additional G protein coupled receptor present in platelets. The use of clopidogrel, a P2Y₁₂ antagonist, decreased platelet accumulation in ischemic myocardium and reduced the rupture rate from 45% in control to 10% in clopidogrel treated animals (165, 166). In a rat model of isolated hearts, the perfusion of platelets from acute MI patients enlarges infarct area (179) while the concomitant administration of cangrelor or abciximab decreases the infarct size. As opposed to clopidogrel, aspirin administration did not

reduce infarct size nor the rupture rate in mice (165, 180) suggesting a relative hierarchy in the platelet receptors during MI.

Platelet degranulation, a marker of platelet activation, triggers the inflammatory responses by P-selectin exposure involved in leukocyte recruitment. Nbeal2^{-/-} and Unc13d^{-/-} mice lacking, respectively, alpha and dense granules did not show any alterations in infarct sizes, arguing against a significant role of degranulation in the pathophysiology of myocardial IR injury (167). Nevertheless, blockade or genetic deficiency of P-selectin can lead to smaller infarct sizes after myocardial infarction (181–183) suggesting a possible contribution of the endothelial P-selectin. Critical to platelet activation is calcium mobilization. Cytosolic Ca²⁺ concentration is regulated by two major proteins: STIM1 (endoplasmic reticulum Ca²⁺ sensor) and Orai-1 (Ca²⁺ channel). Genetic depletion in hematopoietic cells of STIM1 or Orai-1 did also not reduce the INF/AAR ratio (167) hinting at alternative platelet activation pathways.

The final step of platelet activation is their aggregation and is mainly driven by α IIB β 3 activation. The inhibition of this integrin via Fab anti- α IIB β 3 administration did not alter the INF/AAR ratio when compared with control mice (167). In another study, α IIB β 3 inhibition by abciximab seems to reduce myocardial injury in isolated rat hearts through a reduction of platelet adhesion to the endothelium or leukocytes (179). This discrepancy can be probably explained by the different experimental models which can have a different impact on platelet activation. Platelet mechanisms in the MI model have been summarized in **Table 3A** and **Figure 2**.

In humans, several clinical trials tested platelet inhibitors in the context of MI. Currently, guidelines for the management of NSTEMI-ACS and STEMI patients recommend aspirin intake for long-term treatment for all patients without contraindications (148, 155, 156). Moreover, the dual antiplatelet treatment (DAPT) composed of aspirin plus a P2Y₁₂ inhibitor is recommended. Indeed, the CURE (Clopidogrel in Unstable Angina to Prevent Recurrent Events) trial was designed to assess the efficacy of the combination of aspirin and clopidogrel compared to aspirin alone (184). Patients with NSTEMI-ACS were enrolled within 24 h after symptom onset, and either treated with the combination treatment or aspirin alone. The DAPT significantly reduced the composite rate of death from cardiovascular causes, non-fatal MI and stroke. The rate of each component of this composite outcome also tended to be lower in the DAPT group. However, the risk of major bleeding is increased among patients treated with clopidogrel. TRITON-TIMI-38 (Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel—Thrombolysis in Myocardial Infarction-38) is a phase III trial which enrolled patients with ACS (NSTEMI-ACS and STEMI) within 72 h after symptom onset (185). This trial was designed to compare the efficacy of prasugrel with clopidogrel, both associated with aspirin. The percentage of non-fatal MI (Clopidogrel: 9.5%; Prasugrel: 7.3%) and stent thrombosis (Clopidogrel: 2.4%; Prasugrel: 1.1%) was significantly reduced in patients treated with prasugrel, but it caused more life-threatening bleedings than clopidogrel treatment (from 0.9 to 1.4%). In conclusion, this trial showed that prasugrel is more effective at preventing ischemic events than clopidogrel in patients with NSTEMI-ACS

or STEMI. However, this beneficial effect is accompanied by an increased rate of major bleeding. The PLATO (Study of Platelet Inhibition and Patient Outcomes) clinical trial aimed to determine whether, combined to aspirin, ticagrelor is more efficient than clopidogrel in patients with ACS, enrolled within 24 h after symptom onset (186). Patients receiving ticagrelor had a significantly lower MI event rate (5.8%) compared to clopidogrel-treated patients (6.9%). The rate of death from any cause is also significantly lower for patients treated with ticagrelor (ticagrelor: 4.5%; clopidogrel: 5.9%). It is important to note that no difference in life-threatening bleeding was observed between the two treatment groups (ticagrelor: 5.8%; clopidogrel: 5.8%). This trial found that, in patients who have an NSTEMI-ACS or STEMI, treatment with ticagrelor compared to clopidogrel significantly reduced the rate of death from vascular causes, myocardial infarction, or stroke without an increase in the rate of overall major bleeding.

These results were later confirmed by the PEGASUS-TIMI-54 (Prevention of Cardiovascular Events in Patients With Prior Heart Attack Using Ticagrelor Compared to Placebo on a Background of Aspirin—Thrombolysis in Myocardial Infarction 54) trial which enrolled patient who had a MI 1 to 3 years earlier (187). Patients either received ticagrelor plus aspirin or aspirin alone. The ticagrelor treatment significantly reduced MI event rate (from 5.25 to 4.53%) (188). Moreover, this protective effect is consistent over time and this trial supports the use of prolonged therapy in patients who continue to tolerate this antiplatelet agent (189). Current guidelines recommend delivering DAPT to NSTEMI-ACS and STEMI patients, with aspirin plus ticagrelor or prasugrel (155). Clopidogrel can be administered to ACS patients who cannot receive the two previous antiplatelet agents.

Vorapaxar, a PAR₁ inhibitor, has been tested in clinical trials. The TRA 2P-TIMI 50 (Thrombin Receptor Antagonist in Secondary Prevention of Atherothrombotic Ischemic Events—Thrombolysis in Myocardial Infarction 50) clinical trial enrolled patients who had a history of atherosclerosis within the previous 2 weeks to 12 months (190). Patients were randomly assigned to either vorapaxar treatment or placebo. All concomitant medical therapy, including the use of other antiplatelet agents, was managed by the clinicians who were responsible for the care of the patients. Patients receiving vorapaxar had a reduced rate of MI event (from 6.1 to 5.2%) but presented an increase in major bleeding (from 11.1 to 15.8%). This trial assessed that inhibition of PAR-1 with vorapaxar reduced the risk of cardiovascular death or ischemic events in patients with stable atherosclerosis. However, it increased the risk of moderate or severe bleeding, including intracranial hemorrhage. The findings of TRA 2P-TIMI 50 clinical trial were confirmed by the TRACER (Thrombin Receptor Antagonist for Clinical Event Reduction in Acute Coronary Syndrome) trial. Investigators aimed to compare vorapaxar administration with placebo, in addition to standard therapy, in patients suffering from NSTEMI-ACS (191). The main result observed is a decreased rate of MI event for patients treated with vorapaxar. These data support the use of vorapaxar in MI secondary prevention since it provided net clinical benefit in patients at low risk for bleeding but high risk for ischemic events, as it especially prevented stent thrombosis after MI. However, the safety and efficacy of vorapaxar in STEMI patients have not been

TABLE 3 | (A) A comprehensive analysis of platelet mechanisms in myocardial infarction (MI) mouse model and **(B)** in MI patients (↓ decrease, ↑ increase, = no effect on MI or bleeding risk).

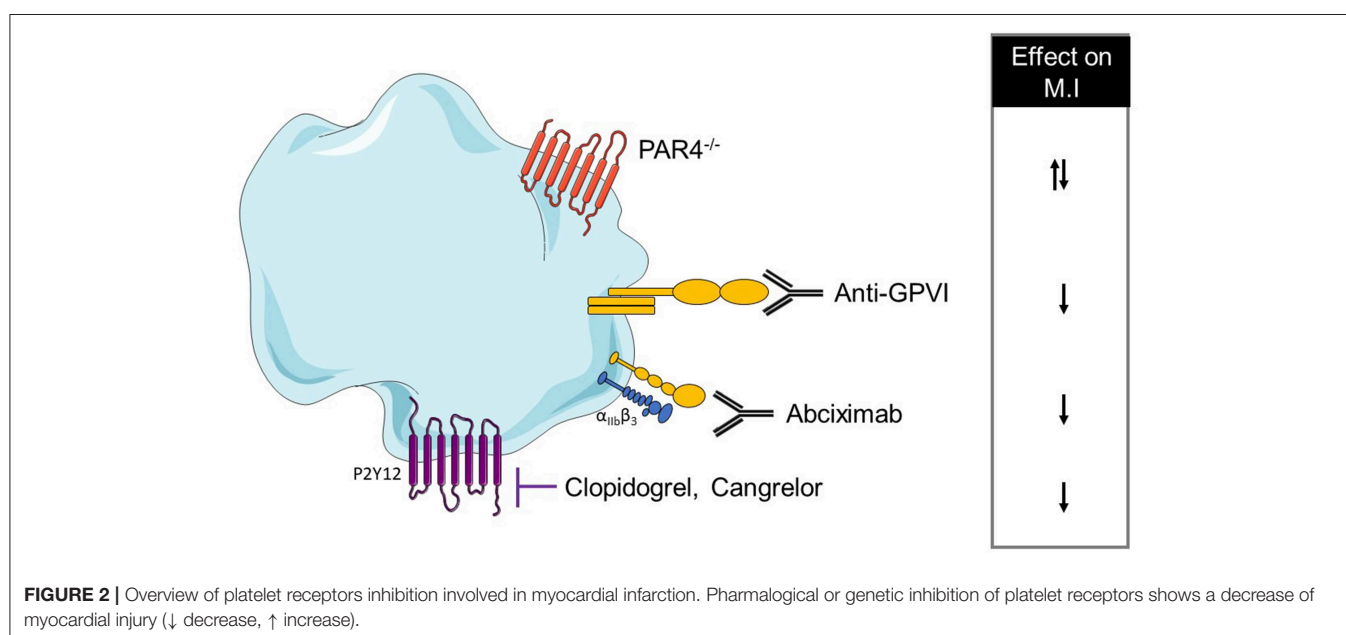
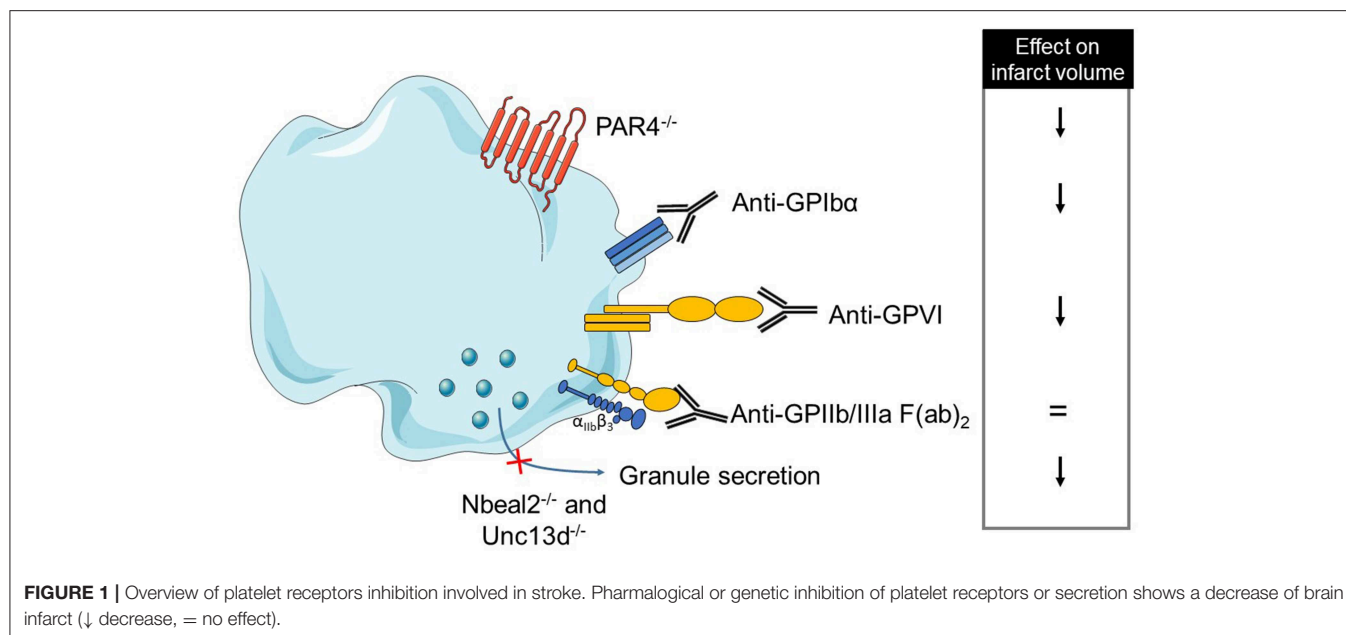
A				
Molecule targeted	Animal model	Effect on M.I.	References	
Platelets	Thrombocytopenia	↓	(166)	
GP1bα	Fab anti-GP1bα	=	(167)	
Pld1	Pld1 ^{-/-}	=	(167)	
GPVI	Antibody anti-GPVI	↓	(167, 169)	
	Soluble GPVI-Fc	↓	(169)	
FcRγ	FcRγ ^{-/-}	↓	(168)	
CLEC-2	Fab anti-CLEC-2	=	(167)	
PAR4	PAR4 ^{-/-}	↑	(176)	
	PAR4 ^{-/-}	↓	(177)	
	PAR4 antagonist	↓	(178)	
Gα ₁₂	Gα ₁₂ ^{-/-}	↓	(124)	
αIIbβ3	Fab anti-GPIIb/IIIa	=	(167)	
	Isolated hearts and perfusion of platelets from acute MI patients	↓	(179)	
	+ Abciximab			
δ granules	Nbeal2 ^{-/-}	=	(167)	
Dense granules	Unc13d ^{-/-}	=	(167)	
Stim1	Stim1 ^{-/-}	=	(167)	
Orai1	Orai1 ^{-/-}	=	(167)	
TXA ₂	Aspirin	=	(165, 180)	
P2Y12	Clopidogrel	↓	(165, 166)	
	Isolated hearts and perfusion of platelets from acute MI patients			
	+ Cangrelor	↓	(179)	
B				
Molecule targeted	Clinical trial	Effect	Bleeding	References
P2Y12	CURE	↓	↑	(184)
	Clopidogrel + aspirin vs. aspirin alone			
	TRITON-TIMI-38	↓ Prasugrel	↑ Prasugrel	(185)
	Prasugrel vs. clopidogrel (+ aspirin)			
	PLATO	↓ Ticagrelor	=	(186)
	Ticagrelor vs. clopidogrel (+ aspirin)			
	PEGASUS-TIMI-54	↓	↑	(187–189)
	Ticagrelor + Aspirin vs. Aspirin alone			
PAR1	The TRA 2P–TIMI 50	↓	↑	(190)
	Vorapaxar vs. placebo (+ concomitant medical therapy)			
	TRACER	↓	↑	(191)
	Vorapaxar vs. placebo (+ standard therapy)			

investigated yet. These clinical findings have been summarized in **Table 3B**.

PLATELETS PARTICIPATE TO THE DEVELOPMENT OF THE ABDOMINAL AORTIC ANEURYSM (AAA)

Abdominal aortic aneurysm (AAA) is a permanent and irreversible localized dilatation of the infrarenal segment of the

abdominal aorta caused by the degradation and remodeling of the layers of the vessel wall and a chronic wall inflammation. AAA can extend along the aorta (fusiform), or be localized (sacciform). Major AAA risk factors are age, atherosclerosis, hypertension, male gender, and smoking. In western countries, AAA incidence is ~0.4–0.67% annually and reaches 5–10% of men and 1% of women over 65 years old (192). This pathology is mainly asymptomatic and aneurysm rupture leads to death. Endovascular and open repair of AAA remains the only effective treatments. Nevertheless, many pharmacological therapies are



still under investigation like statins, angiotensin receptor blockers and anti-platelets drugs (193).

AAA is characterized by chronic inflammation with a large degradation of elastin and collagen fibers. It results in the proteolytic activity of matrix-degrading proteinases including matrix metalloproteinases (MMPs) leading to aorta dilatation. Reduced vascular wall thickness and the lack of tissue repair are associated with vascular smooth muscle cells apoptosis. The adventitia neovascularization induces inflammatory cells (lymphocytes, neutrophils and macrophages) infiltration in the aortic vessel wall, maintaining a continuous level of inflammation. This process contributes to the intra-luminal thrombus (ILT) formation (194), which involves platelets and

coagulation activation. Overall, the ILT thromboinflammatory status contributes to the outward remodeling and eventually to the disruption of wall integrity (195, 196).

The ILT is structured in multilayers. In AAA patients, ILT is often organized in three layers—luminal (in contact with the blood), medial and abluminal (in contact with the wall). Luminal ILT layer is biologically active and enriched in platelets, neutrophils, red blood cells and a dense fibrin network (197). The ILT has also been shown to contain weak pathogens like *Porphyromonas gingivalis* (Pg) which contribute to leukocyte recruitment (198). On the opposite side, the abluminal layer has a marked fibrinolytic activity and contains few cells (199, 200). ILT evolution can lead to vessel wall weakness due to the

high concentrations of reactive oxygen species (ROS), proteases and cytokines. Indeed, a study showed that ILT thickness is correlated with AAA diameter and MMP9 expression (201). The main specificity of the ILT in AAA is its non-healing property. The continuous release of neutrophil-derived proteases from these thrombi prevents vascular healing. Indeed, the re-endothelialization and adherence of mesenchymal stem cells are prevented by neutrophil proteases (202). This protease-rich thrombus is considered as the driving force in vessel wall rupture leading to death (203). However, ILT formation mechanisms in AAA are so far unknown.

TABLE 4 | (A) A comprehensive analysis of platelet mechanisms in abdominal aortic aneurysm (AAA) animal models and **(B)** in AAA patients (↓ decrease, ↑ increase, = no effect on intraluminal thrombus ILT or aneurysm diameter).

A			
Molecule targeted	Animal model	Effect on ILT	References
$\alpha\text{IIb}\beta_3$	Xenograft rats model + abciximab	↓	(197)
P2Y ₁₂	Xenograft model + AZD6140	↓	(209)
	Angiotensin II + Clopidogrel	↓	(210)
P2Y ₁₂ and TXA ₂	Angiotensin II + Clopidogrel and Aspirin	↓	(211)
B			
Molecule targeted	Clinical trial	Effect on aneurysm diameter	References
TXA ₂	Aspirin	↓ (between 40 and 49 mm)	(212)
P2Y ₁₂	Ticagrelor	=	(213)

Observational studies based on human tissue samples from AAA patients provide information at the late stage of the disease. In order to understand the mechanisms in the early steps, different animal models have been used, including mice and rats. The role of platelets and coagulation in ILT formation during AAA has been recently reviewed elsewhere (194). To study the role of platelets in AAA, two major models were used in mice and rats. The hypertension model, induced by angiotensin II in ApoE^{-/-} or Ldlr^{-/-} mice, reproduces important features of human AAA with inflammation, smooth muscle cells apoptosis and macrophage infiltration. However, aneurysms formed have a suprarenal location and abluminal thrombus formation occurs after an aortic dissection due to a false channel (204).

The main model in rats consists of elastin degradation via elastase perfusion and presents the same characteristics as the first model. However, in this model, aneurysms have an infrarenal location and do not present a thrombus and hypertension (205). This model was also developed in mice (206). A recent study showed that administration of beta 3-aminopropionitrile fumarate salt (BAPN, inhibitor of lysyl oxidase) in the drinking water of elastase-treated mice resulted in ILT formation (207). Both of these models (elastase and angiotensin II) do not recapitulate all human characteristics but they contribute to better understand the disease.

A xenograft rat model which consists of grafting decellularized aorta of guinea pig into rat aorta has been shown to present an ILT (208). With this model, abciximab treatment (platelet aggregation inhibitor) reduces the aneurysmal diameter and ILT activities accompanied by fewer P-selectin expression and reduced vessel wall degradation. These results suggest that platelets are involved in the thrombus biological activity and aneurysm development (197). Similar results were observed in rats after 10 and 42 days of AZD6140 treatment, a P2Y₁₂ receptor antagonist. A reduced ILT was observed as well as decreased MMP-9, MMP-2 expression, leukocyte infiltration, media and elastin preservation (209).

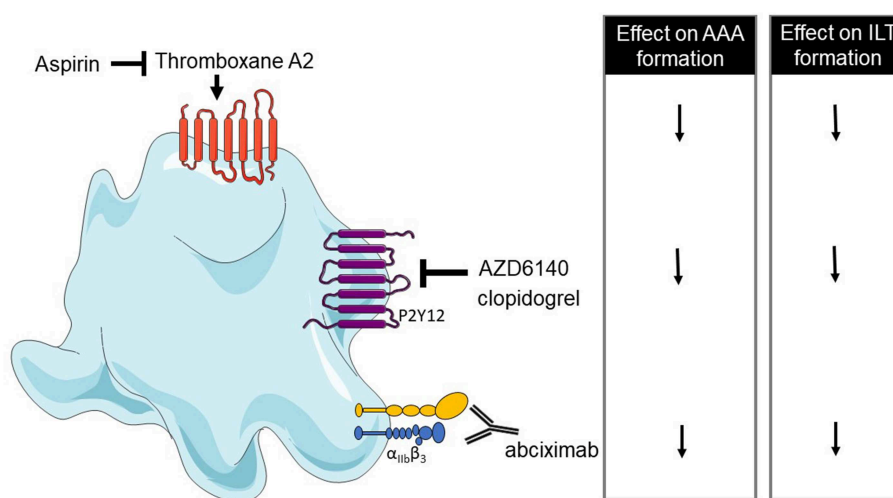


FIGURE 3 | Overview of platelet receptors inhibition involved in abdominal aortic aneurysm (AAA). Pharmacological inhibition of platelet receptors shows a decrease of AAA formation and intraluminal thrombus (ILT) formation (↓ decrease).

Other studies with angiotensin II mouse model have shown that clopidogrel treatment (inhibitor of P2Y₁₂), or aspirin (inhibitor of COX-2) reduces the macrophage infiltration, MMP2 and ROS production, suggesting that platelets play a role in vascular inflammation during AAA progression (210, 211). A reduction of thrombi, uPA, t-PA, and PF4 in the aorta was also observed in aspirin or clopidogrel-treated mice but these treatments have no effect on aorta diameter on established AngII-aneurysm model (211). However, clopidogrel administration in the early steps of AAA decreases the aorta diameter (210). The same treatments in patients emphasize that anti-platelet treatments can reduce AAA progression and rupture or dissection (211). Low-dose of aspirin can prevent the progression of AAA measuring from 40 to 49 mm and no decrease of AAA growth was observed in AAAs measuring <40 mm (212). The use of a P2Y₁₂ receptor inhibitor as ticagrelor treatment revealed a lack of difference in AAA size compared to placebo-treated subjects, suggesting that ticagrelor has no effect on the development of small AAAs. However, in this study, most of the patients did not present an ILT (213). These results have been summarized in **Table 4** and **Figure 3**.

Nowadays, there is no definite treatment to decelerate or stop AAA progression. Nevertheless, clinical and animal studies mentioned above provide additional information on the effect of platelets in AAA development, opening up treatment prospects that may be in the long term substitutes of heavy and invasive surgery.

CONCLUSIONS AND PERSPECTIVES

Over the past years, the field of platelets gained a lot of attention in their contribution to vascular diseases. Platelet biology is at the crossroads of several clinical specialties (cardiology, neurology,

pulmonology). The current use of antithrombotic drugs, aspirin, and P2Y₁₂ antagonists, is based on their inhibitory effect on platelet aggregation. While those drugs show a beneficial effect in CVD, they are still associated with some bleeding risks. Recent studies provided a new understanding of the role of platelets in vascular inflammation that extends beyond their role in aggregation. The development of future anti-platelet drugs will need to take into account the role of platelets in inflammation. In addition, as most of the thrombosis and inflammatory studies are conducted in healthy vessels, it is important to keep in mind that the identified mechanisms need to be validated in models relevant to CVD. Importantly, there is significant inter-individual variability of antiplatelet therapy responses among patients with CVD highlighting the need for tailored therapies to each individual. Central to this approach is the development of robust assays that can determine platelet reactivity in a patient-specific manner.

AUTHOR CONTRIBUTIONS

HL did the literature research and wrote the first draft of the review. KY wrote a part of the manuscript. RM designed the tables. YB contributed to the idea of the manuscript, wrote parts of the manuscript, and provided critical feedback. All the authors reviewed the manuscript and approved the submitted version.

FUNDING

This work was funded by a predoctoral fellowship from the Doctoral School Hématologie – Oncogénèse – Biothérapies ED 561 (to KY) and by the European Research Council grant 759880 and Fondation de France grant 00075823 (to YB).

REFERENCES

- Osler W. An account of certain organisms occurring in the liquor sanguinis. *Monthly Microscopical J.* (1874) 12:141–8. doi: 10.1111/j.1365-2818.1874.tb01798.x
- Bizzozero J. Ueber einen neuen formbestandtheil des blutes und dessen rolle bei der thrombose und der blutgerinnung. *Arch Pathol Anat.* (1882) 90:261–332. doi: 10.1007/BF01931360
- Brill A, Fuchs TA, Chauhan AK, Yang JJ, De Meyer SE, Köllnberger M, et al. von Willebrand factor-mediated platelet adhesion is critical for deep vein thrombosis in mouse models. *Blood.* (2011) 117:1400–7. doi: 10.1182/blood-2010-05-287623
- von Brühl ML, Stark K, Steinhart A, Chandraratne S, Konrad I, Lorenz M, et al. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice *in vivo*. *J Exp Med.* (2012) 209:819–35. doi: 10.1084/jem.20112322
- van der Meijden PEJ, Heemskerk JWM. Platelet biology and functions: new concepts and clinical perspectives. *Nat Rev Cardiol.* (2019) 16:166–79. doi: 10.1038/s41569-018-0110-0
- Harper MT, Poole AW. Diverse functions of protein kinase C isoforms in platelet activation and thrombus formation. *J Thromb Haemost.* (2010) 8:454–62. doi: 10.1111/j.1538-7836.2009.03722.x
- Jin J, Daniel JL, Kunapuli SP. Molecular basis for ADP-induced platelet activation. II. The P2Y₁ receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. *J Biol Chem.* (1998) 273:2030–4. doi: 10.1074/jbc.273.4.2030
- Hollopeter G, Jantzen HM, Vincent D, Li G, England L, Ramakrishnan V, et al. Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature.* (2001) 409:202–7. doi: 10.1038/35051599
- Nieswandt B, Bergmeier W, Eckly A, Schulte V, Ohlmann P, Cazenave JP, et al. Evidence for cross-talk between glycoprotein VI and Gi-coupled receptors during collagen-induced platelet aggregation. *Blood.* (2001) 97:3829–35. doi: 10.1182/blood.V97.12.3829
- Violi F, Basili S, Berger JS, Hiatt WR. Antiplatelet therapy in peripheral artery disease. *Handb Exp Pharmacol.* (2012) 210:547–63. doi: 10.1007/978-3-642-29423-5_22
- van der Meijden PE, Munnix IC, Auger JM, Govers-Riemsag JW, Cosemans JM, Kuijpers MJ, et al. Dual role of collagen in factor XII-dependent thrombus formation. *Blood.* (2009) 114:881–90. doi: 10.1182/blood-2008-07-171066
- Kahn ML, Nakanishi-Matsui M, Shapiro MJ, Ishihara H, Coughlin SR. Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *J Clin Invest.* (1999) 103:879–87. doi: 10.1172/JCI6042
- Inoue O, Suzuki-Inoue K, McCarty OJ, Moroi M, Ruggeri ZM, Kunicki TJ, et al. Laminin stimulates spreading of platelets through integrin α 6 β 1-dependent activation of GPVI. *Blood.* (2006) 107:1405–12. doi: 10.1182/blood-2005-06-2406

14. Schaff M, Tang C, Maurer E, Bourdon C, Receveur N, Eckly A, et al. Integrin $\alpha 6 \beta 1$ is the main receptor for vascular laminins and plays a role in platelet adhesion, activation, and arterial thrombosis. *Circulation*. (2013) 128:541–52. doi: 10.1161/CIRCULATIONAHA.112.000799
15. Voors-Pette C, Lebozec C, Dogterom P, Jullien L, Billiald P, Ferlan P, et al. Safety and tolerability, pharmacokinetics, and pharmacodynamics of ACT017, an antiplatelet GPVI (Glycoprotein VI) Fab. *Arterioscler Thromb Vasc Biol*. (2019) 39:956–64. doi: 10.1161/ATVBAHA.118.312314
16. May F, Hagedorn I, Pleines I, Bender M, Vögtle T, Eble J, et al. CLEC-2 is an essential platelet-activating receptor in hemostasis and thrombosis. *Blood*. (2009) 114:3464–72. doi: 10.1182/blood-2009-05-222273
17. Suzuki-Inoue K, Inoue O, Ding G, Nishimura S, Hokamura K, Eto K, et al. Essential *in vivo* roles of the C-type lectin receptor CLEC-2: embryonic/neonatal lethality of CLEC-2-deficient mice by blood/lymphatic misconnections and impaired thrombus formation of CLEC-2-deficient platelets. *J Biol Chem*. (2010) 285:24494–507. doi: 10.1074/jbc.M110.130575
18. Hughes CE, Navarro-Núñez L, Finney BA, Mourão-Sá D, Pollitt AY, Watson SP. CLEC-2 is not required for platelet aggregation at arteriolar shear. *J Thromb Haemost*. (2010) 8:2328–32. doi: 10.1111/j.1538-7836.2010.04006.x
19. Ward LSC, Sheriff L, Marshall JL, Manning JE, Brill A, Nash GB, et al. Podoplanin regulates the migration of mesenchymal stromal cells and their interaction with platelets. *J Cell Sci*. (2019) 132:jcs222067. doi: 10.1242/jcs.222067
20. Petri B, Broermann A, Li H, Khandoga AG, Zarbock A, Krombach F, et al. von Willebrand factor promotes leukocyte extravasation. *Blood*. (2010) 116:4712–9. doi: 10.1182/blood-2010-03-276311
21. Hara T, Shimizu K, Ogawa F, Yanaba K, Iwata Y, Muroi E, et al. Platelets control leukocyte recruitment in a murine model of cutaneous arthritis reaction. *Am J Pathol*. (2010) 176:259–69. doi: 10.2353/ajpath.2010.081117
22. Hidalgo A, Chang J, Jang JE, Peired AJ, Chiang EY, Frenette PS. Heterotypic interactions enabled by polarized neutrophil microdomains mediate thromboinflammatory injury. *Nat Med*. (2009) 15:384–91. doi: 10.1038/nm.1939
23. Kuligowski MP, Kitching AR, Hickey MJ. Leukocyte recruitment to the inflamed glomerulus: a critical role for platelet-derived P-selectin in the absence of rolling. *J Immunol*. (2006) 176:6991–9. doi: 10.4049/jimmunol.176.11.6991
24. Devi S, Kuligowski MP, Kwan RY, Westein E, Jackson SP, Kitching AR, et al. Platelet recruitment to the inflamed glomerulus occurs via an α IIb β 3/GPVI-dependent pathway. *Am J Pathol*. (2010) 177:1131–42. doi: 10.2353/ajpath.2010.091143
25. Schulz C, von Brühl ML, Barocke V, Cullen P, Mayer K, Okrojek R, et al. EMMPRIN (CD147/basigin) mediates platelet-monocyte interactions *in vivo* and augments monocyte recruitment to the vascular wall. *J Thromb Haemost*. (2011) 9:1007–19. doi: 10.1111/j.1538-7836.2011.04235.x
26. Pamuk GE, Nuri Pamuk O, Örlüm H, Arican O, Turgut B, Demir M. Elevated platelet-monocyte complexes in patients with psoriatic arthritis. *Platelets*. (2009) 20:493–7. doi: 10.3109/09537100903165174
27. Nicu EA, Van der Velden U, Nieuwland R, Everts V, Loos BG. Elevated platelet and leukocyte response to oral bacteria in periodontitis. *J Thromb Haemost*. (2009) 7:162–70. doi: 10.1111/j.1538-7836.2008.03219.x
28. Bunesco A, Seideman P, Lenkei R, Levin K, Egberg N. Enhanced Fc γ receptor I, α IIb β 2 integrin receptor expression by monocytes and neutrophils in rheumatoid arthritis: interaction with platelets. *J Rheumatol*. (2004) 31:2347–55.
29. Dole VS, Bergmeier W, Mitchell HA, Eichenberger SC, Wagner DD. Activated platelets induce Weibel-Palade-body secretion and leukocyte rolling *in vivo*: role of P-selectin. *Blood*. (2005) 106:2334–9. doi: 10.1182/blood-2005-04-1530
30. Nachman RL, Rafii S. Platelets, petechiae, and preservation of the vascular wall. *N Engl J Med*. (2008) 359:1261–70. doi: 10.1056/NEJMra0800887
31. Cloutier N, Paré A, Farndale RW, Schumacher HR, Nigrovic PA, Lacroix S, et al. Platelets can enhance vascular permeability. *Blood*. (2012) 120:1334–43. doi: 10.1182/blood-2012-02-413047
32. Moon DG, van der Zee H, Weston LK, Gudewicz PW, Fenton JW, Kaplan JE. Platelet modulation of neutrophil superoxide anion production. *Thromb Haemost*. (1990) 63:91–6. doi: 10.1055/s-0038-1645693
33. Bazzoni G, Dejana E, Del Maschio A. Platelet-neutrophil interactions. Possible relevance in the pathogenesis of thrombosis and inflammation. *Haematologica*. (1991) 76:491–9.
34. Clark SR, Ma AC, Tavener SA, McDonald B, Goodarzi Z, Kelly MM, et al. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat Med*. (2007) 13:463–9. doi: 10.1038/nm1565
35. Kim S-J, Jenne CN. Role of platelets in neutrophil extracellular trap (NET) production and tissue injury. *Semin Immunol*. (2016) 28:546–54. doi: 10.1016/j.smim.2016.10.013
36. Vivekananthan DP, Bhatt DL, Chew DP, Zidar FJ, Chan AW, Moliterno DJ, et al. Effect of clopidogrel pretreatment on periprocedural rise in C-reactive protein after percutaneous coronary intervention. *Am J Cardiol*. (2004) 94:358–60. doi: 10.1016/j.amjcard.2004.04.035
37. Nidorf SM, Eikelboom JW, Budgeon CA, Thompson PL. Low-dose colchicine for secondary prevention of cardiovascular disease. *J Am Coll Cardiol*. (2013) 61:404–10. doi: 10.1016/j.jacc.2012.10.027
38. Deftereos S, Giannopoulos G, Efremidis M, Kossyvakis C, Katsivas A, Panagopoulou V, et al. Colchicine for prevention of atrial fibrillation recurrence after pulmonary vein isolation: mid-term efficacy and effect on quality of life. *Heart Rhythm*. (2014) 11:620–8. doi: 10.1016/j.hrthm.2014.02.002
39. Frostegård J. Immunity, atherosclerosis and cardiovascular disease. *BMC Med*. (2013) 11:117. doi: 10.1186/1741-7015-11-117
40. Ross R, Glomset J, Harker L. Response to injury and atherogenesis. *Am J Pathol*. (1977) 86:675–84.
41. Glagov S. *Hemodynamic Risk Factors: Mechanical Stress, Mural Architecture, Medial Nutrition and the Vulnerability of Arteries to Atherosclerosis* (1972).
42. Franck G, Even G, Gautier A, Salinas M, Lose A, Procopio E, et al. Haemodynamic stress-induced breaches of the arterial intima trigger inflammation and drive atherogenesis. *Eur Heart J*. (2019) 40:928–37. doi: 10.1093/eurheartj/ehy822
43. Massberg S, Brand K, Grüner S, Page S, Müller E, Müller I, et al. A critical role of platelet adhesion in the initiation of atherosclerotic lesion formation. *J Exp Med*. (2002) 196:887–96. doi: 10.1084/jem.20012044
44. Fujimura Y, Titani K, Holland LZ, Russell SR, Roberts JR, Elder JH, et al. von Willebrand Factor a reduced and alkylated 52/48-kDa fragment beginning at amino acid residue 449 contains the domain interacting with platelet glycoprotein Ib. *J Biol Chem*. (1986) 261:381–5.
45. Romo GM, Dong JF, Schade AJ, Gardiner EE, Kansas GS, Li CQ, et al. The glycoprotein Ib-IX-V complex is a platelet counterreceptor for P-selectin. *J Exp Med*. (1999) 190:803–14. doi: 10.1084/jem.190.6.803
46. Ruggeri ZM, Zarpellon A, Roberts JR, Mc Clintock RA, Jing H, Mendolicchio GL. Unravelling the mechanism and significance of thrombin binding to platelet glycoprotein Ib. *Thromb Haemost*. (2010) 104:894–902. doi: 10.1160/TH10-09-0578
47. Simon DI, Chen Z, Xu H, Li CQ, Dong JF, McIntire LV, et al. Platelet glycoprotein Ib is a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18). *J Exp Med*. (2000) 192:193–204. doi: 10.1084/jem.192.2.193
48. Koltsova E, Sundt P, Zarpellon A, Ouyang H, Mikulski Z, Zampolli A, et al. Genetic deletion of platelet glycoprotein Ib α but not its extracellular domain protects from atherosclerosis. *Thromb Haemost*. (2014) 112:1252–63. doi: 10.1160/th14-02-0130
49. Methia N, André P, Denis CV, Economopoulos M, Wagner DD. Localized reduction of atherosclerosis in von Willebrand factor-deficient mice. *Blood*. (2001) 98:1424–8. doi: 10.1182/blood.V98.5.1424
50. Ghasemzadeh M, Kaplan ZS, Alwis I, Schoenwaelder SM, Ashworth KJ, Westein E, et al. The CXCR1/2 ligand NAP-2 promotes directed intravascular leukocyte migration through platelet thrombi. *Blood*. (2013) 121:4555–66. doi: 10.1182/blood-2012-09-459636
51. Wang Y, Sakuma M, Chen Z, Ustinov V, Shi C, Croce K, et al. Leukocyte engagement of platelet glycoprotein Ib α via the integrin Mac-1 is critical for the biological response to vascular injury. *Circulation*. (2005) 112:2993–3000. doi: 10.1161/CIRCULATIONAHA.105.571315
52. Strassel C, Hechler B, Bull A, Gachet C, Lanza F. Studies of mice lacking the GPIb-V-IX complex question the role of this receptor in atherosclerosis. *J Thromb Haemost*. (2009) 7:1935–8. doi: 10.1111/j.1538-7836.2009.03608.x
53. Massberg S, Schürzinger K, Lorenz M, Konrad I, Schulz C, Plesnila N, et al. Platelet adhesion via glycoprotein IIb integrin is critical

- for atheroprogession and focal cerebral ischemia: an *in vivo* study in mice lacking glycoprotein IIb. *Circulation*. (2005) 112:1180–8. doi: 10.1161/CIRCULATIONAHA.105.539221
54. Stalker TJ, Newman DK, Ma P, Wannemacher KM, Brass LF. Platelet signaling. *Handb Exp Pharmacol*. (2012) 210:59–85. doi: 10.1007/978-3-642-29423-5_3
 55. Bültmann A, Li Z, Wagner S, Peluso M, Schönberger T, Weis C, et al. Impact of glycoprotein VI and platelet adhesion on atherosclerosis—A possible role of fibronectin. *J Mol Cell Cardiol*. (2010) 49:532–42. doi: 10.1016/j.yjmcc.2010.04.009
 56. Fitzgerald DJ, Roy L, Catella F, FitzGerald GA. Platelet activation in unstable coronary disease. *N Engl J Med*. (1986) 315:983–9. doi: 10.1056/NEJM198610163151602
 57. Tschöepe D, Roesen P, Schwippert B, Gries F. Platelets in diabetes: the role in the hemostatic regulation in atherosclerosis. *Semin Thromb Hemostasis*. (1993) 19:122–8. doi: 10.1055/s-2007-994015
 58. Huo Y, Schober A, Forlow SB, Smith DE, Hyman MC, Jung S, et al. Circulating activated platelets exacerbate atherosclerosis in mice deficient in apolipoprotein E. *Nat Med*. (2003) 9:61–7. doi: 10.1038/nm810
 59. Page C, Pitchford S. Neutrophil and platelet complexes and their relevance to neutrophil recruitment and activation. *Int Immunopharmacol*. (2013) 17:1176–84. doi: 10.1016/j.intimp.2013.06.004
 60. Dotsenko O, Chaturvedi N, Thom SA, Wright AR, Mayet J, Shore A, et al. Platelet and leukocyte activation, atherosclerosis and inflammation in European and South Asian men. *J Thromb Haemost*. (2007) 5:2036–42. doi: 10.1111/j.1538-7836.2007.02711.x
 61. Finsterbusch M, Schrottmaier WC, Kral-Pointner JB, Salzmann M, Assinger A. Measuring and interpreting platelet-leukocyte aggregates. *Platelets*. (2018) 29:677–85. doi: 10.1080/09537104.2018.1430358
 62. Tekelioglu Y, Uzun H, Güçer H. Circulating platelet-leukocyte aggregates in patients with inflammatory bowel disease. *J Chin Med Assoc*. (2013) 76:182–5. doi: 10.1016/j.jcma.2012.12.015
 63. Burger PC, Wagner DD. Platelet P-selectin facilitates atherosclerotic lesion development. *Blood*. (2003) 101:2661–6. doi: 10.1182/blood-2002-07-2209
 64. Dong ZM, Brown AA, Wagner DD. Prominent role of P-selectin in the development of advanced atherosclerosis in ApoE-deficient mice. *Circulation*. (2000) 101:2290–5. doi: 10.1161/01.CIR.101.19.2290
 65. Li D, Wang Y, Zhang L, Luo X, Li J, Chen X, et al. Roles of purinergic receptor P2Y₁₂, G protein-coupled 12 in the development of atherosclerosis in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol*. (2012) 32:e81–9. doi: 10.1161/ATVBAHA.111.239095
 66. Wihlborg AK, Wang L, Braun OO, Eyjolfsson A, Gustafsson R, Gudbjartsson T, et al. ADP receptor P2Y₁₂ is expressed in vascular smooth muscle cells and stimulates contraction in human blood vessels. *Arterioscler Thromb Vasc Biol*. (2004) 24:1810–5. doi: 10.1161/01.ATV.0000142376.30582.ed
 67. Boulaftali Y, Owens AP, Beale A, Piatt R, Casari C, Lee RH, et al. CalDAG-GEFI deficiency reduces atherosclerotic lesion development in mice. *Arterioscler Thromb Vasc Biol*. (2016) 36:792–9. doi: 10.1161/ATVBAHA.115.306347
 68. Afek A, Kogan E, Maysel-Auslender S, Mor A, Regev E, Rubinstein A, et al. Clopidogrel attenuates atheroma formation and induces a stable plaque phenotype in apolipoprotein E knockout mice. *Microvasc Res*. (2009) 77:364–9. doi: 10.1016/j.mvr.2009.01.009
 69. Heim C, Gebhardt J, Ramsperger-Gleixner M, Jacobi J, Weyand M, Ensminger SM. Clopidogrel significantly lowers the development of atherosclerosis in ApoE-deficient mice *in vivo*. *Heart Vessels*. (2016) 31:783–94. doi: 10.1007/s00380-015-0696-7
 70. Schulz C, Konrad I, Sauer S, Orschiedt L, Koellnberger M, Lorenz R, et al. Effect of chronic treatment with acetylsalicylic acid and clopidogrel on atheroprogession and atherothrombosis in ApoE-deficient mice *in vivo*. *Thromb Haemost*. (2008) 99:190–5. doi: 10.1160/TH07-03-0235
 71. Ganbaatar B, Fukuda D, Salim HM, Nishimoto S, Tanaka K, Higashikuni Y, et al. Ticagrelor, a P2Y₁₂ antagonist, attenuates vascular dysfunction and inhibits atherogenesis in apolipoprotein-E-deficient mice. *Atherosclerosis*. (2018) 275:124–32. doi: 10.1016/j.atherosclerosis.2018.05.053
 72. Xia X, Li J, Liang X, Zhang S, Liu T, Liu J, et al. Ticagrelor suppresses oxidized low-density lipoprotein-induced endothelial cell apoptosis and alleviates atherosclerosis in ApoE^{-/-} mice via downregulation of PCSK9. *Mol Med Rep*. (2018) 19:1453–62. doi: 10.3892/mmr.2018.9779
 73. Preusch MR, Rusnak J, Staudacher K, Mogler C, Uhlmann L, Sievers P, et al. Ticagrelor promotes atherosclerotic plaque stability in a mouse model of advanced atherosclerosis. *Drug Des Dev Ther*. (2016) 10:2691–9. doi: 10.2147/DDDT.S105718
 74. Hamilton JR, Cornelissen I, Mountford JK, Coughlin SR. Atherosclerosis proceeds independently of thrombin-induced platelet activation in ApoE^{-/-} mice. *Atherosclerosis*. (2009) 205:427–32. doi: 10.1016/j.atherosclerosis.2009.01.018
 75. Lutgens E, Gorelik L, Daemen MJ, de Muinck ED, Grewal IS, Kotliansky VE, et al. Requirement for CD154 in the progression of atherosclerosis. *Nat Med*. (1999) 5:1313–6. doi: 10.1038/15271
 76. Sachai B, Turrentine T, Dawicki McKenna JM, Rux AH, Rader D, Kowalska MA. Elimination of platelet factor 4 (PF4) from platelets reduces atherosclerosis in C57Bl/6 and apoE^{-/-} mice. *Thromb Haemost*. (2007) 98:1108–13. doi: 10.1160/TH07-04-0271
 77. Brauersreuther V, Steffens S, Arnaud C, Pelli G, Burger F, Proudfoot A, et al. A novel RANTES antagonist prevents progression of established atherosclerotic lesions in mice. *Arterioscler Thromb Vasc Biol*. (2008) 28:1090–6. doi: 10.1161/ATVBAHA.108.165423
 78. Veillard NR, Kwak B, Pelli G, Mulhaupt F, James RW, Proudfoot AE, et al. Antagonism of RANTES receptors reduces atherosclerotic plaque formation in mice. *Circ Res*. (2004) 94:253–61. doi: 10.1161/01.RES.0000109793.17591.4E
 79. Gerdes N, Seijkens T, Lievens D, Kuijpers MJ, Winkels H, Projahn D, et al. Platelet CD40 exacerbates atherosclerosis by transcellular activation of endothelial cells and leukocytes. *Arterioscler Thromb Vasc Biol*. (2016) 36:482–90. doi: 10.1161/ATVBAHA.115.307074
 80. Cayatte AJ, Du Y, Oliver-Krasinski J, Lavielle G, Verbeuren TJ, Cohen RA. The thromboxane receptor antagonist S18886 but not aspirin inhibits atherogenesis in apo E-deficient mice: evidence that eicosanoids other than thromboxane contribute to atherosclerosis. *Arterioscler Thromb Vasc Biol*. (2000) 20:1724–8. doi: 10.1161/01.ATV.20.7.1724
 81. Kobayashi T, Tahara Y, Matsumoto M, Iguchi M, Sano H, Murayama T, et al. Roles of thromboxane A₂ and prostacyclin in the development of atherosclerosis in apoE-deficient mice. *J Clin Invest*. (2004) 114:784–94. doi: 10.1172/JCI21446
 82. McClelland S, Gawaz M, Kennerknecht E, Konrad CS, Sauer S, Schuerzinger K, et al. Contribution of cyclooxygenase-1 to thromboxane formation, platelet-vessel wall interactions and atherosclerosis in the ApoE null mouse. *Atherosclerosis*. (2009) 202:84–91. doi: 10.1016/j.atherosclerosis.2008.04.016
 83. Cyrus T, Sung S, Zhao L, Funk CD, Tang S, Praticò D. Effect of low-dose aspirin on vascular inflammation, plaque stability, and atherogenesis in low-density lipoprotein receptor-deficient mice. *Circulation*. (2002) 106:1282–7. doi: 10.1161/01.CIR.0000027816.54430.96
 84. Kraus S, Naumov I, Shapira S, Kazanov D, Aroch I, Afek A, et al. Aspirin but not meloxicam attenuates early atherosclerosis in apolipoprotein E knockout mice. *Israel Med Assoc J*. (2014) 16:233–8.
 85. Paul A, Calleja L, Camps J, Osada J, Vilella E, Ferré N, et al. The continuous administration of aspirin attenuates atherosclerosis in apolipoprotein E-deficient mice. *Life Sci*. (2000) 68:457–65. doi: 10.1016/S0024-3205(00)00950-4
 86. Cyrus T, Yao Y, Tung LX, Praticò D. Stabilization of advanced atherosclerosis in low-density lipoprotein receptor-deficient mice by aspirin. *Atherosclerosis*. (2006) 184:8–14. doi: 10.1016/j.atherosclerosis.2004.10.047
 87. Tous M, Ferré N, Vilella E, Riu F, Camps J, Joven J. Aspirin attenuates the initiation but not the progression of atherosclerosis in apolipoprotein E-deficient mice fed a high-fat, high-cholesterol diet. *Basic Clin Pharmacol Toxicol*. (2004) 95:15–9. doi: 10.1111/j.1742-7843.2004.pto950104.x
 88. Crittenden JR, Bergmeier W, Zhang Y, Piffath CL, Liang Y, Wagner DD, et al. CalDAG-GEFI integrates signaling for platelet aggregation and thrombus formation. *Nat Med*. (2004) 10:982–6. doi: 10.1038/nm1098
 89. Stefanini L, Roden RC, Bergmeier W. CalDAG-GEFI is at the nexus of calcium-dependent platelet activation. *Blood*. (2009) 114:2506–14. doi: 10.1182/blood-2009-04-218768
 90. Stefanini L, Bergmeier W. CalDAG-GEFI and platelet activation. *Platelets*. (2010) 21:239–43. doi: 10.3109/09537101003639931

91. Mann J, Davies MJ. Mechanisms of progression in native coronary artery disease: role of healed plaque disruption. *Heart*. (1999) 82:265–8. doi: 10.1136/hrt.82.3.265
92. Kuijpers MJ, Gilio K, Reitsma S, Nergiz-Unal R, Prinzen L, Heeneman S, et al. Complementary roles of platelets and coagulation in thrombus formation on plaques acutely ruptured by targeted ultrasound treatment: a novel intravital model. *J Thromb Haemost*. (2009) 7:152–61. doi: 10.1111/j.1538-7836.2008.03186.x
93. Hechler B, Gachet C. Comparison of two murine models of thrombosis induced by atherosclerotic plaque injury. *Thromb. Haemost.* (2011) 105 (Suppl. 1):S3–12. doi: 10.1160/THS10-11-0730
94. Nergiz-Unal R, Cosemans JM, Feijge MA, van der Meijden PE, Storey RF, van Giezen JJ, et al. Stabilizing role of platelet P2Y₁₂ receptors in shear-dependent thrombus formation on ruptured plaques. *PLoS ONE*. (2010) 5:e10130. doi: 10.1371/journal.pone.0010130
95. Feigin VL, Norrving B, Mensah GA. Global burden of stroke. *Circ Res*. (2017) 120:439–48. doi: 10.1161/CIRCRESAHA.116.308413
96. Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, et al. Executive Summary: Heart Disease and Stroke Statistics—2016 Update. *Circulation*. (2016) 133:447–54. doi: 10.1161/CIR.0000000000000366
97. Gorelick PB, Wong KS, Bae H-J, Pandey DK. Large artery intracranial occlusive disease: a large worldwide burden but a relatively neglected frontier. *Stroke*. (2008) 39:2396–9. doi: 10.1161/STROKEAHA.107.505776
98. Banerjee C, Chimowitz MI. Stroke caused by atherosclerosis of the major intracranial arteries. *Circ Res*. (2017) 120:502–13. doi: 10.1161/CIRCRESAHA.116.308441
99. NINDS. Tissue plasminogen activator for acute ischemic stroke. *N Engl J Med*. (1995) 333:1581–7. doi: 10.1056/NEJM199512143332401
100. Broderick JP, Berkhemer OA, Palesch YY, Dippel DW, Foster LD, Roos YB, et al. Endovascular therapy is effective and safe for patients with severe ischemic stroke: pooled analysis of interventional management of stroke III and multicenter randomized clinical trial of endovascular therapy for acute ischemic stroke in the Netherlands data. *Stroke*. (2015) 46:3416–22. doi: 10.1161/STROKEAHA.115.011397
101. Ducroux C, Di Meglio L, Loyau S, Delbosc S, Boisseau W, Deschildre C, et al. Thrombus neutrophil extracellular traps content impair tPA-induced thrombolysis in acute ischemic stroke. *Stroke*. (2018) 49:754–7. doi: 10.1161/STROKEAHA.117.019896
102. Di Meglio L, Desilles JP, Mazighi M, Ho-Tin-Noé B. Thrombolysis-resistant intracranial clot. *Neurology*. (2018) 90:1075. doi: 10.1212/WNL.0000000000005645
103. Rha J-H, Saver JL. The impact of recanalization on ischemic stroke outcome: a meta-analysis. *Stroke*. (2007) 38:967–73. doi: 10.1161/01.STR.0000258112.14918.24
104. Alexandrov AV, Grotta JC. Arterial reocclusion in stroke patients treated with intravenous tissue plasminogen activator. *Neurology*. (2002) 59:862–7. doi: 10.1212/WNL.59.6.862
105. Rubiera M, Alvarez-Sabin J, Ribo M, Montaner J, Santamarina E, Arenillas JF, et al. Predictors of early arterial reocclusion after tissue plasminogen activator-induced recanalization in acute ischemic stroke. *Stroke*. (2005) 36:1452–6. doi: 10.1161/01.STR.0000170711.43405.81
106. Saqqur M, Molina CA, Salam A, Siddiqui M, Ribo M, Uchino K, et al. Clinical deterioration after intravenous recombinant tissue plasminogen activator treatment: a multicenter transcranial doppler study. *Stroke*. (2007) 38:69–74. doi: 10.1161/01.STR.0000251800.01964.f6
107. George T, Ho-Tin-Noe B, Carbo C, Benarafa C, Remold-O'Donnell E, Zhao BQ, et al. Inflammation induces hemorrhage in thrombocytopenia. *Blood*. (2008) 111:4958–64. doi: 10.1182/blood-2007-11-123620
108. Kleinschnitz C, Pozgajova M, Pham M, Bendszus M, Nieswandt B, Stoll G. Targeting platelets in acute experimental stroke: impact of glycoprotein Ib, VI, and IIb/IIIa blockade on infarct size, functional outcome, and intracranial bleeding. *Circulation*. (2007) 115:2323–30. doi: 10.1161/CIRCULATIONAHA.107.691279
109. Zhao BQ, Chauhan AK, Canault M, Patten IS, Yang JJ, Dockal M, et al. von Willebrand factor-cleaving protease ADAMTS13 reduces ischemic brain injury in experimental stroke. *Blood*. (2009) 114:3329–34. doi: 10.1182/blood-2009-03-213264
110. Goebel S, Li Z, Vogelmann J, Holthoff HP, Degen H, Hermann DM, et al. The GPVI-Fc fusion protein revacept improves cerebral infarct volume and functional outcome in stroke. *PLoS ONE*. (2013) 8:e66960. doi: 10.1371/journal.pone.0066960
111. Schuhmann MK, Kraft P, Bieber M, Kollikowski AM, Schulze H, Nieswandt B, et al. Targeting platelet GPVI Plus rt-PA administration but not $\alpha 2\beta 1$ -mediated collagen binding protects against ischemic brain damage in mice. *IJMS*. (2019) 20:2019. doi: 10.3390/ijms20082019
112. Dumont B, Lasne D, Rothschild C, Bouabdelli M, Ollivier V, Oudin C, et al. Absence of collagen-induced platelet activation caused by compound heterozygous GPVI mutations. *Blood*. (2009) 114:1900–3. doi: 10.1182/blood-2009-03-213504
113. Stegner D, Nieswandt B. Platelet receptor signaling in thrombus formation. *J Mol Med*. (2011) 89:109–21. doi: 10.1007/s00109-010-0691-5
114. Jiang P, Jandrot-Perrus M. New advances in treating thrombotic diseases: GPVI as a platelet drug target. *Drug Discov Today*. (2014) 19:1471–5. doi: 10.1016/j.drudis.2014.06.005
115. Kraft P, Schuhmann MK, Fluri F, Lorenz K, Zernecke A, Stoll G, et al. Efficacy and safety of platelet glycoprotein receptor blockade in aged and comorbid mice with acute experimental stroke. *Stroke*. (2015) 46:3502–6. doi: 10.1161/STROKEAHA.115.011114
116. Vinik AI, Erbas T, Park TS, Nolan R, Pittenger GL. Platelet dysfunction in type 2 diabetes. *Diabetes Care*. (2001) 24:1476–85. doi: 10.2337/diacare.24.8.1476
117. Standley PR, Ali S, Bapna C, Sowers JR. Increased platelet cytosolic calcium responses to low density lipoprotein in type II diabetes with and without hypertension. *Am J Hypertens*. (1993) 6:938–43. doi: 10.1093/ajh/6.11.938
118. Akai T, Naka K, Okuda K, Takemura T, Fujii S. Decreased sensitivity of platelets to prostacyclin in patients with diabetes mellitus. *Horm Metab Res*. (1983) 15:523–6. doi: 10.1055/s-2007-1018778
119. Grundy SM. Hypertriglyceridemia, atherogenic dyslipidemia, and the metabolic syndrome. *Am J Cardiol*. (1998) 81:18B–25B. doi: 10.1016/S0002-9149(98)00033-2
120. Tschöppe D, Roesen P, Kaufmann L, Schauseil S, Kehrel B, Ostermann H, et al. Evidence for abnormal platelet glycoprotein expression in diabetes mellitus. *Eur J Clin Invest*. (1990) 20:166–70. doi: 10.1111/j.1365-2362.1990.tb02264.x
121. Winocour PD, Bryszewska M, Watala C, Rand ML, Epand RM, Kinlough-Rathbone RL, et al. Reduced membrane fluidity in platelets from diabetic patients. *Diabetes*. (1990) 39:241–4. doi: 10.2337/diab.39.2.241
122. Strigrow F, Riek-Burchardt M, Kiesel A, Schmidt W, Henrich-Noack P, Breder J, et al. Four different types of protease-activated receptors are widely expressed in the brain and are up-regulated in hippocampus by severe ischemia. *Eur J Neurosci*. (2001) 14:595–608. doi: 10.1046/j.0953-816x.2001.01676.x
123. Mao Y, Zhang M, Tuma RF, Kunapuli SP. Deficiency of PAR4 attenuates cerebral ischemia/reperfusion injury in mice. *J Cereb Blood Flow Metab*. (2010) 30:1044–52. doi: 10.1038/jcbfm.2009.283
124. Devanathan V, Hagedorn I, Köhler D, Pexa K, Cherpokova D, Kraft P, et al. Platelet Gi protein G α 2 is an essential mediator of thrombo-inflammatory organ damage in mice. *Proc Natl Acad Sci USA*. (2015) 112:6491–6. doi: 10.1073/pnas.1505887112
125. Schuhmann MK, Kraft P, Bieber M, Haarmann A, Homola GA, Pham M, et al. Influence of thrombolysis on the safety and efficacy of blocking platelet adhesion or secretory activity in acute ischemic stroke in mice. *Transl Stroke Res*. (2018) 9:493–8. doi: 10.1007/s12975-017-0606-7
126. Schuhmann MK, Guthmann J, Stoll G, Nieswandt B, Kraft P, Kleinschnitz C. Blocking of platelet glycoprotein receptor Ib reduces “thrombo-inflammation” in mice with acute ischemic stroke. *J Neuroinflamm*. (2017) 14:18. doi: 10.1186/s12974-017-0792-y
127. Uchiyama S, Ishizuka N, Shimada K, Teramoto T, Yamazaki T, Oikawa S, et al. Aspirin for stroke prevention in elderly patients with vascular risk factors: Japanese primary prevention project. *Stroke*. (2016) 47:1605–11. doi: 10.1161/STROKEAHA.115.012461
128. Gaziano JM, Brotons C, Coppolecchia R, Cricelli C, Darius H, Gorelick PB, et al. Use of aspirin to reduce risk of initial vascular events in patients at moderate risk of cardiovascular disease (ARRIVE):

- a randomised, double-blind, placebo-controlled trial. *Lancet*. (2018) 392:1036–46. doi: 10.1016/S0140-6736(18)31924-X
129. McNeil JJ, Wolfe R, Woods RL, Tonkin AM, Donnan GA, Nelson MR, et al. Effect of aspirin on cardiovascular events and bleeding in the healthy elderly. *N Engl J Med*. (2018) 379:1509–18. doi: 10.1056/NEJMoa1803955
 130. Zinkstok SM, Roos YB, ARTIS Investigators. Early administration of aspirin in patients treated with alteplase for acute ischaemic stroke: a randomised controlled trial. *Lancet*. (2012) 380:731–7. doi: 10.1016/S0140-6736(12)60949-0
 131. Zinkstok SM, Beenen LF, Majoie CB, Marquering HA, de Haan RJ, Roos YB. Early deterioration after thrombolysis plus aspirin in acute stroke: a post hoc analysis of the antiplatelet therapy in combination with recombinant t-PA thrombolysis in ischemic stroke trial. *Stroke*. (2014) 45:3080–2. doi: 10.1161/STROKEAHA.114.006268
 132. CAPRIE Steering Committee. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). *Lancet*. (1996) 348:1329–39. doi: 10.1016/S0140-6736(96)09457-3
 133. Bhatt DL, Fox KA, Hacke W, Berger PB, Black HR, Boden WE, et al. Clopidogrel and aspirin versus aspirin alone for the prevention of atherothrombotic events. *N Engl J Med*. (2006) 354:1706–17. doi: 10.1056/NEJMoa060989
 134. Diener HC, Bogousslavsky J, Brass LM, Cimminiello C, Csiba L, Kaste M, et al. Aspirin and clopidogrel compared with clopidogrel alone after recent ischaemic stroke or transient ischaemic attack in high-risk patients (MATCH): randomised, double-blind, placebo-controlled trial. *Lancet*. (2004) 364:331–7. doi: 10.1016/S0140-6736(04)16721-4
 135. Wang Y, Wang Y, Zhao X, Liu L, Wang D, Wang C, et al. Clopidogrel with aspirin in acute minor stroke or transient ischemic attack. *N Engl J Med*. (2013) 369:11–9. doi: 10.1056/NEJMoa1215340
 136. Wang Y, Pan Y, Zhao X, Li H, Wang D, Johnston SC, et al. Clopidogrel with aspirin in acute minor stroke or transient ischemic attack (CHANCE) trial: one-year outcomes. *Circulation*. (2015) 132:40–6. doi: 10.1161/CIRCULATIONAHA.114.014791
 137. Hong KS, Lee SH, Kim EG, Cho KH, Chang DI, Rha JH, et al. Recurrent ischemic lesions after acute atherothrombotic stroke: clopidogrel plus aspirin versus aspirin alone. *Stroke*. (2016) 47:2323–30. doi: 10.1161/STROKEAHA.115.012293
 138. Johnston SC, Easton SC, Farrant M, Barsan W, Conwit RA, Elm JJ, et al. Clopidogrel and aspirin in acute ischemic stroke and high-risk TIA. *N Engl J Med*. (2018) 379:215–25. doi: 10.1056/NEJMoa1800410
 139. Tillman H, Johnston SC, Farrant M, Barsan W, Elm JJ, Kim AS, et al. Risk for major hemorrhages in patients receiving clopidogrel and aspirin compared with aspirin alone after transient ischemic attack or minor ischemic stroke: a secondary analysis of the POINT randomized clinical trial. *JAMA Neurol*. (2019) 76:774–82. doi: 10.1001/jamaneurol.2019.0932
 140. Johnston SC, Amarenco P, Albers GW, Denison H, Easton JD, Evans SR, et al. Ticagrelor versus aspirin in acute stroke or transient ischemic attack. *N Engl J Med*. (2016) 375:35–43. doi: 10.1056/NEJMoa1603060
 141. Amarenco P, Albers GW, Denison H, Easton JD, Evans SR, Held P, et al. Efficacy and safety of ticagrelor versus aspirin in acute stroke or transient ischaemic attack of atherosclerotic origin: a subgroup analysis of SOCRATES, a randomised, double-blind, controlled trial. *Lancet Neurol*. (2017) 16:301–10. doi: 10.1016/S1474-4422(17)30038-8
 142. Wang Y, Lin Y, Meng X, Chen W, Chen G, Wang Z, et al. Effect of ticagrelor with clopidogrel on high on-treatment platelet reactivity in acute stroke or transient ischemic attack (PRINCE) trial: rationale and design. *Int J Stroke*. (2017) 12:321–5. doi: 10.1177/1747493017694390
 143. Wang Y, Johnston SC, Bath PM, Grotta JC, Pan Y, Amarenco P, et al. Acute dual antiplatelet therapy for minor ischaemic stroke or transient ischaemic attack. *BMJ*. (2019) 364:l895. doi: 10.1136/bmj.l895
 144. Bath PM, Woodhouse LJ, Appleton JP, Beridze M, Christensen H, Dineen RA, et al. Antiplatelet therapy with aspirin, clopidogrel, and dipyridamole versus clopidogrel alone or aspirin and dipyridamole in patients with acute cerebral ischaemia (TARDIS): a randomised, open-label, phase 3 superiority trial. *Lancet*. (2018) 391:850–9. doi: 10.1016/S0140-6736(17)32849-0
 145. Adams HP, Effron MB, Torner J, Dávalos A, Frayne J, Teal P, et al. Emergency administration of abciximab for treatment of patients with acute ischemic stroke: results of an international phase III trial: abciximab in emergency treatment of stroke trial (AbESTT-II). *Stroke*. (2008) 39:87–99. doi: 10.1161/STROKEAHA.106.476648
 146. Siebler M, Hennerici MG, Schneider D, von Reutern GM, Seitz RJ, Röther J, et al. Safety of tirofiban in acute ischemic stroke: the SaTIS trial. *Stroke*. (2011) 42:2388–92. doi: 10.1161/STROKEAHA.110.599662
 147. Li L, Geraghty OC, Mehta Z, Rothwell PM. Age-specific risks, severity, time course, and outcome of bleeding on long-term antiplatelet treatment after vascular events: a population-based cohort study. *Lancet*. (2017) 390:490–9. doi: 10.1016/S0140-6736(17)30770-5
 148. Antithrombotic Trialists (ATT) Collaboration, Baigent C, Blackwell L, Collins R, Emberson J, Godwin J, et al. Aspirin in the primary and secondary prevention of vascular disease: collaborative meta-analysis of individual participant data from randomised trials. *Lancet*. (2009) 373:1849–60. doi: 10.1016/S0140-6736(09)60503-1
 149. Antithrombotic Trialists (ATT) Collaboration. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. *BMJ*. (2002) 324:71–86. doi: 10.1136/bmj.324.7329.71
 150. Gorelick PB, Weisman SM. Risk of hemorrhagic stroke with aspirin use: an update. *Stroke*. (2005) 36:1801–7. doi: 10.1161/01.STR.0000174189.81153.85
 151. Sandercock PA, Counsell C, Tseng M-C, Cecconi E. Oral antiplatelet therapy for acute ischaemic stroke. *Cochrane Database Syst Rev*. (2014) 26:CD000029. doi: 10.1002/14651858.CD000029.pub3
 152. Powers WJ, Rabinstein AA, Ackerson T, Adeoye OM, Bambakidis NC, Becker K, et al. 2018 Guidelines for the early management of patients with acute ischemic stroke: a guideline for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke*. (2018) 49:e46–99. doi: 10.1161/STR.0000000000000172
 153. Reed GW, Rossi JE, Cannon CP. Acute myocardial infarction. *Lancet*. (2017) 389:197–210. doi: 10.1016/S0140-6736(16)30677-8
 154. Yeh RW, Sidney S, Chandra M, Sorel M, Selby JV, Go AS. Population trends in the incidence and outcomes of acute myocardial infarction. *N Engl J Med*. (2010) 362:2155–65. doi: 10.1056/NEJMoa0908610
 155. Roffi M, Patrono C, Collet JP, Mueller C, Valgimigli M, Andreotti F, et al. 2015 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: Task Force for the Management of Acute Coronary Syndromes in Patients Presenting without Persistent ST-Segment Elevation of the European Society of Cardiology (ESC). *Eur Heart J*. (2016) 37:267–315. doi: 10.1093/eurheartj/ehv320
 156. Ibanez B, James S, Agewall S, Antunes MJ, Bucciarelli-Ducci C, Bueno H, et al. 2017 ESC Guidelines for the management of acute myocardial infarction in patients presenting with ST-segment elevation. *Eur Heart J*. (2018) 39:119–77. doi: 10.5603/KP.2018.0041
 157. Ndrepepa G, Colleran R, Kastrati A. Reperfusion injury in ST-segment elevation myocardial infarction: the final frontier. *Coronary Artery Dis*. (2017) 28:253–62. doi: 10.1097/MCA.0000000000000468
 158. Amraotkar AR, Song DD, Otero D, Trainor PJ, Ismail I, Kothari V, et al. Platelet count and mean platelet volume at the time of and after acute myocardial infarction. *Clin Appl Thromb/Hemostasis*. (2017) 23:1052–9. doi: 10.1177/1076029616683804
 159. Yaghoubi A, Golmohamadi Z, Alizadehasl A, Azarfarin R. Role of platelet parameters and haematological indices in myocardial infarction and unstable angina. *J Pak Med Assoc*. (2013) 63:5.
 160. van der Zee PM, Biró E, Ko Y, de Winter RJ, Hack CE, Sturk A, et al. P-selectin- and CD63-exposing platelet microparticles reflect platelet activation in peripheral arterial disease and myocardial infarction. *Clin Chem*. (2006) 52:657–64. doi: 10.1373/clinchem.2005.057414
 161. Goto S, Sakai H, Goto M, Ono M, Ikeda Y, Handa S, et al. Enhanced shear-induced platelet aggregation in acute myocardial infarction. *Circulation*. (1999) 99:608–13. doi: 10.1161/01.CIR.99.5.608
 162. Mauler M, Herr N, Schoenichen C, Witsch T, Marchini T, Härdtner C, et al. Platelet serotonin aggravates myocardial ischemia/reperfusion injury via neutrophil degranulation. *Circulation*. (2019) 139:918–31. doi: 10.1161/CIRCULATIONAHA.118.033942
 163. Furman MI, Barnard MR, Krueger LA, Fox ML, Shilale EA, Lessard DM, et al. Circulating monocyte-platelet aggregates are an early marker

- of acute myocardial infarction. *J Am Coll Cardiol.* (2001) 38:1002–6. doi: 10.1016/S0735-1097(01)01485-1
164. Ren F, Mu N, Zhang X, Tan J, Li L, Zhang C, et al. Increased platelet-leukocyte aggregates are associated with myocardial no-reflow in patients with ST elevation myocardial infarction. *Am J Med Sci.* (2016) 352:261–6. doi: 10.1016/j.amjms.2016.05.034
 165. Du XJ, Shan L, Gao XM, Kiriazis H, Liu Y, Lobo A, et al. Role of intramural platelet thrombus in the pathogenesis of wall rupture and intra-ventricular thrombosis following acute myocardial infarction. *Thromb Haemost.* (2011) 105:356–64. doi: 10.1160/TH10-07-0449
 166. Liu Y, Gao XM, Fang L, Jennings NL, Su Y, Q X, et al. Novel role of platelets in mediating inflammatory responses and ventricular rupture or remodeling following myocardial infarction. *Arterioscler Thromb Vasc Biol.* (2011) 31:834–41. doi: 10.1161/ATVBAHA.110.220467
 167. Pachel C, Mathes D, Arias-Loza AP, Heitzmann W, Nordbeck P, Deppermann C, et al. Inhibition of platelet GPVI protects against myocardial ischemia-reperfusion injury. *Arterioscler Thromb Vasc Biol.* (2016) 36:629–35. doi: 10.1161/ATVBAHA.115.305873
 168. Takaya N, Katoh Y, Iwabuchi K, Hayashi I, Konishi H, Itoh S, et al. Platelets activated by collagen through the immunoreceptor tyrosine-based activation motif in the Fc receptor γ -chain play a pivotal role in the development of myocardial ischemia-reperfusion injury. *J Mol Cell Cardiol.* (2005) 39:856–64. doi: 10.1016/j.yjmcc.2005.07.006
 169. Schönberger T, Ziegler M, Borst O, Konrad I, Nieswandt B, Massberg S, et al. The dimeric platelet collagen receptor GPVI-Fc reduces platelet adhesion to activated endothelium and preserves myocardial function after transient ischemia in mice. *Am J Physiol Cell Physiol.* (2012) 303:C757–66. doi: 10.1152/ajpcell.00060.2012
 170. Vélez P, Ocaranza-Sánchez R, López-Otero D, Grigorian-Shamagian L, Rosa I, Guitián E, et al. Alteration of platelet GPVI signaling in ST-elevation myocardial infarction patients demonstrated by a combination of proteomic, biochemical, and functional approaches. *Sci Rep.* (2016) 6:39603. doi: 10.1038/srep39603
 171. Bigalke B, Haap M, Stellos K, Geisler T, Seizer P, Kremmer E, et al. Platelet glycoprotein VI (GPVI) for early identification of acute coronary syndrome in patients with chest pain. *Thromb Res.* (2010) 125:e184–9. doi: 10.1016/j.thromres.2010.01.005
 172. Chien PT-Y, Hsieh H-L, Chi P-L, Yang C-M. PAR1-dependent COX-2/PGE₂ production contributes to cell proliferation via EP₂ receptors in primary human cardiomyocytes: thrombin induces COX-2 expression. *Br J Pharmacol.* (2014) 171:4504–19. doi: 10.1111/bph.12794
 173. Sabri A, Guo J, Elouardighi H, Darrow AL, Andrade-Gordon P, Steinberg SF. Mechanisms of protease-activated receptor-4 Actions in cardiomyocytes: role of Src tyrosine kinase. *J Biol Chem.* (2003) 278:11714–20. doi: 10.1074/jbc.M213091200
 174. Pawlinski R, Tencati M, Hampton CR, Shishido T, Bullard TA, Casey LM, et al. Protease-activated receptor-1 contributes to cardiac remodeling and hypertrophy. *Circulation.* (2007) 116:2298–306. doi: 10.1161/CIRCULATIONAHA.107.692764
 175. Strande JL, Hsu A, Su J, Fu X, Gross GJ, Baker JE. SCH 79797, a selective PAR1 antagonist, limits myocardial ischemia/reperfusion injury in rat hearts. *Basic Res Cardiol.* (2007) 102:350–8. doi: 10.1007/s00395-007-0653-4
 176. Antoniuk S, Pawlinski R, Mackman N. Protease-activated receptors and myocardial infarction. *IUBMB Life.* (2011) 63:383–9. doi: 10.1002/iub.441
 177. Kolpakov MA, Rafiq K, Guo X, Hooshdaran B, Wang T, Vlasenko L, et al. Protease-activated receptor 4 deficiency offers cardioprotection after acute ischemia reperfusion injury. *J Mol Cell Cardiol.* (2016) 90:21–9. doi: 10.1016/j.yjmcc.2015.11.030
 178. Strande JL, Hsu A, Su J, Fu X, Gross GJ, Baker JE. Inhibiting protease-activated receptor 4 limits myocardial ischemia/reperfusion injury in rat hearts by unmasking adenosine signaling. *J Pharmacol Exp Therapeut.* (2008) 324:1045–54. doi: 10.1124/jpet.107.133595
 179. Barrabés JA, Inserre J, Mirabet M, Quiroga A, Hernando V, Figueras J, et al. Antagonism of P2Y₁₂ or GPIIb/IIIa receptors reduces platelet-mediated myocardial injury after ischemia and reperfusion in isolated rat hearts. *Thromb Haemost.* (2010) 104:128–35. doi: 10.1160/TH09-07-0440
 180. Adamek A, Hu K, Bayer B, Wagner H, Ertl G, Bauersachs J, et al. High dose aspirin and left ventricular remodeling after myocardial infarction: aspirin and myocardial infarction. *Basic Res Cardiol.* (2007) 102:334–40. doi: 10.1007/s00395-007-0647-2
 181. Xu XR, Carrim N, Neves MA, McKeown T, Stratton TW, Coelho RM, et al. Platelets and platelet adhesion molecules: novel mechanisms of thrombosis and anti-thrombotic therapies. *Thromb J.* (2016) 14:29. doi: 10.1186/s12959-016-0100-6
 182. Hohlfeld T, Braun M, Strobach H, Schrör K. Protection of reperfused ischemic pig myocardium by nexopamil, a new combined Ca²⁺ and serotonin antagonist. *J Cardiovasc Pharmacol.* (1994) 23:922–31. doi: 10.1097/00005344-199406000-00010
 183. Oostingh GJ, Pozgajova M, Ludwig RJ, Krahn T, Boehncke WH, Nieswandt B, et al. Diminished thrombus formation and alleviation of myocardial infarction and reperfusion injury through antibody- or small-molecule-mediated inhibition of selectin-dependent platelet functions. *Haematologica.* (2007) 92:502–12. doi: 10.3324/haematol.10741
 184. The CURE Trial Investigators. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST segment elevation. *BMJ.* (2007) 334:1265–9. doi: 10.1136/bmj.39220.618646.AE
 185. Wiviott SD, Braunwald E, McCabe CH, Montalescot G, Ruzyllo W, Gottlieb S, et al. Prasugrel versus clopidogrel in patients with acute coronary syndromes. *N Engl J Med.* (2007) 357:2001–15. doi: 10.1056/NEJMoa0706482
 186. Wallentin L, Becker RC, Budaj A, Cannon CP, Emanuelsson H, Held C, et al. Ticagrelor versus clopidogrel in patients with acute coronary syndromes. *N Engl J Med.* (2009) 361:1045–57. doi: 10.1056/NEJMoa0904327
 187. Bonaca MP, Bhatt DL, Cohen M, Steg PG, Storey RF, Jensen EC, et al. Long-term use of ticagrelor in patients with prior myocardial infarction. *N Engl J Med.* (2015) 372:1791–800. doi: 10.1056/NEJMoa1500857
 188. Bonaca MP, Wiviott SD, Morrow DA, Steg PG, Hamm C, Bhatt DL, et al. Reduction in subtypes and sizes of myocardial infarction with ticagrelor in PEGASUS-TIMI 54. *J Am Heart Assoc.* (2018) 7:e009260. doi: 10.1161/JAHA.118.009260
 189. Bonaca MP, Storey RF, Theroux P, Steg PG, Bhatt DL, Cohen MC, et al. Efficacy and safety of ticagrelor over time in patients with prior MI in PEGASUS-TIMI 54. *J Am Coll Cardiol.* (2017) 70:1368–75. doi: 10.1016/j.jacc.2017.07.768
 190. Morrow DA, Braunwald E, Bonaca MP, Ameriso SF, Dalby AJ, Fish MP, et al. Vorapaxar in the secondary prevention of atherothrombotic events. *N Engl J Med.* (2012) 366:1404–13. doi: 10.1056/NEJMoa1200933
 191. Leonardi S, Tricoci P, White HD, Armstrong PW, Huang Z, Wallentin L, et al. Effect of vorapaxar on myocardial infarction in the thrombin receptor antagonist for clinical event reduction in acute coronary syndrome (TRA.CER) trial. *Eur Heart J.* (2013) 34:1723–31. doi: 10.1093/eurheartj/ehd104
 192. Eckstein HH, Böckler D, Flessenkämper I, Schmitz-Rixen T, Debus S, Lang W. Ultrasonographic screening for the detection of abdominal aortic aneurysms. *Dtsch Arztebl Int.* (2009) 106:657–63. doi: 10.3238/arztebl.2009.0657
 193. Wang Y-D, Liu Z-J, Ren J, Xiang M-X. Pharmacological therapy of abdominal aortic aneurysm: an update. *Curr Vasc Pharmacol.* (2018) 16:114–24. doi: 10.2174/1570161115666170413145705
 194. Cameron SJ, Russell HM, Owens AP. Antithrombotic therapy in abdominal aortic aneurysm: beneficial or detrimental? *Blood.* (2018) 132:2619–28. doi: 10.1182/blood-2017-08-743237
 195. Kazi M, Thyberg J, Religa P, Roy J, Eriksson P, Hedin U, et al. Influence of intraluminal thrombus on structural and cellular composition of abdominal aortic aneurysm wall. *J Vasc Surg.* (2003) 38:1283–92. doi: 10.1016/S0741-5214(03)00791-2
 196. Behr-Rasmussen C, Grøndal N, Bramsen MB, Thomsen MD, Lindholt JS. Mural thrombus and the progression of abdominal aortic aneurysms: a large population-based prospective cohort study. *Eur J Vasc Endovasc Surg.* (2014) 48:301–7. doi: 10.1016/j.ejvs.2014.05.014
 197. Touat Z, Ollivier V, Dai J, Huisse MG, Bezeaud A, Sebbag U, et al. Renewal of mural thrombus releases plasma markers and is involved in aortic abdominal aneurysm evolution. *Am J Pathol.* (2006) 168:1022–30. doi: 10.2353/ajpath.2006.050868
 198. Delbosc S, Alsac JM, Journe C, Louedec L, Castier Y, Bonnaure-Mallet M, et al. *Porphyromonas gingivalis* participates in pathogenesis

- of human abdominal aortic aneurysm by neutrophil activation. Proof of concept in rats. *PLoS ONE*. (2011) 6:e18679. doi: 10.1371/journal.pone.0018679
199. Adolph R, Vorp DA, Steed DL, Webster MW, Kameneva MV, Watkins SC. Cellular content and permeability of intraluminal thrombus in abdominal aortic aneurysm. *J Vasc Surg*. (1997) 25:916–26. doi: 10.1016/S0741-5214(97)70223-4
 200. Michel JB, Martin-Ventura JL, Egido J, Sakalihasan N, Treska V, Lindholt J, et al. Novel aspects of the pathogenesis of aneurysms of the abdominal aorta in humans. *Cardiovasc Res*. (2011) 90:18–27. doi: 10.1093/cvr/cvq337
 201. Khan JA, Abdul Rahman MN, Mazari FA, Shahin Y, Smith G, Madden L, et al. Intraluminal thrombus has a selective influence on matrix metalloproteinases and their inhibitors (tissue inhibitors of matrix metalloproteinases) in the wall of abdominal aortic aneurysms. *Ann Vasc Surg*. (2012) 26:322–9. doi: 10.1016/j.avsg.2011.08.015
 202. Fontaine V, Touat Z, Mtairag el M, Vranckx R, Louedec L, Houard X, et al. Role of leukocyte elastase in preventing cellular re-colonization of the mural thrombus. *Am J Pathol*. (2004) 164:2077–87. doi: 10.1016/S0002-9440(10)63766-2
 203. Swedenborg J, Eriksson P. The intraluminal thrombus as a source of proteolytic activity. *Ann N Y Acad Sci*. (2006) 1085:133–8. doi: 10.1196/annals.1383.044
 204. Saraff K, Babamusta F, Cassis LA, Daugherty A. Aortic dissection precedes formation of aneurysms and atherosclerosis in angiotensin II-infused, apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol*. (2003) 23:1621–6. doi: 10.1161/01.ATV.0000085631.76095.64
 205. Anidjar S, Salzmänn JL, Gentric D, Lagneau P, Camilleri JP, Michel JB. Elastase-induced experimental aneurysms in rats. *Circulation*. (1990) 82:973–81. doi: 10.1161/01.CIR.82.3.973
 206. Pyo R, Lee JK, Shipley JM, Curci JA, Mao D, Ziporin SJ, et al. Targeted gene disruption of matrix metalloproteinase-9 (gelatinase B) suppresses development of experimental abdominal aortic aneurysms. *J Clin Invest*. (2000) 105:1641–9. doi: 10.1172/JCI8931
 207. Lu G, Su G, Davis JP, Schaheen B, Downs E, Roy RJ, et al. A novel chronic advanced stage abdominal aortic aneurysm murine model. *J Vasc Surg*. (2017) 66:232–42.e4. doi: 10.1016/j.jvs.2016.07.105
 208. Allaire E, Muscatelli-Groux B, Guinault AM, Pages C, Goussard A, Mandet C, et al. Vascular smooth muscle cell endovascular therapy stabilizes already developed aneurysms in a model of aortic injury elicited by inflammation and proteolysis. *Ann Surg*. (2004) 239:417–27. doi: 10.1097/01.sla.00000114131.79899.82
 209. Dai J, Louedec L, Philippe M, Michel JB, Houard X. Effect of blocking platelet activation with AZD6140 on development of abdominal aortic aneurysm in a rat aneurysmal model. *J Vasc Surg*. (2009) 49:719–27. doi: 10.1016/j.jvs.2008.09.057
 210. Liu O, Jia L, Liu X, Wang Y, Wang X, Qin Y, et al. Clopidogrel, a platelet P2Y12 receptor inhibitor, reduces vascular inflammation and angiotensin II induced-abdominal aortic aneurysm progression. *PLoS ONE*. (2012) 7:e51707. doi: 10.1371/journal.pone.0051707
 211. Owens AP, Edwards TL, Antoniak S, Geddings JE, Jahangir E, Wei WQ, et al. Platelet inhibitors reduce rupture in a mouse model of established abdominal aortic aneurysm. *Arterioscler Thromb Vasc Biol*. (2015) 35:2032–41. doi: 10.1161/ATVBAHA.115.305537
 212. Lindholt JS, Sørensen HT, Michel JB, Thomsen HF, Henneberg EW. Low-dose aspirin may prevent growth and later surgical repair of medium-sized abdominal aortic aneurysms. *Vasc Endovasc Surg*. (2008) 42:329–34. doi: 10.1177/1538574408315205
 213. Wanhainen A, Mani K, Kullberg J, Svensjö S, Bersztel A, Karlsson L, et al. The effect of ticagrelor on growth of small abdominal aortic aneurysms—a randomized controlled trial. *Cardiovasc. Res*. (2019) 2019:cvz133. doi: 10.1093/cvr/cvz133

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Lebas, Yahiaoui, Martos and Boulaftali. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Advances in Platelet Subpopulation Research

Gabriela Lesyk^{1,2} and Paul Jurasz^{1,2,3,4*}

¹ Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, AB, Canada, ² Cardiovascular Research Centre, University of Alberta, Edmonton, AB, Canada, ³ Department of Pharmacology, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, AB, Canada, ⁴ Mazankowski Alberta Heart Institute, University of Alberta, Edmonton, AB, Canada

OPEN ACCESS

Edited by:

Christian Schulz,
Ludwig Maximilian University of
Munich, Germany

Reviewed by:

George L. Dale,
University of Oklahoma Health
Sciences Center, United States
Benoît Ho-Tin-Noé,
Institut National de la Santé et de la
Recherche Médicale (INSERM), France

*Correspondence:

Paul Jurasz
jurasz@ualberta.ca

Specialty section:

This article was submitted to
Atherosclerosis and Vascular
Medicine,
a section of the journal
Frontiers in Cardiovascular Medicine

Received: 24 July 2019

Accepted: 29 August 2019

Published: 13 September 2019

Citation:

Lesyk G and Jurasz P (2019)
Advances in Platelet
Subpopulation Research.
Front. Cardiovasc. Med. 6:138.
doi: 10.3389/fcvm.2019.00138

Although lacking a nucleus, platelets are increasingly recognized not only for their complexity, but also for their diversity. Some 50 years ago platelet subpopulations were characterized by size and density, and these characteristics were thought to reflect platelet aging. Since, our knowledge of platelet heterogeneity has grown to recognize that differences in platelet biochemistry and function exist. This includes the identification of vanguard and follower platelets, platelets with differing procoagulant ability including “COAT-platelets” which enhance procoagulant protein retention on their surface, and most recently, the identification of platelet subpopulations with a differential ability to generate and respond to nitric oxide. Hence, in this mini-review, we summarize the current knowledge of platelet subpopulation diversity focusing on their physical, biochemical, and functional heterogeneity. In addition, we review how platelet subpopulations may change between health and disease and how differences among platelets may influence response to anti-platelet therapy. Finally, we look forward and discuss some of the future directions and challenges for this growing field of platelet research.

Keywords: platelet subpopulations, vanguard and follower platelets, COAT-platelets, eNOS-based platelet subpopulations, thrombosis, hemostasis

INTRODUCTION

Compared to leukocytes, which exist as functionally distinct subpopulations, platelets have often been considered simple. This general apathy for platelet complexity may stem from the fact that platelets lack nuclei and because they are so widely recognized for their roles in hemostasis and thrombosis. However, increasingly platelets are recognized for their biochemical and functional complexity despite being anucleate. Platelet proteome and transcriptome studies have revealed that platelets contain nearly 4,000 different proteins (1, 2) and an abundance of mRNA (3, 4) and miRNA (5–7). It's also appreciated within the field that platelets play diverse roles beyond hemostasis including contributing to wound healing, angiogenesis, and immunity and inflammation (8–11). Pathophysiologically, they are also recognized to contribute to cancer metastasis (12, 13), as well as to various inflammatory disorders (14, 15). To fulfill these diverse roles the question arises whether there is predetermined biochemical diversity and functional heterogeneity among platelets? That is, do platelets subpopulations with predetermined specialized functions exist?

HISTORICAL PERSPECTIVES

Initial studies into platelet subpopulations occurred 50 years ago and focused on characterizing human platelet subpopulations based on size and buoyant density and largely isolated these subpopulations by density gradient centrifugation. Studies by Karpatkin (16) showed that large, dense platelets have greater glycogen, orthophosphate and adenine nucleotide content than smaller less dense platelets. The biochemical processes of glycolysis, glycogenolysis, and protein synthesis were reported to be greater in large-dense platelets but no differences were observed in lipid content or synthesis. Large-dense platelets were shown to aggregate more than small-light platelets largely due to greater ADP release and lower ADPase activity (17). Subsequently, large-dense platelets were shown to adhere to collagen at a faster rate and this was thought to reflect more surface GPIa/IIa (integrin $\alpha_2\beta_1$) on this subpopulation (18). Electron microscopy examination of light platelets demonstrated lower granule content than that of dense platelets, but not fewer mitochondria (19). Although, currently it's recognized that an anuclear programmed cell death limits platelet lifespan (20, 21), the biochemical, ultrastructural, and functional differences among platelets with differing size and density were thought to reflect platelet aging with large-heavy platelets representing young platelets recently released into the circulation while light-small platelets represented older platelets that have circulated for a number of days. Using rabbit platelets, Rand et al. (22) demonstrated that the least dense platelets contained less sialic acid than the densest. They suggested loss of surface sialic acid from the least dense platelets was a mechanism by which old platelets are recognized and removed from the circulation.

Studies by Penington et al. (23, 24) argued that in fact megakaryocyte heterogeneity was responsible for platelet heterogeneity and that platelet density does not change with aging. Based on morphometric studies, they contended that the three-ploidy classes of megakaryocytes (8n, 16n, 32n) differ in their organelle content concentration and relate to the density of their platelet progeny. Large-dense platelets would be expected to arise from 8n megakaryocytes with greater granule content, while small-light platelets arise from 32n megakaryocytes. This view was more in line with that of Paulus that thrombopoiesis is likely responsible for platelet heterogeneity and not aging in circulation, but that only a single platelet population exists and the only size heterogeneity is that inherent to the log normally distributed population (25). Consistent with this thesis, other studies demonstrated that in the steady state platelet density does not change with circulatory age (26, 27), while others still showed that platelet density may increase with time due to accumulation of 5-hydroxytryptamine (5-HT) (28).

In addition to categorizing platelets into subpopulations based on size and density, platelets were also separated into subpopulations based on volume using counterflow centrifugation. Platelets with larger volume were shown to have more rapid and complete aggregation, and to be slightly denser (29). These studies argued that fundamentally platelets of different volume have similar function but the absolute abilities to aggregate and secrete granular contents correlates

with their volume (30). Further, it was argued that platelet size heterogeneity likely results from their production from megakaryocytes and that this influences platelet function independently of time within circulation (30, 31).

Several groups attempted to unify the hypotheses that both megakaryocyte heterogeneity and platelet time in circulation may contribute to platelet heterogeneity and the formation of size and density-based subpopulations (32–34). However, it's also important to note that some discrepancies in characterizing size and density-based platelet subpopulations may have occurred due to species differences. It appears rabbit platelets decrease in density with circulatory age, while in humans high-density platelets may be enriched with those that may have circulated longer and accumulated more 5-HT (28). Other characterization discrepancies may have also occurred due to methodology, and theoretical aspects of density separation need to be considered when interpreting early platelet subpopulation studies. As explained by Martin and Trowbridge if centrifugation is stopped before equilibrium is reached platelets may be separated based on a mixture of volume and density variation (35). This problem appears to be greater for discontinuous vs. continuous gradients as a greater time is needed to reach equilibrium necessary for separation of density-based subpopulations. Further, it has been suggested that platelets held at equilibrium between the opposing forces of the gravitational field and medium buoyancy may cause trauma to the platelet potentially causing secretion of granular contents; thus, potentially changing the characteristics of the isolated subpopulations (35).

CHARACTERIZATION OF PLATELET SUBPOPULATIONS BASED ON DIFFERENTIAL FUNCTION AND BIOCHEMISTRY

In addition to being characterized by size and density, platelet subpopulations have also been characterized based on function. Using a biotinylation technique to label dog platelets *in vivo*, it was demonstrated that reactivity to thrombin declines with platelet circulatory age (36). Similarly, utilizing ^{35}S -labeled rabbit platelets Hirsh et al. (37) demonstrated that younger platelets with lower ^{35}S -specific activity adhered to collagen fibers more readily than older platelets. Alike, two human platelet subpopulations were shown to have different collagen adhering kinetics, with 20% of platelets adhering within 1 min and with a second larger subpopulation accounting for 80% of platelets adhering more slowly between 1 and 60 min (38). The biochemical basis of this finding was unknown; however, several explanations were proposed. The one favored was the existence of functionally discrete subpopulations due to the sharp discontinuity between the two adhesion phases and the simple kinetic behavior of the second phase. Using time-lapse videomicroscopy and a microchamber model, Patel et al. (39) defined platelets that first adhered and spread on collagen as “vanguard” platelets and those that subsequently tether to and spread on vanguard platelets or nearby collagen as “followers.” Whether vanguard and follower platelets represent functionally

and biochemically distinct platelets was not investigated, but analysis of platelet deposition showed that adhesion events occurred randomly.

In addition to characterizing based on function, some studies characterized platelet subpopulations based on biochemical differences including the ability to bind adenosine and release 5-HT (40), as well as the presence or absence of acid phosphatase although no functional differences were observed (41). However, biochemical comparison of low vs. high density human platelets by Opper et al. (42) revealed that low density platelets have an enhanced intracellular Ca^{2+} response to thrombin, increased ADP-ribosylation of the inhibitory G-protein ($\text{G}_{i\alpha 1-3}$) and rho A, and decreased ADP-ribosylation of the stimulatory G-protein ($\text{G}_{s\alpha}$), as well as lower levels of the phosphorylated form of vasodilator-stimulated protein compared to high density platelets. These biochemical differences likely further explain the authors' findings that low density platelets demonstrate enhanced aggregation in response to thrombin, weaker inhibitory effects and a smaller rise in cGMP to the nitric oxide (NO) donor sodium nitroprusside, and a smaller increase in cAMP in response to prostaglandin E1 compared to high density platelets (43, 44). The authors proposed that the biochemical basis of the functional heterogeneity between low and high density platelets depends on differences in their G- and phospho-protein signaling within stimulatory and inhibitory signaling pathways (42). Others suggested that the increased reactivity of low density platelets may be attributed in part due to elevated α -granule content (45), although another study suggested α -granule content is greater in high density platelets (46).

Similar to the findings of Opper et al. (42), which suggested differences in NO-signaling between low and high density platelet subpopulations, recently we identified human platelet subpopulations based on the presence or absence of endothelial nitric oxide synthase (eNOS-positive and -negative platelets) and the differential ability to produce NO (47). We showed that eNOS-negative platelets fail to produce NO, have a down-regulated soluble guanylate cyclase-protein kinase G-vasodilator-stimulated protein (sGC-PKG-VASP) signaling

pathway, primarily initiate adhesion to collagen, more readily activate integrin $\alpha_{\text{IIb}}\beta_3$, and secrete more of the platelet activating protease matrix metalloproteinase-2 (MMP-2) than their eNOS-positive counterparts. eNOS-positive platelets have an intact eNOS-sGC-PKG-VASP signaling pathway, are more abundant (~80% of total platelets) and form the bulk of an aggregate via greater cyclooxygenase-1-mediated signaling. However, eNOS-positive platelets ultimately limit aggregate size via NO generation. It appears that eNOS-based platelet subpopulations are of the same circulatory age as the levels of activated caspase-3, the downstream effector of the platelet internal apoptotic clock (21), within them are equal. Moreover, eNOS-negative and -positive platelet volume was found to be equal also (47).

Based on these data, we proposed a novel model of thrombosis and hemostasis called the seed platelet hypothesis, in which eNOS-negative platelets initiate adhesion and aggregation reactions (Figure 1) (47). This initial response arises from their enhanced adhesiveness and reactivity due to an absence of endogenous NO generation (48, 49). Decreased sGC-PKG signaling within eNOS-negative platelets also facilitates refractoriness to endothelial-derived NO and increases integrin $\alpha_{\text{IIb}}\beta_3$ activation (50, 51), which stabilizes initial rolling and adhesion (52). We further proposed that enhanced MMP-2 secretion by eNOS-negative platelets promotes eNOS-negative platelet activation and recruitment of eNOS-positive platelets to the forming aggregate (53–55). eNOS-positive platelets then form the bulk of an aggregate/thrombus due to their higher COX-1 content and greater thromboxane A_2 generation. However, eNOS-positive platelets ultimately limit aggregate/thrombus size via NO generation as both increasing the ratio of eNOS^{neg} to eNOS^{pos} platelets and pharmacologically inhibiting eNOS enhances aggregation.

The differential ability of platelets to generate NO has been shown by others (56). However, how eNOS-based platelet subpopulations and the seed-platelet model of thrombosis and hemostasis relate to other models, such as a core and shell model of hemostatic plug formation by Stalker et al. (57), still

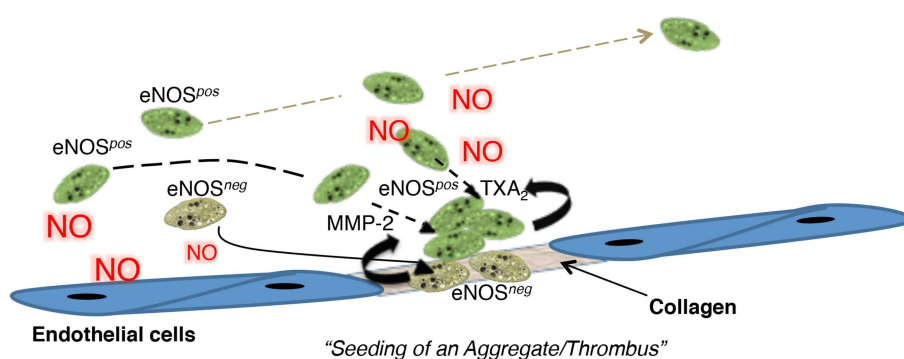


FIGURE 1 | A cartoon summarizing the “seed platelet” hypothesis in which NO-refractory eNOS^{neg} platelets preferentially initiate platelet adhesion and aggregation, while eNOS^{pos} platelets form the bulk of an aggregate and limit its size. Figure S21. Radziwon-Balicka et al. (47), by permission of Oxford University Press. MMP-2, matrix metalloproteinase-2; NO, nitric oxide, TXA_2 , Thromboxane A_2 .

needs to be determined. Similarly, what role these subpopulations play in coagulation and how they relate to subpopulations with differential procoagulant ability also remains to be seen (58).

CHARACTERIZATION OF PLATELET SUBPOPULATIONS BASED ON DIFFERENTIAL PROCOAGULANT ABILITY

For a thorough review of procoagulant platelets we refer readers to a paper by Reddy and Rand within this series of articles on the Established and Novel Roles of Platelets in Health and Disease. However, briefly, it has been long recognized that a subpopulation of platelets becomes procoagulant exposing a phosphatidylserine (PS)-surface upon activation (59). Alberio et al. (60) described a platelet subpopulation demonstrating α -granule-derived surface-bound factor V (FV), which they named COAT-platelets, due to their appearance upon collagen and thrombin stimulation. This subpopulation accounts for approximately 30% of platelets and was shown to utilize 5-HT to retain other α -granule-derived procoagulant proteins such as fibrinogen and thrombospondin on the platelet surface (58, 61). COAT-platelets may also retain factors VIII, IX, and X (62), and they may be generated via stimulation of platelets with thrombin alone (63). These platelets are also sometimes referred to as “coat” or “coated” platelets due to their surface retention of procoagulant proteins. Additionally, heterogeneity within the procoagulant subpopulation itself may exist wherein procoagulant platelets that are highly PS-positive and have a sustained increase in Ca^{2+} -signaling surprisingly aggregate poorly, due in part to a lack of active integrin $\alpha_{\text{IIb}}\beta_3$. Conversely, a COAT-platelet subpopulation with lower intracellular Ca^{2+} -signaling exhibits greater proaggregatory potential (64). Using mathematical modeling to support their experimental observations, Yakimenko Alena et al. (65) and Abaeva et al. (66) suggested that highly PS-positive COAT-platelets are recruited into developing aggregates by non-COAT platelets, as a result of a high surface density of α -granule-derived fibrinogen/fibrin retained on the COAT-platelet surface, and largely do not bind each other. Others have demonstrated that in fact integrin $\alpha_{\text{IIb}}\beta_3$ may initially activate but subsequently deactivate, while PS surface exposure occurs more slowly in these COAT-platelets (67). Nonetheless, a model of platelet-based coagulation was proposed by Heemskerk et al. (68) wherein two different subpopulations of platelets with differential roles exist (procoagulant vs. aggregating platelets). Collagen-adhered platelets, and later in the growing thrombus thrombin-activated platelets, expose PS on their membranes serving as a substrate for coagulation factors, thrombin generation, and fibrin coat formation. Aggregating platelets, due to their activated integrin $\alpha_{\text{IIb}}\beta_3$, on the other hand are proposed to be responsible for contracting and retracting the clot by interacting with fibrin.

Whether all platelets have the capacity to become procoagulant/COAT vs. aggregating platelet subpopulations or whether these subpopulations are predetermined requires further investigation. Studying platelet adhesion to glass some have argued that all platelets can form these subpopulations

and that they simply reflect snapshots in time of a dynamic platelet activation process (69). However, multi-parameter flow cytometry studies of platelet responses to increasing concentrations of thrombin and CRP-XL showed that only a fraction of platelets can take on the procoagulant phenotype supporting the theory of distinct platelet subpopulations (70).

CHANGES IN PLATELET SUBPOPULATIONS BETWEEN HEALTH AND DISEASE

Relatively little is known about how platelet subpopulations change between physiological and pathological conditions, although recently a number of studies have investigated procoagulant/COAT“ed” platelet levels in stroke and transient ischemic attack (71–78). Coated-platelets were demonstrated to be elevated in patients with large-artery atherosclerotic stroke compared to small artery lacunar strokes with the authors suggesting this reflects distinct pathological processes of ischemic stroke subtypes (71). Similarly, elevated coated-platelet levels were reported during transient ischemic attacks (TIA) (79). High coated-platelet levels were also reported to be associated with early stroke recurrence in large-artery stroke patients (72, 74); and in patients with asymptomatic carotid artery stenosis high coat-platelet levels ($\geq 45\%$ of platelets) improved stroke and TIA prediction (75). Conversely, a pilot study reported that non-lacunar stroke patients with early haemorrhagic transformation exhibited lower coated-platelet levels (78). Likewise, among subarachnoid hemorrhage and spontaneous intracerebral hemorrhage patients low coated-platelet levels were associated with increased mortality at 1-month (76, 77). The association of low levels of coated-platelets with bleeding events was also noted in a study aimed at evaluating the diagnostic utility of platelet flow cytometry analysis in haemorrhagic diathesis patients with normal standard laboratory workup (80).

Interestingly, a recent study utilizing a microfabricated chip capable of measuring individual platelet contractile forces identified platelet subpopulations with varying contractility (81). This platelet-contraction cytometry revealed generally high levels of highly-contractile platelets in healthy donors. These were absent in patients with Wiskott-Aldrich syndrome, and a subpopulation of platelets with low-contractility was also noted among a subset of patients with chronic bleeding but normal clinical haemostasis tests. Whether subpopulations of high- and low-contractile platelets correspond to subpopulations of coated- or aggregating-platelet subpopulations remains to be studied.

Older studies characterizing platelets based on buoyant density and/or volume also demonstrated differences between platelets of healthy controls and patients. Using continuous gradients of Percoll to isolate platelets, it was shown that compared to healthy controls insulin-dependent diabetics with poor glycemic control have lower density platelets but with apparently normal granule levels (82). The change in density profiles was proposed to be due to abnormal platelet subpopulations, although what appeared to be log normally

distributed populations were observed (83). A shift toward an increase in low-density platelet subpopulations was also observed in patients with hypercholesterolemia and it was argued that these platelets are more reactive in response to agonist stimulation (83). Conversely, a small increase in platelet size with increased surface exposure of integrin $\alpha_{IIb}\beta_3$ was noted following acute myocardial infarction (84).

Interestingly, recent preliminary studies suggest that FACS separated platelets into the smallest and largest 10% may differ in some of their RNA transcripts (85). A sub-analysis of the RNA profiles of these platelet subpopulations demonstrated a trend in which the large-platelet subpopulation RNA profile was associated with hemostasis and wound healing, while the small-platelet subpopulation profile was associated with vascular cell function likely reflecting a potential differential ability to uptake RNA from vascular cells. The differential uptake of RNA by platelet subpopulations may allow for these subpopulations to serve as novel biomarkers for various diseases. In this context, tumor-educated platelets (platelets containing tumor-associated mRNA) have been identified as a potential platform for blood-based liquid biopsies for cancer (86, 87). How platelet subpopulations change in cancer and how these changes impact their mRNA content is unknown but likely of important significance to liquid biopsy utility.

DIFFERENTIAL RESPONSES OF PLATELET SUBPOPULATIONS TO ANTI-PLATELET DRUGS

Less is still known about how various platelet subpopulations respond to anti-platelet drugs. Using counterflow centrifugation to isolate platelet subpopulations of different volume, Jakubowski et al. (88) demonstrated a correlation between increasing mean platelet volume and prostacyclin concentration necessary to inhibit aggregation suggesting greater platelet mass is associated with decreased inhibitory effect of prostacyclin. Similar, a study of FACS-sorted platelets into the 20% smallest and largest subpopulations showed that following incubation with acetylsalicylic acid (ASA) large platelets generate more thromboxane B₂ (TXB₂) compared to the small platelet subpopulation (89). However, the percent reduction in TXB₂ generation caused by ASA tended to be greater in the small platelet subpopulation. Potential differences in response to anti-platelet therapy by platelet subpopulations may be clinically important as Hoefer et al. (90) have shown that subpopulations of drug-free/uninhibited platelets can either intermingle with ASA-inhibited platelets within an aggregate or form aggregate cores around which ASA- and P2Y₁₂-inhibited platelets may activate potentially seeding thrombus formation.

CHALLENGES AND FUTURE DIRECTIONS

Some of the challenges with studying platelet subpopulations are their identification, labeling and separating for functional

TABLE 1 | List of cytometry techniques conducive to platelet subpopulation studies.

Technique	Some major advantages for platelet subpopulation studies	References
Confocal/epifluorescence microscopy	• Visualization of discrete subpopulations • Compatible with functional studies	(47, 56)
Flow cytometry (FC)	• Rapid, multi-parameter characterization of subpopulations	(47, 58)
Fluorescence activated cell sorting (FACS)	• Allows for separation of platelet subpopulations for further analysis	(47, 85)
Laser scanning cytometry (LSC)	• Rapid measurement of fluorescence and light scatter of slide-based specimens • Microscope-based enables visualization and morphological assessment	(93)
Interfacial platelet cytometry (iPC)	• Minimal sample preparation • Allows for studying subpopulation interactions with protein surfaces	(94)
Platelet contraction cytometry	• Enables measurement of contractile forces of individual platelets adhering on substrates	(81)
Mass cytometry	• Detects multiple heavy metal isotope-conjugated antibodies on platelet surface • Overcomes challenges with spectral overlap and eliminates need for compensation associated with FC • Greatly increases number of parameters that can be analyzed	(95)
FlowRNA	• Enables RNA measurement within specific cell subpopulations due to concurrent measurement of protein expression • Capacity to multiplex RNA measurement	(96)

studies. In the past these challenges were met with advances in research methodology and technology, such as the application of flow cytometry to platelet studies (91, 92). Recent advances in cytometry (Table 1) that have enhanced our ability to study platelet subpopulations include that application of fluorescence activated cell sorting and confocal microscopy (47), laser scanning cytometry (93), interfacial platelet cytometry (94), platelet-contraction cytometry (81), and mass cytometry (95). Moreover, the application of flowRNA technology to platelets studies may further help with RNA profiling of platelet subpopulations (96). These technologies will not only be crucial in aiding to delineate the roles of various platelet subpopulations in hemostasis and thrombosis, but also to understanding their potential differing roles in wound healing, angiogenesis, malignancy, and immunity and inflammation. Lastly, isolation of functionally distinct platelet subpopulations may be desirable for platelet concentrate preparation and various transfusion medicine applications (97, 98).

AUTHOR CONTRIBUTIONS

GL and PJ contributed to manuscript generation, editing, and approval of the submitted manuscript.

FUNDING

This work was funded by grants from the Canadian Institutes of Health Research (MOP-162128) to PJ.

ACKNOWLEDGMENTS

We thank Dr. M. Rand for discussions and critical review of our manuscript.

REFERENCES

- Burkhart JM, Vaudel M, Gambaryan S, Radau S, Walter U, Martens L, et al. The first comprehensive and quantitative analysis of human platelet protein composition allows the comparative analysis of structural and functional pathways. *Blood*. (2012) 120:e73–82. doi: 10.1182/blood-2012-04-416594
- Senis YA, Tomlinson MG, García Á, Dumon S, Heath VL, Herbert J, et al. A comprehensive proteomics and genomics analysis reveals novel transmembrane proteins in human platelets and mouse megakaryocytes including G6b-B, a novel immunoreceptor tyrosine-based inhibitory motif protein. *Mol Cell Proteomics*. (2007) 6:548–64. doi: 10.1074/mcp.D600007-MCP200
- Gnatenko DV, Dunn JJ, McCorkle SR, Weissmann D, Perrotta PL, Bahou WF. Transcript profiling of human platelets using microarray and serial analysis of gene expression. *Blood*. (2003) 101:2285. doi: 10.1182/blood-2002-09-2797
- Rowley JW, Oler AJ, Tolley ND, Hunter BN, Low EN, Nix DA, et al. Genome-wide RNA-seq analysis of human and mouse platelet transcriptomes. *Blood*. (2011) 118:e101. doi: 10.1182/blood-2011-03-339705
- Landry P, Plante I, Ouellet DL, Perron MP, Rousseau G, Provost P. Existence of a microRNA pathway in anucleate platelets. *Nat Struct Mol Biol*. (2009) 16:961–6. doi: 10.1038/nsmb.1651
- Sunderland N, Skroblin P, Barwari T, Huntley Rachael P, Lu R, Joshi A, et al. MicroRNA biomarkers and platelet reactivity. *Circ Res*. (2017) 120:418–35. doi: 10.1161/CIRCRESAHA.116.309303
- McManus DD, Freedman JE. MicroRNAs in platelet function and cardiovascular disease. *Nat Rev Cardiol*. (2015) 12:711. doi: 10.1038/nrcardio.2015.101
- Golebiewska EM, Poole AW. Platelet secretion: from haemostasis to wound healing and beyond. *Blood Rev*. (2015) 29:153–62. doi: 10.1016/j.blre.2014.10.003
- Nurden AT. The biology of the platelet with special reference to inflammation, wound healing and immunity. *Front Biosci Landmark Ed*. (2018) 23:726–51. doi: 10.2741/4613
- Radziwon-Balicka A, Moncada de la Rosa C, Jurasz P. Platelet-associated angiogenesis regulating factors: a pharmacological perspective. *Can J Physiol Pharmacol*. (2012) 90:679–88. doi: 10.1139/y2012-036
- Jenne CN, Kubes P. Platelets in inflammation and infection. *Platelets*. (2015) 26:286–92. doi: 10.3109/09537104.2015.1010441
- Yan M, Jurasz P. The role of platelets in the tumor microenvironment: from solid tumors to leukemia. *Biochim Biophys Acta (BBA) Mol Cell Res*. (2016) 1863:392–400. doi: 10.1016/j.bbamcr.2015.07.008
- Menter DG, Kopetz S, Hawk E, Sood AK, Loree JM, Gresele P, et al. Platelet “first responders” in wound response, cancer, and metastasis. *Cancer Metastasis Rev*. (2017) 36:199–213. doi: 10.1007/s10555-017-9682-0
- Thachil J. Platelets in inflammatory disorders: a pathophysiological and clinical perspective. *Semin Thromb Hemost*. (2015) 41:572–81. doi: 10.1055/s-0035-1556589
- Middleton EA, Weyrich AS, Zimmerman GA. Platelets in pulmonary immune responses and inflammatory lung diseases. *Physiol Rev*. (2016) 96:1211–59. doi: 10.1152/physrev.00038.2015
- Karparkin S. Heterogeneity of human platelets. I. Metabolic and kinetic evidence suggestive of young and old platelets. *J Clin Invest*. (1969) 48:1073–82. doi: 10.1172/JCI106063
- Karparkin S. Heterogeneity of human platelets. II. Functional evidence suggestive of young and old platelets. *J Clin Invest*. (1969) 48:1083–7. doi: 10.1172/JCI106064
- Polanowska-Grabowska R, Raha S, Gear ARL. Adhesion efficiency, platelet density and size. *Br J Haematol*. (1992) 82:715–20. doi: 10.1111/j.1365-2141.1992.tb06949.x
- Corash L, Tan H, Gralnick HR. Heterogeneity of human whole blood platelet subpopulations. I. Relationship between buoyant density, cell volume, and ultrastructure. *Blood*. (1977) 49:71.
- Dowling MR, Josefsson EC, Henley KJ, Hodgkin PD, Kile BT. Platelet senescence is regulated by an internal timer, not damage inflicted by hits. *Blood*. (2010) 116:1776–8. doi: 10.1182/blood-2009-12-259663
- Mason KD, Carpinelli MR, Fletcher JJ, Collinge JE, Hilton AA, Ellis S, et al. Programmed anuclear cell death delimits platelet life span. *Cell*. (2007) 128:1173–86. doi: 10.1016/j.cell.2007.01.037
- Rand ML, Greenberg JP, Packham MA, Mustard JF. Density subpopulations of rabbit platelets: size, protein, and sialic acid content, and specific radioactivity changes following labeling with ³⁵S-sulfate *in vivo*. *Blood*. (1981) 57:741–6. doi: 10.1111/j.1365-2141.1976.tb03611.x
- Penington DG, Streatfield K, Roxburgh AE. Megakaryocytes and the heterogeneity of circulating platelets. *Br J Haematol*. (1976) 34:639–53. doi: 10.1111/j.1365-2141.1976.tb03583.x
- Penington DG, Lee NLY, Roxburgh AE, McGready JR. Platelet density and size: the interpretation of heterogeneity. *Br J Haematol*. (1976) 34:365–76. doi: 10.1111/j.1365-2141.1976.tb03583.x
- Paulus JM. Platelet size in man. *Blood*. (1975) 46:321.
- Boneu B, Boneu A, Raison CL, Guiraud R, Bierme R. Kinetics of platelet populations in the stationary state. *Thromb Res*. (1973) 3:605–11. doi: 10.1016/0049-3848(73)90009-1
- Caranobe C, Sie P, Boneu B. Serotonin uptake and storage in human platelet density subpopulations. *Br J Haematol*. (1982) 52:253–8. doi: 10.1111/j.1365-2141.1982.tb03887.x
- Mezzano D, Aranda E, Rodriguez S, Foradori A, Lira P. Increase in density and accumulation of serotonin by human aging platelets. *Am J Hematol*. (1984) 17:11–21. doi: 10.1002/ajh.2830170103
- Thompson CB, Eaton KA, Princiotto SM, Rushin CA, Valeri CR. Size dependent platelet subpopulations: relationship of platelet volume to ultrastructure, enzymatic activity, and function. *Br J Haematol*. (1982) 50:509–19. doi: 10.1111/j.1365-2141.1982.tb01947.x
- Thompson CB, Love DG, Quinn PG, Valeri CR. Platelet size does not correlate with platelet age. *Blood*. (1983) 62:487–94.
- Thompson CB, Jakubowski JA, Quinn PG, Deykin D, Valeri CR. Platelet size and age determine platelet function independently. *Blood*. (1984) 63:1372–5. doi: 10.1111/j.1365-2141.1982.tb01947.x
- Corash L, Shafer B, Perlow M. Heterogeneity of human whole blood platelet subpopulations. II. Use of a subhuman primate model to analyze the relationship between density and platelet age. *Blood*. (1978) 52:726.
- Corash L, Costa JL, Shafer B, Donlon JA, Murphy D. Heterogeneity of human whole blood platelet subpopulations. III. Density-dependent differences in subcellular constituents. *Blood*. (1984) 64:185.
- Rand ML, Packham MA, Mustard JF. Survival of density subpopulations of rabbit platelets: use of ⁵¹Cr- or ¹¹¹In-labeled platelets to measure survival of least dense and most dense platelets concurrently. *Blood*. (1983) 61:362.
- Martin JE, Trowbridge EA. Theoretical requirements for the density separation of platelets with comparison of continuous and discontinuous gradients. *Thromb Res*. (1982) 27:513–22. doi: 10.1016/0049-3848(82)90298-5
- Peng J, Friese P, Heilmann E, George JN, Burstein SA, Dale GL. Aged platelets have an impaired response to thrombin as quantitated by P-selectin expression. *Blood*. (1994) 83:161.
- Hirsh J, Glynn MF, Mustard JF. The effect of platelet age on platelet adherence to collagen. *J Clin Invest*. (1968) 47:466–73. doi: 10.1172/JCI105743
- Dolowy K, Cunningham LW. Adhesion of human platelets to collagen: evidence for two states of platelet activation. *Coll Relat Res*. (1984) 4:111–8. doi: 10.1016/S0174-173X(84)80019-9
- Patel D, Vaananen H, Jirouskova M, Hoffmann T, Bodian C, Collier BS. Dynamics of GPIIb/IIIa-mediated platelet-platelet interactions in platelet adhesion/thrombus formation on collagen *in vitro* as revealed by videomicroscopy. *Blood*. (2003) 101:929–36. doi: 10.1182/blood.V101.3.929

40. Zhao B, Filler TJ, Rickert CH, Liu JF, Skholl-Engberts AD, Gurevich V. Different adhesion types and active sensitivity of platelet subpopulations. *Am J Hematol.* (1996) 52:322–4.
41. Behnke O, Forer A. Blood platelet heterogeneity: evidence for two classes of platelets in man and rat. *Br J Haematol.* (1993) 84:686–93. doi: 10.1111/j.1365-2141.1993.tb03147.x
42. Oppel C, Schuessler G, Kuschel M, Clement H-W, Gear ARL, Hinsch E, et al. Analysis of GTP-binding proteins, phosphoproteins, and cytosolic calcium in functional heterogeneous human blood platelet subpopulations. *Biochem Pharmacol.* (1997) 54:1027–35. doi: 10.1016/S0006-2952(97)00317-1
43. Oppel C, Fett C, Capito B, Raha S, Wesemann W. Plasma membrane properties in heterogeneous human blood platelet subfractions modulate the cellular response at the second messenger level. *Thromb Res.* (1993) 72:39–47. doi: 10.1016/0049-3848(93)90171-J
44. Oppel C, Schrumph E, Gear ARL, Wesemann W. Involvement of guanylate cyclase and phosphodiesterases in the functional heterogeneity of human blood platelet subpopulations. *Thromb Res.* (1995) 80:461–70. doi: 10.1016/0049-3848(95)00201-4
45. Milovanovic M, Lysen J, Ramström S, Lindahl TL, Richter A, Järemo P. Identification of low-density platelet populations with increased reactivity and elevated α -granule content. *Thromb Res.* (2003) 111:75–80. doi: 10.1016/j.thromres.2003.08.019
46. van Oost BA, Timmermans AP, Sixma JJ. Evidence that platelet density depends on the alpha-granule content in platelets. *Blood.* (1984) 63:482.
47. Radziwon-Balicka A, Lesyk G, Back V, Fong T, Loreda-Calderon EL, Dong B, et al. Differential eNOS-signalling by platelet subpopulations regulates adhesion and aggregation. *Cardiovasc Res.* (2017) 113:1719–31. doi: 10.1093/cvr/cvx179
48. Radomski MW, Palmer RM, Moncada S. An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. *Proc Natl Acad Sci USA.* (1990) 87:5193–7. doi: 10.1073/pnas.87.13.5193
49. Freedman JE, Sauter R, Battinelli EM, Ault K, Knowles C, Huang PL, et al. Deficient platelet-derived nitric oxide and enhanced hemostasis in mice lacking the NOSIII gene. *Circ Res.* (1999) 84:1416–21. doi: 10.1161/01.RES.84.12.1416
50. Roberts W, Michno A, Aburima A, Naseem KM. Nitric oxide inhibits von Willebrand factor-mediated platelet adhesion and spreading through regulation of integrin α IIb β 3 and myosin light chain. *J Thromb Haemost.* (2009) 7:2106–15. doi: 10.1111/j.1538-7836.2009.03619.x
51. Jurasz P, Stewart MW, Radomski A, Khadour F, Duszyk M, Radomski MW. Role of von Willebrand factor in tumour cell-induced platelet aggregation: differential regulation by NO and prostacyclin. *Br J Pharmacol.* (2001) 134:1104–12. doi: 10.1038/sj.bjp.0704343
52. Savage B, Almus-Jacobs F, Ruggeri ZM. Specific synergy of multiple substrate-receptor interactions in platelet thrombus formation under flow. *Cell.* (1998) 94:657–66. doi: 10.1016/S0092-8674(00)81607-4
53. Sawicki G, Salas E, Murat J, Miszta-Lane H, Radomski MW. Release of gelatinase A during platelet activation mediates aggregation. *Nature.* (1997) 386:616–9. doi: 10.1038/386616a0
54. Jurasz P, Chung AWY, Radomski A, Radomski MW. Nonremodeling properties of matrix metalloproteinases: the platelet connection. *Circ Res.* (2002) 90:1041–3. doi: 10.1161/01.RES.0000021398.28936.1D
55. Falcinelli E, Guglielmini G, Torti M, Gesele P. Intraplatelet signaling mechanisms of the priming effect of matrix metalloproteinase-2 on platelet aggregation. *J Thromb Haemost.* (2005) 3:2526–35. doi: 10.1111/j.1538-7836.2005.01614.x
56. Cozzi MR, Guglielmini G, Battiston M, Momi S, Lombardi E, Miller EC, et al. Visualization of nitric oxide production by individual platelets during adhesion in flowing blood. *Blood.* (2014) 125:697–705. doi: 10.1182/blood-2014-06-579474
57. Stalker TJ, Traxler EA, Wu J, Wannemacher KM, Cermignano SL, Voronov R, et al. Hierarchical organization in the hemostatic response and its relationship to the platelet-signaling network. *Blood.* (2013) 121:1875. doi: 10.1182/blood-2012-09-457739
58. Dale GL, Friese P, Batar P, Hamilton SF, Reed GL, Jackson KW, et al. Stimulated platelets use serotonin to enhance their retention of procoagulant proteins on the cell surface. *Nature.* (2002) 415:175–9. doi: 10.1038/415175a
59. Dachary-Prigent J, Freyssinet JM, Pasquet JM, Carron JC, Nurden AT. Annexin V as a probe of aminophospholipid exposure and platelet membrane vesiculation: a flow cytometry study showing a role for free sulfhydryl groups. *Blood.* (1993) 81:2554.
60. Alberio L, Safa O, Clemetson KJ, Esmon CT, Dale GL. Surface expression and functional characterization of α -granule factor V in human platelets: effects of ionophore A23187, thrombin, collagen, and convulxin. *Blood.* (2000) 95:1694.
61. Szasz R, Dale GL. Thrombospondin and fibrinogen bind serotonin-derivatized proteins on COAT-platelets. *Blood.* (2002) 100:2827. doi: 10.1182/blood-2002-02-0354
62. Kempton CL, Hoffman M, Roberts HR, Monroe DM. Platelet heterogeneity. *Arterioscler Thromb Vasc Biol.* (2005) 25:861–6. doi: 10.1161/01.ATV.0000155987.26583.9b
63. Pantelev MA, Ananyeva NM, Greco NJ, Ataullakhanov FI, Saenko EL. Two subpopulations of thrombin-activated platelets differ in their binding of the components of the intrinsic factor X-activating complex. *J Thromb Haemost.* (2005) 3:2545–53. doi: 10.1111/j.1538-7836.2005.01616.x
64. Topalov Nikolay N, Yakimenko Alena O, Canault M, Artemenko Elena O, Zakharova Natalia V, Abaeva Anastasia A, et al. Two types of procoagulant platelets are formed upon physiological activation and are controlled by integrin α IIb β 3. *Arterioscler Thromb Vasc Biol.* (2012) 32:2475–83. doi: 10.1161/ATVBAHA.112.253765
65. Yakimenko Alena O, Verholomova Faina Y, Kotova Yana N, Ataullakhanov Fazoil I, Pantelev Mikhail A. Identification of different proaggregatory abilities of activated platelet subpopulations. *Biophys J.* (2012) 102:2261–9. doi: 10.1016/j.bpj.2012.04.004
66. Abaeva AA, Canault M, Kotova YN, Obyednyy SI, Yakimenko AO, Podoplelova NA, et al. Procoagulant platelets form an α -granule protein-covered “cap” on their surface that promotes their attachment to aggregates. *J Biol Chem.* (2013) 288:29621–32. doi: 10.1074/jbc.M113.474163
67. Mattheij NJA, Swieringa F, Mastenbroek TG, Berny-Lang MA, May F, Baaten CCFMJ, et al. Coated platelets function in platelet-dependent fibrin formation via integrin α IIb β 3 and transglutaminase factor XIII. *Haematologica.* (2016) 101:427. doi: 10.3324/haematol.2015.131441
68. Heemskerk JWM, Mattheij NJA, Cosemans JMEM. Platelet-based coagulation: different populations, different functions. *J Thromb Haemost.* (2013) 11:2–16. doi: 10.1111/jth.12045
69. Donati A, Gupta S, Reviakine I. Subpopulations in purified platelets adhering on glass. *Biointerphases.* (2016) 11:029811. doi: 10.1116/1.4953866
70. Södergren AL, Ramström S. Platelet subpopulations remain despite strong dual agonist stimulation and can be characterised using a novel six-colour flow cytometry protocol. *Sci Rep.* (2018) 8:1441. doi: 10.1038/s41598-017-19126-8
71. Prodan CI, Joseph PM, Vincent AS and Dale GL. Coated-platelets in ischemic stroke: differences between lacunar and cortical stroke. *J Thromb Haemost.* (2008) 6:609–14. doi: 10.1111/j.1538-7836.2008.02890.x
72. Prodan CI, Stoner JA, Cowan LD, Dale GL. Higher coated-platelet levels are associated with stroke recurrence following nonlacunar brain infarction. *J Cereb Blood Flow Metab.* (2012) 33:287–92. doi: 10.1038/jcbfm.2012.168
73. Prodan CI, Vincent AS, Dale GL. Higher coated-platelet levels are associated with chronic hypertension in patients with transient ischemic attack. *Platelets.* (2013) 24:316–9. doi: 10.3109/09537104.2012.696747
74. Kirkpatrick AC, Stoner JA, Dale GL, Prodan CI. Elevated coated-platelets in symptomatic large-artery stenosis patients are associated with early stroke recurrence. *Platelets.* (2014) 25:93–6. doi: 10.3109/09537104.2013.775570
75. Kirkpatrick AC, Tafur AJ, Vincent AS, Dale GL, Prodan CI. Coated-platelets improve prediction of stroke and transient ischemic attack in asymptomatic internal carotid artery stenosis. *Stroke.* (2014) 45:2995–3001. doi: 10.1161/STROKEAHA.114.006492
76. Prodan CI, Stoner JA, Dale GL. Lower coated-platelet levels are associated with increased mortality after spontaneous intracerebral hemorrhage. *Stroke.* (2015) 46:1819–25. doi: 10.1161/STROKEAHA.115.009068
77. Prodan CI, Vincent AS, Kirkpatrick AC, Hoover SL, Dale GL. Higher levels of coated-platelets are observed in patients with subarachnoid hemorrhage but lower levels are associated with increased mortality at 30 days. *J Neurol Sci.* (2013) 334:126–9. doi: 10.1016/j.jns.2013.08.008
78. Prodan CI, Stoner JA, Cowan LD, Dale GL. Lower coated-platelet levels are associated with early hemorrhagic transformation in patients

- with non-lacunar brain infarction. *J Thromb Haemost.* (2010) 8:1185–90. doi: 10.1111/j.1538-7836.2010.03851.x
79. Prodan CI, Vincent AS, Dale GL. Coated-platelet levels are elevated in patients with transient ischemic attack. *Transl Res.* (2011) 158:71–5. doi: 10.1016/j.trsl.2011.02.010
 80. Daskalakis M, Colucci G, Keller P, Rochat S, Silzle T, Biasiutti FD, et al. Decreased generation of procoagulant platelets detected by flow cytometric analysis in patients with bleeding diathesis. *Cytometry Part B.* (2014) 86B:397–409. doi: 10.1002/cyto.b.21157
 81. Myers DR, Qiu Y, Fay ME, Tennenbaum M, Chester D, Cuadrado J, et al. Single-platelet nanomechanics measured by high-throughput cytometry. *Nat Mater.* (2016) 16:230. doi: 10.1038/nmat4772
 82. Collier A, Watson HHK, Matthews DM, Strain L, Ludlam CA, Clarke BF. Platelet-density analysis and intraplatelet granule content in young insulin-dependent diabetics. *Diabetes.* (1986) 35:1081. doi: 10.2337/diabetes.35.10.1081
 83. Oppen C, Clement C, Schwarz H, Krappe J, Steinmetz A, Schneider J, et al. Increased number of high sensitive platelets in hypercholesterolemia, cardiovascular diseases, and after incubation with cholesterol. *Atherosclerosis.* (1995) 113:211–7. doi: 10.1016/0021-9150(94)05448-R
 84. Giles H, Smith REA, Martin JF. Platelet glycoprotein IIb-IIIa and size are increased in acute myocardial infarction. *Eur J Clin Invest.* (1994) 24:69–72. doi: 10.1111/j.1365-2362.1994.tb02062.x
 85. Clancy L, Beaulieu LM, Tanriverdi K, Freedman JE. The role of RNA uptake in platelet heterogeneity. *Thromb Haemost.* (2017) 117:948–61. doi: 10.1160/TH16-11-0873
 86. Best MG, Sol N, Kooi I, Tannous J, Westerman Bart A, Rustenburg F, et al. RNA-seq of tumor-educated platelets enables blood-based pan-cancer, multiclass, and molecular pathway cancer diagnostics. *Cancer Cell.* (2015) 28:666–76. doi: 10.1016/j.ccell.2015.09.018
 87. Best MG, Wesseling P and Wurdinger T. Tumor-educated platelets as a noninvasive biomarker source for cancer detection and progression monitoring. *Cancer Res.* (2018) 78:3407. doi: 10.1158/0008-5472.CAN-18-0887
 88. Jakubowski JA, Alder B, Thompson CB, Valeri CR, Deykin D. Influence of platelet volume on the ability of prostacyclin to inhibit platelet aggregation and the release reaction. *J Lab Clin Med.* (1985) 105:271–6.
 89. Mangalpally KKR, Siqueiros-Garcia A, Vaduganathan M, Dong J-F, Kleiman NS, Guthikonda S. Platelet activation patterns in platelet size sub-populations: differential responses to aspirin *in vitro*. *J Thromb Thrombol.* (2010) 30:251–62. doi: 10.1007/s11239-010-0489-x
 90. Hoefer T, Armstrong PC, Finsterbusch M, Chan MV, Kirkby NS, Warner TD. Drug-free platelets can act as seeds for aggregate formation during antiplatelet therapy. *Arterioscler Thromb Vasc Biol.* (2015) 35:2122–33. doi: 10.1161/ATVBAHA.115.306219
 91. Michelson AD. Flow cytometric analysis of platelet surface glycoproteins: phenotypically distinct subpopulations of platelets in children with chronic myeloid leukemia. *J Lab Clin Med.* (1987) 110:346–54.
 92. Frojmovic M, Wong T. Dynamic measurements of the platelet membrane glycoprotein IIb-IIIa receptor for fibrinogen by flow cytometry. II. Platelet size-dependent subpopulations. *Biophys J.* (1991) 59:828–37. doi: 10.1016/S0006-3495(91)82295-0
 93. Claytor RB, Li JM, Furman MI, Garnette CS, Rohrer MJ, Barnard MR, et al. Laser scanning cytometry: a novel method for the detection of platelet–endothelial cell adhesion. *Cytometry.* (2001) 43:308–13. doi: 10.1002/1097-0320(20010401)43:4<308::AID-CYTO1063>3.0.CO;2-9
 94. Basabe-Desmonts L, Ramstrom S, Meade G, O'Neill S, Riaz A, Lee LP, et al. Single-step separation of platelets from whole blood coupled with digital quantification by interfacial platelet cytometry (iPC). *Langmuir.* (2010) 26:14700–6. doi: 10.1021/la9039682
 95. Blair TA, Michelson AD, Frelinger AL. Mass cytometry reveals distinct platelet subtypes in healthy subjects and novel alterations in surface glycoproteins in glanzmann thrombasthenia. *Sci Rep.* (2018) 8:10300. doi: 10.1038/s41598-018-28211-5
 96. Soh KT, Wallace PK. RNA flow cytometry using the branched DNA technique. *Methods Mol Biol.* (2018) 1678:49–77. doi: 10.1007/978-1-4939-7346-0_4
 97. Handtke S, Steil L, Greinacher A, Thiele T. Toward the relevance of platelet subpopulations for transfusion medicine. *Front Med.* (2018) 5:17. doi: 10.3389/fmed.2018.00017
 98. Vučetić D, Ilić V, Vojvodić D, Subota V, Todorović M, Balint B. Flow cytometry analysis of platelet populations: usefulness for monitoring the storage lesion in pooled buffy-coat platelet concentrates. *Blood Transfus.* (2018) 16:83–92. doi: 10.2450/2016.0193-16

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Lesyk and Jurasz. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Effect on Platelet Function of Metal-Based Nanoparticles Developed for Medical Applications

Nadhim Kamil Hante^{1,2}, Carlos Medina^{1*} and Maria Jose Santos-Martinez^{1,3*}

¹ The School of Pharmacy and Pharmaceutical Sciences, Trinity Biomedical Sciences Institute, Trinity College Dublin, The University of Dublin, Dublin, Ireland, ² College of Pharmacy, University of Kufa, Najaf, Iraq, ³ School of Medicine, Trinity College Dublin, The University of Dublin, Dublin, Ireland

OPEN ACCESS

Edited by:

Marie Lordkipanidzé,
Université de Montréal, Canada

Reviewed by:

Christoph Eugen Hagemeyer,
Monash University, Australia
Matthew Dean Linden,
University of Western
Australia, Australia

*Correspondence:

Carlos Medina
carlos.medina@tcd.ie
Maria Jose Santos-Martinez
santosmm@tcd.ie

Specialty section:

This article was submitted to
Atherosclerosis and Vascular
Medicine,
a section of the journal
Frontiers in Cardiovascular Medicine

Received: 14 June 2019

Accepted: 03 September 2019

Published: 18 September 2019

Citation:

Hante NK, Medina C and
Santos-Martinez MJ (2019) Effect on
Platelet Function of Metal-Based
Nanoparticles Developed for Medical
Applications.
Front. Cardiovasc. Med. 6:139.
doi: 10.3389/fcvm.2019.00139

Nanomaterials have been recently introduced as potential diagnostic and therapeutic tools in the medical field. One of the main concerns in relation to the use of nanomaterials in humans is their potential toxicity profile and blood compatibility. In fact, and due to their small size, NPs can translocate into the systemic circulation even after dermal contact, inhalation, or oral ingestion. Once in the blood stream, nanoparticles become in contact with the different components of the blood and can potentially interfere with normal platelet function leading to bleeding or thrombosis. Metallic NPs have been already used for diagnosis and treatment purposes due to their unique characteristics. However, the potential interactions between metallic NPs and platelets has not been widely studied and reported. This review focuses on the factors that can affect platelet activation and aggregation by metal NPs and the nature of such interactions, providing a summary of the effect of various metal NPs on platelet function available in the literature.

Keywords: platelets, metallic nanoparticles, nanoparticles, activation, aggregation, cytotoxicity, nanoparticles charge, nanoparticles shape

INTRODUCTION

Nanoparticles (NPs) are defined as particles which range from 1 to 100 nm in size in at least one dimension (length, width, or depth) (1, 2). Although, the development of nanotechnology is a modern science, NPs have always existed in the natural environment and throughout the ages, humans have been continually exposed to airborne NPs (3). However, with the onset of the industrial revolution and the more recent explosion of interest in NPs for scientific development, the exposure to different types of NPs is on the rise. In fact, the use of engineered nanomaterials from a wide range of compositions and sizes together with the study of their potential hazards is continuously increasing.

This review will focus on the interactions of metallic NPs developed for medical applications with blood platelets. The search has been conducted using PubMed, Scopus, and Google Scholar and relevant full text original and review articles published in English up to April 2019 have been included.

Factors Associated to Nanoparticle's Toxicity

Since the introduction of nanomedicine to describe the application of nanotechnology to benefit patients by The Royal Society and Royal Academy Engineering in 2004 (4), engineered NPs have become promising tools for the monitoring, diagnosis and treatment of human diseases. Despite the ability of humans to avoid, tolerate, and adapt to naturally occurring NPs,

the exposure and accumulation of engineered NPs in our body can lead to plausible side effects (4). Multiple studies have already demonstrated that NPs interactions with biological systems depend on size, shape, charge, and the constituent material of the nanomaterial. In fact, those parameters can have a profound effect on cellular uptake and toxicity (5) and therefore on platelet's function (6–8).

Nanoparticles Size

Alveolar clearance by macrophages in the lungs constitutes an important barrier for inhaled NPs. However, the efficacy of this mechanism decreases with NPs size leading to a local increased deposition, greater access of NPs to the circulatory system and to potential cardiovascular side effects (9). It has been previously demonstrated that the degree of cytotoxicity and platelet activation and aggregation is inversely correlated with the NPs size (6, 10–12). Therefore, to dampen their potential toxic effect, while improving their stability, various types of surface coatings have been used (7, 13, 14). Surface modification using polyethylene glycol (PEG) for example, has been successfully employed to improve the platelet compatibility of gold NPs (15).

Nanoparticles Shape

The physical dimensions of nanomaterials are also strongly correlated with their potential toxicity profiles when compared with the bulk materials of the same composition (16, 17). For example, although carbon black is non-toxic, inhaled carbon nanotubes can result highly toxic (18). One of the factors that strongly influences NPs toxicity is the NPs shape. However, for a given geometric shape, NPs size largely determines the cellular uptake (19–22). Gratton et al. have demonstrated that nanorods larger than 100 nm exhibit the highest rate of cellular uptake followed by spheres, cylinders, and cubes of similar size (23). In contrast, studies using particles smaller than 100 nm have found that spheres show a greater cellular uptake than nanorods (19, 24). However, little is known about the influence of NPs shape on platelet function. Holzer et al. have shown that carbon-based nanomaterials could induce thrombus formation in rodents independently of their shape (25). In contrast, He et al. have recently demonstrated that cuboidal cyclodextrin frameworks enhanced platelet aggregation when compared with their spherical counterpart (26).

Protein Binding

When NPs become in contact with biological fluids, electrostatic, dispersive, and covalent interactions will regulate the adsorption of proteins onto NPs, leading to the formation of a dynamic “protein corona” (27, 28). Binding of plasma proteins induce changes in the biophysical properties of the NPs modifying their biocompatibility. In fact, the nature and the concentration of the proteins adsorbed on the NPs can affect their bio

uptake, biodistribution, and potential side effects (29–34). The adsorption of some types of plasma proteins may result in an inflammatory response and platelet activation and aggregation. For instance, carbon nanotubes have been reported to bind complement proteins leading to complement activation via both classical and alternative pathways. In contrast, adsorption of complement proteins on the surface of gold colloids has not been associated with complement activation (35–37). The ability of PEG-coatings to protect against platelet aggregation corroborates the hypothesis that physical barriers around NPs may also contribute to the loss of their pro-aggregatory effect (15, 38). However, although the adsorption of fibrinogen onto NPs can contribute to platelet adhesion and initiate thrombogenesis, it has been also demonstrated that the composition of the protein corona on PEGylated NPs doesn't predict their hemocompatibility (39).

Nanoparticle Charge

NPs charge plays also an important role in NP-plasma protein interactions (29, 40) and therefore in the blood compatibility of NPs. Positively charge NPs can potentially interact with the negatively charged platelet surface and induce platelet aggregation (41, 42). However, Love et al. found that gold NPs with the same size and opposite charge did not induced platelet aggregation (43). Marginal variation in charge may contribute to a significant difference in protein binding to the NP surface and that may be the case with albumin binding to colloidal gold NPs (37).

METALLIC NANOPARTICLES AND THEIR APPLICATIONS IN MEDICINE

Metallic NPs can be easily synthesized and modified with various chemical functional groups which allow them to be conjugated with antibodies, ligands, and drugs. Therefore, the potential applications of metallic NPs in a variety of health-related applications over conventional pharmaceuticals, including targeted drug delivery systems, vehicles for gene and drug delivery, and imaging has dramatically increased (44).

Medical diagnostic applications of metal NPs are plentiful, owing to their ability to interact with external stimuli, including infra-red radiation, ultrasonic waves, and magnetic fields (45–48). Paramagnetic and superparamagnetic NPs have shown a great potential for cancer detection, as evidenced, for example, by the superior ability of iron oxide NPs to detect liver metastases and metastatic lymph nodes (49, 50). Metallic NPs can be also used as therapeutics agents. The antibacterial nature of silver has been already well-established and gold NPs have been found to induce cell toxicity when delivered orally. In fact, silver and gold NPs have been demonstrated to be ideal candidates for the treatment of both, multi drug resistant infections and cancer (51–55). In addition, gold NPs have also shown to exert some anti-angiogenic effect by inhibiting the VEGF activity in collagen-induced arthritis in rats and in human umbilical vein endothelial cells (56–58). The use of multifunctional NPs that integrate diagnostic and

Abbreviations: NPs, Nanoparticles; VEGF, Vascular endothelial growth factor; PEG, polyethylene glycol; LTA, light transmission Aggregometry; QCM-D, Quartz Crystal Microbalance with Dissipation; PEI, polyethyleneimine; PVP, polyvinylpyrrolidone; PMA, Phorbol-myristate-acetate; PAA, polyacrylic acid; ROS, reactive oxygen species; SNAP, S-nitroso-N-acetylpenicillamine; HAS, human serum albumin.

therapeutic functions in the same system (theranostic) has been gaining increasing momentum in the nanomedical research and development (R&D). For the management of patients suffering from cancer, for example, nanotheranostic platforms could comprise advanced diagnostics, hyperthermia treatment, and targeted delivery of anticancer drugs. Actually, magnetic iron oxide NPs constitute a good example of such “multitasking” platforms (59).

METALLIC NANOPARTICLES-PLATELETS INTERACTIONS

In 1977, Berry et al. described for the first time the translocation of nano-sized particles across the alveolar epithelium following intratracheal instillations of 30 nm gold particles in rats. They found large amounts of these particles in platelets of pulmonary capillaries and assumed that there might be a pre-disposing factor for platelet aggregation and microthrombi formation even when those NPs are coated with a biocompatible material (60). In fact, NPs which are not intended for systemic use can, due to their ability to cross epithelial barriers, reach the systemic circulation and interfere with physiological platelet function increasing the risk of cardiovascular disease and vascular thrombosis (61–63). Although, some NPs have been developed for therapeutic purposes aimed to target the injured vascular site mimicking platelet function (64) or to enhance blood clotting (65), the potential unwanted, pro- and/or anti-aggregating, properties of NPs are of significant concern in the field of nanomedicine and may impede the progression of promising engineered NPs to the clinical setting.

Induction of platelet aggregation by metallic NPs has been found by multiple research groups. However, some studies also indicate that metal NPs can inhibit or not affect platelet function (Table 1). The extent to which NPs induce platelet aggregation may depend on multiple NPs factors, as explained in the previous sections, but also on the physiological state of platelets prior exposure to NPs. When platelets are in resting state most metallic NPs seem to be inert (7) becoming more sensitive to NPs in the presence of a threshold shear force or “pre-activation” by critical concentrations of ADP (10, 66, 85). However, non-metallic NPs such as carbon nano-tubes or polymer-based NPs seem to induce platelet aggregation in the absence of any “pre-activating factor” (42, 62).

The exact molecular mechanisms by which metallic NPs influence platelet function is not well-understood. However, their effect on platelet granules and integrin receptors seems to be crucial during the process. Metallic NP-induced platelet aggregation is inhibited when ADP pathway is blocked by an appropriate concentration of clopidogrel or apyrase inhibiting therefore the secondary wave of platelet aggregation which depends mainly on granule release. GPIIb/IIIa seems to play also an important role in metallic NP-induced platelet aggregation as in the presence of Arg-Gly-Asp-Ser (RGDS), a tetra-peptide which binds and inhibits activated but not resting GpIIb/IIIa, platelet aggregation is attenuated (86).

Silver NPs

The broad-spectrum antimicrobial properties of silver NPs are well-documented. Their widespread use in both commercial and biomedical applications, due to their large surface-area-to-volume ratio which increases their efficacy against bacteria in comparison to common antibiotics, has raised them to the status of the most commercialized NPs.

The blood compatibility of silver NPs, remains controversial, with several studies that report contradictory results. Spherical silver NPs (10–100 nm) have been found to induce platelet aggregation by increasing intraplatelet Ca^{2+} levels, upregulating GPIIb/IIIa and P-selectin and serotonin secretion (76, 78). Laloy et al. reported that silver NPs increased platelet adhesion but did not exert any further effect on platelet aggregation (77). Coating with PEG inhibits platelet aggregation induced by collagen, ADP, thrombin, and arachidonic acid in a concentration dependent manner (79). Silver NPs spherical in shape, 10–15 nm in diameter and monodispersed, have also been shown to have antiplatelet properties (67). Some variability between studies can be due to the use of different dispersing media (87–89) and Deb et al. have argued that the inhibition of platelet function by silver NPs may be due to the presence of the citric acid used for coating their surface (7). In addition, most of the studies use light transmission aggregometry (LTA) for measuring platelet aggregation and LTA has some limitations: (1) it may not be sensitive enough for studying NP-induced platelet aggregation (90). (2) As silver NPs have light absorbance properties, which are significant from 10 $\mu\text{g/mL}$, the use of concentrations over this threshold can have a profound effect on the results obtained (77). On the other hand, Smock et al. performed a prospective, placebo controlled in healthy human volunteers to assess the effect of commercially available oral colloidal silver NPs on platelet aggregation *ex vivo* using LTA. They found that platelet activation was not enhanced at peak silver serum concentrations (<10 $\mu\text{g/mL}$) (80).

Gold NPs

Colloidal gold was first described as a novel NP vector for tumor directed drug delivery back to 2004 (91). Gold NPs are considered to be one of the safest and most attractive drug-delivery agents due of their inert, non-toxic, and highly permeable properties (92). Still, there are some studies that document toxicity of gold NPs depending on the physical dimensions, surface chemistry, and shape of the NPs studied (19, 20, 93–97).

The compatibility of gold NPs with blood components and their effect on platelet function is not well-established. Although, gold NPs >60 nm in size have no effect on platelet function, smaller NPs (20 nm) can activate platelets. Those NPs could trigger platelet aggregation by a molecular mechanism which involves the platelet canalicular system and the activation of intracellular signaling mechanisms that comprise tyrosine phosphorylation, alpha granule release and P-selectin translocation (10, 98). On the other hand, it has been shown that spherical gold NPs of ~30 nm do not affect platelet aggregation, probably due to the total surface contact and/or the formation of the protein corona (37, 43).

Santos-Martinez et al. have investigated the effect that different NPs may exert on platelet function under flow

TABLE 1 | Metallic nanoparticles-platelets interactions.

Composition	Size (nm)	Charge	Concentration tested	Nanoparticle shape	Effect on platelet function	References
Copper oxide	10	Negative	1.6×10^{19} atoms/mL	Rods and spheres	No effect	(66)
Gold	13,20,29	Negative	50 μ M	Spheres	No effect	(67)
Gold	5–30	Negative	5–40 μ M	Spheres	No effect	(68)
Gold	>60	Negative	5–40 μ M	Spheres	Inhibition of ADP induced platelet aggregation	(68)
Gold	20	Negative	5–40 μ M	Spheres	Platelet Aggregation	(68)
Gold	40	Negative	5–40 μ M	Spheres	Platelet aggregation	(68)
Gold	30	Negative	0.45 mg/mL	Spheres	Inhibition of collagen induced platelet aggregation	(37)
Gold	50	Negative	0.420 mg/mL	Spheres	Inhibition of collagen-induced platelet aggregation	(37)
Gold	18	Negative	5 μ g/mL	Spheres	Platelet aggregation	(69)
Gold	9.509 ± 0.56	Negative	2.144×10^{21} atoms/mL	Rods and spheres	Platelet aggregation	(66)
Gold	30	Negative and positive	5, 15, 25, and 50 μ g/mL	Spheres	No effect	(43)
Gold (PEI-)	20	Negative	50 ppm	Spheres	Platelet aggregation	(70)
Gold (PVP-)	50	Negative	50 ppm	Spheres	Platelet aggregation	(70)
Iron	10–50	Negative	2×10^{19} atoms/mL	Rods and spheres	Platelet aggregation	(66)
Iron	10	Not specified	5 mg/mL	Spheres	Inhibition of PMA and arachidonic acid induced platelet aggregation	(71)
Iron oxide (Fe ₂ O ₃)	55–65	Not specified-	50 μ g/mL	Rods	Platelet aggregation	(72)
Iron oxide (Ferucarbotran)	90	Negative	0.5 mM	Spheres	No effect	(73)
Iron oxide (Ferucarbotran)	90	Negative	10 mM	Spheres	Platelet aggregation	(73)
Iron oxide (Ferucarbotran)	90	Negative	5 mM	Spheres	No effect	(73)
Iron oxide (QD-labeled Ferucarbotran)	90	Negative	0.5 mM	Spheres	No effect	(73)
Iron carbide (PEG)	30	Neutral	1 mg/mL	Spheres	No effect	(74)
Iron oxide (Fe ₃ O ₄)	20–30	Not specified-	50 μ g/ml	Spheres	Platelet aggregation	(72, 75)
Iron oxide (Ferucarbotran (HSA-))	90	Negative	0.5 mM	Spheres	No effect	(73)
Nickel	62	Neutral	50 μ g/ml	Spheres	Platelet activation	(72)
Silver	10–15	Positive	50 μ M	Spheres	Inhibition of thrombin-induced platelet aggregation	(67)
Silver	20	Neutral	0.05–0.1 mg/kg i.v. or 5–10 mg/kg	Spheres	Platelet aggregation	(76)
Silver	16–71	Positive	50 μ g/ml	Spheres	No effect	(77)
Silver (PVP)	90–240	Neutral	50 μ g/mL	Spheres	Platelet activation	(72)
Silver	12	Positive	200 mg/L	Spheres	Platelet activation	(78)
Silver (PEG)	20	Neutral	125–625 μ M	Spheres	Inhibition of fibrinogen induced platelet aggregation	(79)
Silver (Colloidal)	10–100	Positive	<10 μ g/L	Spheres	No effect	(80)
Silver (citrate coated)	24.3 ± 4.5	Negative	~500 μ g/mL	Spheres	No effect	(81)

(Continued)

TABLE 1 | Continued

Composition	Size (nm)	Charge	Concentration tested	Nanoparticle shape	Effect on platelet function	References
Silver (PVP coated)	21.6 ± 4.8	Negative	~500 µg/mL	Spheres	No effect	(81)
Titanium dioxide (anatase)	101	Negative	1 mg/kg	Not specified	Platelet aggregation	(82)
Titanium dioxide (rutile)	104	Negative	1 mg/kg	Not specified	No effect	(82)
Titanium dioxide	10 × 40	Not specified-	0.4–10 µg/mL	Rods	No effect	(83)
Titanium dioxide	600–4,000	Negative	10 µg/mL	Spheres	Platelet activation	(84)
Titanium dioxide	20–160	Not specified-	50 µg/mL	Rod	No effect	(72)
Zinc oxide	100–1,200	Negative	10 µg/mL	Rod	Platelet aggregation	(84)
Zinc oxide	150	Negative	1 mg/kg	Not specified	No effect	(82)

HAS, Human serum albumin; PEG, polyethylene glycol; PEI, polyethyleneimine; PVP, polyvinylpyrrolidone; PMA, Phorbol-myristate-acetate; QD, Quantum dots.

conditions using a Quartz Crystal Microbalance with Dissipation (QCM-D). The group has demonstrated that QCM-D is more sensitive than the traditional LTA when investigating NP-platelet interactions (69, 99) and found that PEGylation of gold NPs improves their platelet compatibility (15). However, the use of polyethyleneimine (PEI) and polyvinylpyrrolidone (PVP) conjugated NPs can induce platelet activation as revealed by the formation of numerous filopodia and degranulation in equine platelets (70). This effect may be related to the adsorption of fibrinogen onto the NPs surface (5) and could be even more obvious if polyacrylic acid (PAA)-conjugated gold nanoparticles are exposed to platelets as PAA binds to and induce conformational changes of fibrinogen (100) that could potentially have a greater impact on the hemocompatibility of those NPs.

Iron Oxide NPs

Iron oxide NPs have been extensively used as contrast agents. With the introduction of theranostic systems their use has become more attractive as a novel approach for cancer therapy. The use of those NPs loaded with cytotoxic drugs and functionalized to detect and specifically attack malignant cells could potentially reduce significantly both, side effects of cytotoxic drugs and healthy cells damage.

The effect of iron-based NPs on platelet function in the literature is somehow inconsistent. Some iron-based NPs have been found to induce platelet aggregation as demonstrated by morphological changes using scanning electron microscopy (72). Bircher et al. found that carbon-coated iron carbide magnetic NPs incubated with whole blood induced upregulation of GPIIb-IIIa and P-Selectin but this effect was reversed when NPs were PEGylated (74). In another study, the use of dextran-stabilized iron oxide NPs developed for hyperthermia did not affect either platelet function (101). Deb et al. show in their work that starch-stabilized iron oxide NPs do not exert any effect of platelets while citric acid-stabilized iron oxide NPs inhibited platelet aggregation (7).

Platelet labeling can be of great interest for evaluating the influence of different methods on platelet survival when preparing platelet concentrates or when there is a need to distinguishing between donor and recipient platelets. Iron oxide NPs conjugated with quantum dots have been used previously

by Aurich et al. as a non-radioactive alternative for platelet labeling. NPs were successfully internalized in platelets but impaired platelet function at the concentrations needed for labeling. However, this effect was abolished when the NPs were functionalized further with human serum albumin (73).

Nickel NPs

Compared to its elemental state, nickel NPs exhibit exceptional electrochemical properties and show unusual superparamagnetic properties and stability that make them very attractive in the nanotechnology field (102, 103). In addition, nickel NPs have been used in medicine as catalysts in the production of hydrogen nanoparticles (104). Some studies have demonstrated that nickel NPs induce cytotoxic effects *in vitro* (105, 106) and changes in platelet shape (72). However, despite their wide use in industry, their potential toxic effect to humans has not been extensively investigated.

Zinc Oxide NPs

Zinc oxide NPs are commonly used in nanomedicine. Spherical NPs have been used as anti-cancer and anti-bacterial agents due to their ability to produce reactive oxygen species (ROS) and induce apoptosis (107, 108). In fact, tetrapod NPs have been recently studied for their antibacterial activity (109), antiviral activity (110, 111), and as a vaccine adjuvant (112). Composite types of nanostructures are also synthesized in various forms including zinc oxide quantum dots and zinc oxide nanoclusters as anti-cancer and anti-bacterial agents (113–115).

Zinc oxide NPs prepared in dispersion medium, citrate or glucose solution have been shown to induce human and canine platelet activation (84). On the other hand, systemic administration of zinc oxide NPs to mice did not induce thrombus formation (82). However, studies looking at the blood compatibility of those NPs are once again scarce.

Copper NPs

Deb et al. have shown that copper NPs have a pro-aggregatory effect activating purinergic receptors (P2Y₁₂) in the presence of ADP at suboptimal concentrations (66). However, Major et al. using a nitric oxide generating polymeric material (polyurethane containing copper NPs) combined with the intravenous infusion of a nitric oxide donor (S-nitroso-N-acetylpenicillamine-SNAP)

TABLE 2 | Effect of metallic nanoparticles on platelet function.

Nanoparticle type	Main findings
Silver	Silver NPs need to accumulate within platelets to interfere with different intraplatelet pathways (67, 76, 78, 79). Primary NPs size is as important as the stability and monodispersity of the NPs which highly depend on the type of dispersion medium used. Chitosan and PEG prevent agglomeration (122) but also increase NPs size affecting NPs properties and leading to inconsistent results despite using similar or close primary NPs size. PVP has no effect on platelet function but citric acid can inhibit platelet aggregation (7, 77). Silver NPs can also interfere with light absorbance and therefore, light transmission aggregometry, which is the gold standard for studying platelet function, may not be suitable to investigate silver NPs-platelets interactions
Gold	Gold NPs size is important when investigating their effect on platelet function. It seems that gold NPs >60 nm do not modify platelet function. However, when NPs are <50 nm they can be internalized and accumulated in platelets affecting their function (10, 66, 68–70, 93). PEI, PVP, and PAA coatings can induce platelet aggregation (70, 100, 123)
Iron oxide	The effect of iron nanoparticles on platelet function is not consistent and the studies available demonstrate that they can induce (66, 72, 73, 75); have no effect (73, 74) or inhibit platelet aggregation (71). No single factor seems to be determinant but coatings may play an important role as HAS, PEG, and citrate have shown to do not affect platelet function (73, 74) but PAA inhibited platelet aggregation (71)
Titanium dioxide, zinc oxide, nickel, and copper oxide	Most studies refer to pro-aggregatory effect of these NPs. However, information is scarce to draw a robust conclusion regarding their effect on blood platelets

PEI, polyethylenimine; PVP, polyvinylpyrrolidone; PAA, poly acrylic acid; HAS, Human serum albumin; PEG, polyethylene glycol; PMA, Phorbol-myristate-acetate.

observed that platelet function was preserved in rabbit blood exposed to extracorporeal circulation (116).

Titanium Dioxide NPs

Titanium dioxide NPs are widely used in cosmetic products and sunscreens (117) and they are very well-known photocatalysts able to generate different reactive species that could be potentially toxic to microorganisms. In fact, *in vitro* studies have shown deleterious effect of those NPs on different cell lines (118, 119). Titanium dioxide NPs have shown to activate dog and, to a lesser degree, human platelets *in vitro* (84). When administered systemically to rats, titanium dioxide anatase but not titanium dioxide rutile NPs of similar size (around 100 nm) were found to increase murine platelet aggregation (82). In another study, systemic administration of titanium dioxide rutile NPs did not activate platelets or exert prothrombotic effects in mice either (120). However, the intratracheal administration of rutile titanium oxide nanorods has resulted in platelet aggregation in rats (83).

CONCLUSIONS

Medicine is envisaged to be one of the primary beneficiaries of the nanotechnological development. The growth of nanotechnology has fired the “technological boom” in diagnostics, imaging, and drug delivery (121). The scientific community has shown significant interest in further developing the potential medical applications of metal NPs. However, nanomaterial-blood interactions are inevitable regardless of the use NPs are intended to.

The compatibility of NPs with blood elements remains as a controversial topic. Several studies utilizing similar experimental protocols and assessing similar end points report contradictory results and **Table 2** summarizes the main effects of metallic NPs on platelets found in the literature. Although there are multiple “NP dependent” factors that can play an important role on NPs-platelets interaction, other circumstances should be kept on mind when carrying out those experiments as

they may potentially contribute to the conflicting results: (a) platelet preparation/platelet handling; (b) use of different solutions/dispersing media when preparing the NPs/performing the experiments; (c) presence of impurities or additional substances in metallic NPs or their suspensions; (d) interference of the NPs tested with the method used; (e) use of different concentrations of NPs and or platelet agonists in different experimental settings; and (f) lack of sensibility for detecting platelet microaggregates with the method used.

Unwanted side effects of NPs is of significant concern in the field of nanomedicine and often hampers the progression of promising nanomaterials to the clinical setting. Knowledge regarding NPs toxic potential is still limited along with an absence of appropriated regulatory policies for their use and testing (124, 125). In fact, not all commercially available nanomaterials have a Safety Data Sheet and novel nanomaterials are sometimes manipulated without full assessment of their potential health risk (124).

There is no doubt that the development of nanomaterials intended for human use should be always carried out in tandem with an extensive toxicological evaluation. Those studies must include the investigation of the effect of nanomaterials on platelet function as they can lead to secondary effects (bleeding or thrombosis) that can affect individuals involved in their production, manipulation or use during medical applications.

AUTHOR CONTRIBUTIONS

NH, MS-M, and CM designed the structure of the different sections of the manuscript. NH did the search and wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

NH was funded from Ministry of Higher Education and Scientific Research in Iraq (MoHER).

REFERENCES

- Morimoto Y, Kobayashi N, Shinohara N, Myojo T, Tanaka I, Nakanishi J. Hazard assessments of manufactured nanomaterials. *J Occup Health*. (2010) 52:325–34. doi: 10.1539/joh.R10003
- Scuri M, Chen BT, Castranova V, Reynolds JS, Johnson VJ, Samsell L, et al. Effects of titanium dioxide nanoparticle exposure on neuroimmune responses in rat airways. *J Toxicol Environ Health*. (2010) 73:1353–69. doi: 10.1080/15287394.2010.497436
- Buffle J. The key role of environmental colloids/nanoparticles for the sustainability of life. *Environ Chem*. (2006) 3:155–8. doi: 10.1071/ENv3n3_ES
- Magaye R, Zhao J, Bowman L, Ding M. Genotoxicity and carcinogenicity of cobalt-, nickel- and copper-based nanoparticles. *Exp Ther Med*. (2012) 4:551–61. doi: 10.3892/etm.2012.656
- Albanese A, Tang PS, Chan WC. The effect of nanoparticle size, shape, and surface chemistry on biological systems. *Annu Rev Biomed Eng*. (2012) 14:1–16. doi: 10.1146/annurev-bioeng-071811-150124
- Mayer A, Vadon M, Rinner B, Novak A, Wintersteiger R, Frohlich E. The role of nanoparticle size in hemocompatibility. *Toxicology*. (2009) 258:139–47. doi: 10.1016/j.tox.2009.01.015
- Deb S, Raja SO, Dasgupta AK, Sarkar R, Chattopadhyay AP, Chaudhuri U, et al. Surface tunability of nanoparticles in modulating platelet functions. *Blood Cells Mol Dis*. (2012) 48:36–44. doi: 10.1016/j.bcmd.2011.09.011
- Tomaszewski KA, Radomski MW, Santos-Martinez MJ. Nanodiagnosics, nanopharmacology and nanotoxicology of platelet-vessel wall interactions. *Nanomedicine*. (2015) 10:1451–75. doi: 10.2217/nnm.14.232
- Yamawaki H, Iwai N. Mechanisms underlying nano-sized air-pollution-mediated progression of atherosclerosis: carbon black causes cytotoxic injury/inflammation and inhibits cell growth in vascular endothelial cells. *Circ J*. (2006) 70:129–40. doi: 10.1253/circj.70.129
- Deb S, Patra HK, Lahiri P, Dasgupta AK, Chakrabarti K, Chaudhuri U. Multistability in platelets and their response to gold nanoparticles. *Nanomedicine*. (2011) 7:376–84. doi: 10.1016/j.nano.2011.01.007
- Park MV, Neigh AM, Vermeulen JP, de la Fonteyne LJ, Verharen HW, Briede JJ, et al. The effect of particle size on the cytotoxicity, inflammation, developmental toxicity and genotoxicity of silver nanoparticles. *Biomaterials*. (2011) 32:9810–7. doi: 10.1016/j.biomaterials.2011.08.085
- Shannahan JH, Lai X, Ke PC, Podila R, Brown JM, Witzmann FA. Silver nanoparticle protein corona composition in cell culture media. *PLoS ONE*. (2013) 8:e74001. doi: 10.1371/journal.pone.0074001
- Gilbert B, Huang F, Zhang H, Waychunas GA, Banfield JF. Nanoparticles: strained and stiff. *Science*. (2004) 305:651–4. doi: 10.1126/science.1098454
- Fröhlich E. Action of nanoparticles on platelet activation and plasmatic coagulation. *Curr Med Chem*. (2016) 23:408–30. doi: 10.2174/0929867323666160106151428
- Santos-Martinez M, Rahme K, Corbalan J, Faulkner C, Holmes J, Tajber L, et al. Pegylation increases platelet biocompatibility of gold nanoparticles. *J Biomed Nanotechnol*. (2014) 10:1004–15. doi: 10.1166/jbn.2014.1813
- Borm PJ, Muller-Schulte D. Nanoparticles in drug delivery and environmental exposure: same size, same risks? *Nanomedicine*. (2006) 1:235–49. doi: 10.2217/17435889.1.2.235
- Dunphy Guzmán KA, Taylor MR, Banfield JF. Environmental risks of nanotechnology: national nanotechnology initiative funding, 2000–2004. *Environ Sci Technol*. (2006) 40:1401–7. doi: 10.1021/es0515708
- Carrero-Sanchez JC, Elias AL, Mancilla R, Arrellin G, Terrones H, Laclette JP, et al. Biocompatibility and toxicological studies of carbon nanotubes doped with nitrogen. *Nano Lett*. (2006) 6:1609–16. doi: 10.1021/nl060548p
- Chithrani BD, Ghazani AA, Chan WC. Determining the size and shape dependence of gold nanoparticle uptake into mammalian cells. *Nano Lett*. (2006) 6:662–8. doi: 10.1021/nl052396o
- Chithrani BD, Chan WC. Elucidating the mechanism of cellular uptake and removal of protein-coated gold nanoparticles of different sizes and shapes. *Nano Lett*. (2007) 7:1542–50. doi: 10.1021/nl070363y
- Jin H, Heller DA, Sharma R, Strano MS. Size-dependent cellular uptake and expulsion of single-walled carbon nanotubes: single particle tracking and a generic uptake model for nanoparticles. *ACS Nano*. (2009) 3:149–58. doi: 10.1021/nn800532m
- Lu F, Wu SH, Hung Y, Mou CY. Size effect on cell uptake in well-suspended, uniform mesoporous silica nanoparticles. *Small*. (2009) 5:1408–13. doi: 10.1002/smll.200900005
- Gratton SE, Ropp PA, Pohlhaus PD, Luft JC, Madden VJ, Napier ME, et al. The effect of particle design on cellular internalization pathways. *Proc Natl Acad Sci USA*. (2008) 105:11613–8. doi: 10.1073/pnas.0801763105
- Qiu Y, Liu Y, Wang L, Xu L, Bai R, Ji Y, et al. Surface chemistry and aspect ratio mediated cellular uptake of Au nanorods. *Biomaterials*. (2010) 31:7606–19. doi: 10.1016/j.biomaterials.2010.06.051
- Holzer M, Bihari P, Praetner M, Uhl B, Reichel C, Fent J, et al. Carbon-based nanomaterials accelerate arteriolar thrombus formation in the murine microcirculation independently of their shape. *J Appl Toxicol*. (2014) 34:1167–76. doi: 10.1002/jat.2996
- He Y, Xu J, Sun X, Ren X, Maharjan A, York P, et al. Cuboidal tethered cyclodextrin frameworks tailored for hemostasis and injured vessel targeting. *Theranostics*. (2019) 9:2489–504. doi: 10.7150/thno.31159
- Gebauer JS, Malissek M, Simon S, Knauer SK, Maskos M, Stauber RH, et al. Impact of the nanoparticle-protein corona on colloidal stability and protein structure. *Langmuir*. (2012) 28:9673–9. doi: 10.1021/la301104a
- Treuel L, Nienhaus GU. Toward a molecular understanding of nanoparticle-protein interactions. *Biophys Rev*. (2012) 4:137–47. doi: 10.1007/s12551-012-0072-0
- Lundqvist M, Stigler J, Elia G, Lynch I, Cedervall T, Dawson KA. Nanoparticle size and surface properties determine the protein corona with possible implications for biological impacts. *Proc Natl Acad Sci USA*. (2008) 105:14265–70. doi: 10.1073/pnas.0805135105
- Lynch I, Salvati A, Dawson KA. Protein-nanoparticle interactions: what does the cell see? *Nat Nanotechnol*. (2009) 4:546–7. doi: 10.1038/nnano.2009.248
- Monopoli MP, Walczyk D, Campbell A, Elia G, Lynch I, Bombelli FB, et al. Physical-chemical aspects of protein corona: relevance to *in vitro* and *in vivo* biological impacts of nanoparticles. *J Am Chem Soc*. (2011) 133:2525–34. doi: 10.1021/ja107583h
- Lesniak A, Salvati A, Santos-Martinez MJ, Radomski MW, Dawson KA, Aberg C. Nanoparticle adhesion to the cell membrane and its effect on nanoparticle uptake efficiency. *J Am Chem Soc*. (2013) 135:1438–44. doi: 10.1021/ja309812z
- Allemann E, Gravel P, Leroux JC, Balant L, Gurny R. Kinetics of blood component adsorption on poly(D,L-lactic acid) nanoparticles: evidence of complement C3 component involvement. *J Biomed Mater Res*. (1997) 37:229–34.
- Monopoli MP, Aberg C, Salvati A, Dawson KA. Biomolecular coronas provide the biological identity of nanosized materials. *Nat Nanotechnol*. (2012) 7:779–86. doi: 10.1038/nnano.2012.207
- Salvador-Morales C, Flahaut E, Sim E, Sloan J, Green ML, Sim RB. Complement activation and protein adsorption by carbon nanotubes. *Mol Immunol*. (2006) 43:193–201. doi: 10.1016/j.molimm.2005.02.006
- Ilinskaya AN, Shah A, Enciso AE, Chan KC, Kaczmarczyk JA, Blonder J, et al. Nanoparticle physicochemical properties determine the activation of intracellular complement. *Nanomedicine*. (2019) 17:266–75. doi: 10.1016/j.nano.2019.02.002
- Dobrovolskaia MA, Patri AK, Zheng J, Clogston JD, Ayub N, Aggarwal P, et al. Interaction of colloidal gold nanoparticles with human blood: effects on particle size and analysis of plasma protein binding profiles. *Nanomedicine*. (2009) 5:106–17. doi: 10.1016/j.nano.2008.08.001
- You J, Zhou J, Zhou M, Liu Y, Robertson JD, Liang D, et al. Pharmacokinetics, clearance, and biosafety of polyethylene glycol-coated hollow gold nanospheres. *Particle Fibre Toxicol*. (2014) 11:26. doi: 10.1186/1743-8977-11-26
- Dobrovolskaia MA, Neun BW, Man S, Ye X, Hansen M, Patri AK, et al. Protein corona composition does not accurately predict hemato-compatibility of colloidal gold nanoparticles. *Nanomedicine*. (2014) 10:1453–63. doi: 10.1016/j.nano.2014.01.009
- Cedervall T, Lynch I, Lindman S, Berggard T, Thulin E, Nilsson H, et al. Understanding the nanoparticle-protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles. *Proc Natl Acad Sci USA*. (2007) 104:2050–5. doi: 10.1073/pnas.0608582104
- Enciso AE, Neun B, Rodriguez J, Ranjan AP, Dobrovolskaia MA, Simanek EE. Nanoparticle effects on human platelets *in vitro*: a

- comparison between PAMAM and triazine dendrimers. *Molecules*. (2016) 21:428. doi: 10.3390/molecules21040428
42. McGuinness C, Duffin R, Brown S, Mills LN, Megson IL, Macnee W, et al. Surface derivatization state of polystyrene latex nanoparticles determines both their potency and their mechanism of causing human platelet aggregation *in vitro*. *Toxicol Sci*. (2011) 119:359–68. doi: 10.1093/toxsci/kfq349
 43. Love SA, Thompson JW, Haynes CL. Development of screening assays for nanoparticle toxicity assessment in human blood: preliminary studies with charged Au nanoparticles. *Nanomedicine*. (2012) 7:1355–64. doi: 10.2217/nnm.12.17
 44. Mody VV, Siwale R, Singh A, Mody HR. Introduction to metallic nanoparticles. *J Pharm Bioallied Sci*. (2010) 2:282–9. doi: 10.4103/0975-7406.72127
 45. Chen J, Saeki F, Wiley BJ, Cang H, Cobb MJ, Li ZY, et al. Gold nanocages: bioconjugation and their potential use as optical imaging contrast agents. *Nano Lett*. (2005) 5:473–7. doi: 10.1021/nl047950t
 46. Fu A, Gu W, Boussett B, Koski K, Gerion D, Manna L, et al. Semiconductor quantum rods as single molecule fluorescent biological labels. *Nano Lett*. (2007) 7:179–82. doi: 10.1021/nl0626434
 47. Gobin AM, Lee MH, Halas NJ, James WD, Drezek RA, West JL. Near-infrared resonant nanoshells for combined optical imaging and photothermal cancer therapy. *Nano Lett*. (2007) 7:1929–34. doi: 10.1021/nl070610y
 48. Lee S, Chon H, Lee M, Choo J, Shin SY, Lee YH, et al. Surface-enhanced Raman scattering imaging of HER2 cancer markers overexpressed in single MCF7 cells using antibody conjugated hollow gold nanospheres. *Biosens Bioelectron*. (2009) 24:2260–3. doi: 10.1016/j.bios.2008.10.018
 49. Pan D, Schmieder AH, Wickline SA, Lanza GM. Manganese-based MRI contrast agents: past, present and future. *Tetrahedron*. (2011) 67:8431–44. doi: 10.1016/j.tet.2011.07.076
 50. Zhu L, Zhou Z, Mao H, Yang L. Magnetic nanoparticles for precision oncology: theranostic magnetic iron oxide nanoparticles for image-guided and targeted cancer therapy. *Nanomedicine*. (2016) 12:73–87. doi: 10.2217/nnm-2016-0316
 51. Bowman M-C, Ballard TE, Ackerson CJ, Feldheim DL, Margolis DM, Melander C. Inhibition of HIV fusion with multivalent gold nanoparticles. *J Am Chem Soc*. (2008) 130:6896–7. doi: 10.1021/ja710321g
 52. Sun L, Singh A, Vig K, Pillai SR, Singh SR. Silver nanoparticles inhibit replication of respiratory syncytial virus. *J Biomed Nanotechnol*. (2008) 4:149–58. doi: 10.1166/jbn.2008.012
 53. Baram-Pinto D, Shukla S, Perkas N, Gedanken A, Sarid R. Inhibition of herpes simplex virus type 1 infection by silver nanoparticles capped with mercaptoethane sulfonate. *Bioconjug Chem*. (2009) 20:1497–502. doi: 10.1021/bc900215b
 54. Lara HH, Ayala-Nuñez NV, Ixtapan-Turrent L, Rodríguez-Padilla C. Mode of antiviral action of silver nanoparticles against HIV-1. *J Nanobiotechnol*. (2010) 8:1. doi: 10.1186/1477-3155-8-1
 55. Mallipeddi R, Rohan LC. Nanoparticle-based vaginal drug delivery systems for HIV prevention. *Expert Opin Drug Deliv*. (2010) 7:37–48. doi: 10.1517/17425240903338055
 56. Bhattacharya R, Mukherjee P, Xiong Z, Atala A, Soker S, Mukhopadhyay D. Gold nanoparticles inhibit VEGF165-induced proliferation of HUVEC cells. *Nano Lett*. (2004) 4:2479–81. doi: 10.1021/nl0483789
 57. Tsai CY, Shiao AL, Chen SY, Chen YH, Cheng PC, Chang MY, et al. Amelioration of collagen-induced arthritis in rats by nanogold. *Arthritis Rheum*. (2007) 56:544–54. doi: 10.1002/art.22401
 58. Arvizo RR, Rana S, Miranda OR, Bhattacharya R, Rotello VM, Mukherjee P. Mechanism of anti-angiogenic property of gold nanoparticles: role of nanoparticle size and surface charge. *Nanomedicine*. (2011) 7:580–7. doi: 10.1016/j.nano.2011.01.011
 59. Gobbo OL, Sjaastad K, Radomski MW, Volkov Y, Prina-Mello A. Magnetic nanoparticles in cancer theranostics. *Theranostics*. (2015) 5:1249–63. doi: 10.7150/thno.11544
 60. Berry JP, Arnoux B, Stanislas G, Galle P, Chretien J. A microanalytic study of particles transport across the alveoli: role of blood platelets. *Biomedicine*. (1977) 27:354–7.
 61. Pope CA III, Burnett RT, Thun MJ, Calle EE, Krewski D, Ito K, et al. Lung cancer, cardiopulmonary mortality, and long-term exposure to fine particulate air pollution. *JAMA*. (2002) 287:1132–41. doi: 10.1001/jama.287.9.1132
 62. Radomski A, Jurasz P, Alonso-Escobedo D, Drews M, Morandi M, Malinski T, et al. Nanoparticle-induced platelet aggregation and vascular thrombosis. *Br J Pharmacol*. (2005) 146:882–93. doi: 10.1038/sj.bjp.0706386
 63. Gaffney AM, Santos-Martinez MJ, Satti A, Major TC, Wynne KJ, Gun'ko YK, et al. Blood biocompatibility of surface-bound multi-walled carbon nanotubes. *Nanomedicine*. (2015) 11:39–46. doi: 10.1016/j.nano.2014.07.005
 64. Anselmo AC, Modery-Pawlowski CL, Menegatti S, Kumar S, Vogus DR, Tian LL, et al. Platelet-like nanoparticles: mimicking shape, flexibility, and surface biology of platelets to target vascular injuries. *ACS Nano*. (2014) 8:11243–53. doi: 10.1021/nn503732m
 65. Roy SC, Paulose M, Grimes CA. The effect of TiO₂ nanotubes in the enhancement of blood clotting for the control of hemorrhage. *Biomaterials*. (2007) 28:4667–72. doi: 10.1016/j.biomaterials.2007.07.045
 66. Deb S, Chatterjee M, Bhattacharya J, Lahiri P, Chaudhuri U, Choudhuri SP, et al. Role of purinergic receptors in platelet-nanoparticle interactions. *Nanotoxicology*. (2007) 1:93–103. doi: 10.1080/17435390600772978
 67. Shrivastava S, Bera T, Singh SK, Singh G, Ramachandrarao P, Dash D. Characterization of antiplatelet properties of silver nanoparticles. *ACS Nano*. (2009) 3:1357–64. doi: 10.1021/nn900277t
 68. Aseychev AV, Azizova OA, Beckman EM, Dudnik LB, Sergienko VI. Effect of gold nanoparticles coated with plasma components on ADP-induced platelet aggregation. *Bull Exp Biol Med*. (2013) 155:685–8. doi: 10.1007/s10517-013-2226-x
 69. Santos-Martinez MJ, Inkiewicz-Stepniak I, Medina C, Rahme K, D'Arcy DM, Fox D, et al. The use of quartz crystal microbalance with dissipation (QCM-D) for studying nanoparticle-induced platelet aggregation. *Int J Nanomed*. (2012) 7:243–55. doi: 10.2147/IJN.S26679
 70. Hecold M, Buczkowska R, Mucha A, Grzesiak J, Rac-Rumijowska O, Teterycz H, et al. The effect of PEI and PVP-stabilized gold nanoparticles on equine platelets activation: potential application in equine regenerative medicine. *J Nanomater*. (2017) 2017:11. doi: 10.1155/2017/8706921
 71. Villegas MG, Ceballos MT, Urquijo J, Torres EY, Ortiz-Reyes BL, Arnache-Olmos OL, et al. Poly(acrylic acid)-coated iron oxide nanoparticles interact with mononuclear phagocytes and decrease platelet aggregation. *Cell Immunol*. (2019) 338:51–62. doi: 10.1016/j.cellimm.2019.03.005
 72. Guildford AL, Poletti T, Osbourne LH, Di Cerbo A, Gatti AM, Santin M. Nanoparticles of a different source induce different patterns of activation in key biochemical and cellular components of the host response. *J R Soc Interf*. (2009) 6:1213–21. doi: 10.1098/rsif.2009.0021
 73. Aurich K, Wesche J, Palankar R, Schluter R, Bakchoul T, Greinacher A. Magnetic nanoparticle labeling of human platelets from platelet concentrates for recovery and survival studies. *ACS Appl Mater Interf*. (2017) 9:34666–73. doi: 10.1021/acsami.7b10113
 74. Bircher L, Theusinger OM, Locher S, Eugster P, Roth-Z'graggen B, Schumacher CM, et al. Characterization of carbon-coated magnetic nanoparticles using clinical blood coagulation assays: effect of PEG-functionalization and comparison to silica nanoparticles. *J Mater Chem B*. (2014) 2:3753–8. doi: 10.1039/C4TB00208C
 75. Aurich K, Spoerl M-C, Füll B, Sietmann R, Greinacher A, Hosten N, et al. Development of a method for magnetic labeling of platelets. *Nanomedicine*. (2012) 8:537–44. doi: 10.1016/j.nano.2011.09.008
 76. Jun EA, Lim KM, Kim K, Bae ON, Noh JY, Chung KH, et al. Silver nanoparticles enhance thrombus formation through increased platelet aggregation and procoagulant activity. *Nanotoxicology*. (2011) 5:157–67. doi: 10.3109/17435390.2010.506250
 77. Laloy J, Minet V, Alpan L, Mullier F, Beken S, Toussaint O, et al. Impact of silver nanoparticles on haemolysis, platelet function and coagulation. *Nanobiomedicine*. (2014) 1:4. doi: 10.5772/59346
 78. Krajewski S, Prucek R, Panacek A, Avci-Adali M, Nolte A, Straub A, et al. Hemocompatibility evaluation of different silver nanoparticle

- concentrations employing a modified Chandler-loop *in vitro* assay on human blood. *Acta Biomater.* (2013) 9:7460–8. doi: 10.1016/j.actbio.2013.03.016
79. Ragaseema VM, Unnikrishnan S, Kalliyana Krishnan V, Krishnan LK. The antithrombotic and antimicrobial properties of PEG-protected silver nanoparticle coated surfaces. *Biomaterials.* (2012) 33:3083–92. doi: 10.1016/j.biomaterials.2012.01.005
 80. Smock KJ, Schmidt RL, Hadlock G, Stoddard G, Grainger DW, Munger MA. Assessment of orally dosed commercial silver nanoparticles on human *ex vivo* platelet aggregation. *Nanotoxicology.* (2014) 8:328–33. doi: 10.3109/17435390.2013.788749
 81. Huang H, Lai W, Cui M, Liang L, Lin Y, Fang Q, et al. An evaluation of blood compatibility of silver nanoparticles. *Sci Rep.* (2016) 6:25518. doi: 10.1038/srep25518
 82. Haberl N, Hirn S, Holzer M, Zuchtriegel G, Rehberg M, Krombach F. Effects of acute systemic administration of TiO₂, ZnO, SiO₂, and Ag nanoparticles on hemodynamics, hemostasis and leukocyte recruitment. *Nanotoxicology.* (2015) 9:963–71. doi: 10.3109/17435390.2014.992815
 83. Nemmar A, Melghit K, Ali BH. The acute proinflammatory and prothrombotic effects of pulmonary exposure to rutile TiO₂ nanorods in rats. *Exp Biol Med.* (2008) 233:610–9. doi: 10.3181/0706-RM-165
 84. Šimundić M, Drašler B, Šuštar V, Zupanc J, Štukelj R, Makovec D, et al. Effect of engineered TiO₂ and ZnO nanoparticles on erythrocytes, platelet-rich plasma and giant unilamellar phospholipid vesicles. *BMC Vet Res.* (2013) 9:7. doi: 10.1186/1746-6148-9-7
 85. Geys J, Nemmar A, Verbeken E, Smolders E, Ratoi M, Hoylaerts MF, et al. Acute toxicity and prothrombotic effects of quantum dots: impact of surface charge. *Environ Health Perspect.* (2008) 116:1607–13. doi: 10.1289/ehp.11566
 86. Deb S, Dasgupta AK. *Thrombotic Inception at Nano-Scale Acute Coronary Syndromes*. Open access peer-reviewed chapter (2012). Available online at: <https://www.intechopen.com/books/acute-coronary-syndromes/thrombotic-inception-at-nano-scale>
 87. Bouwmeester H, Lynch I, Marvin HJ, Dawson KA, Berges M, Braguer D, et al. Minimal analytical characterization of engineered nanomaterials needed for hazard assessment in biological matrices. *Nanotoxicology.* (2011) 5:1–11. doi: 10.3109/17435391003775266
 88. Taurozzi JS, Hackley VA, Wiesner MR. Ultrasonic dispersion of nanoparticles for environmental, health and safety assessment—issues and recommendations. *Nanotoxicology.* (2011) 5:711–29. doi: 10.3109/17435390.2010.528846
 89. Strojan K, Leonardi A, Bregar VB, Križaj I, Svete J, Pavlin M. Dispersion of nanoparticles in different media importantly determines the composition of their protein corona. *PLoS ONE.* (2017) 12:e0169552. doi: 10.1371/journal.pone.0169552
 90. Santos-Martinez MJ, Prina-Mello A, Medina C, Radomski MW. Analysis of platelet function: role of microfluidics and nanodevices. *Analyst.* (2011) 136:5120–6. doi: 10.1039/c1an15445a
 91. Paciotti GF, Myer L, Weinreich D, Goia D, Pavel N, McLaughlin RE, et al. Colloidal gold: a novel nanoparticle vector for tumor directed drug delivery. *Drug Deliv.* (2004) 11:169–83. doi: 10.1080/10717540490433895
 92. Huff TB, Tong L, Zhao Y, Hansen MN, Cheng JX, Wei A. Hyperthermic effects of gold nanorods on tumor cells. *Nanomedicine.* (2007) 2:125–32. doi: 10.2217/17435889.2.1.125
 93. Goodman CM, McCusker CD, Yilmaz T, Rotello VM. Toxicity of gold nanoparticles functionalized with cationic and anionic side chains. *Bioconjug Chem.* (2004) 15:897–900. doi: 10.1021/bc049951i
 94. Pernodet N, Fang X, Sun Y, Bakhtina A, Ramakrishnan A, Sokolov J, et al. Adverse effects of citrate/gold nanoparticles on human dermal fibroblasts. *Small.* (2006) 2:766–73. doi: 10.1002/smll.200500492
 95. Pan Y, Neuss S, Leifert A, Fischler M, Wen F, Simon U, et al. Size-dependent cytotoxicity of gold nanoparticles. *Small.* (2007) 3:1941–9. doi: 10.1002/smll.200700378
 96. Alkilany AM, Murphy CJ. Toxicity and cellular uptake of gold nanoparticles: what we have learned so far? *J Nanopart Res.* (2010) 12:2313–33. doi: 10.1007/s11051-010-9911-8
 97. Malugin A, Ghandehari H. Cellular uptake and toxicity of gold nanoparticles in prostate cancer cells: a comparative study of rods and spheres. *J Appl Toxicol.* (2010) 30:212–7. doi: 10.1002/jat.1486
 98. White JG. Platelets are coverocytes, not phagocytes: uptake of bacteria involves channels of the open canalicular system. *Platelets.* (2005) 16:121–31. doi: 10.1080/09537100400007390
 99. Santos-Martinez MJ, Tomaszewski KA, Medina C, Bazou D, Gilmer JE, Radomski MW. Pharmacological characterization of nanoparticle-induced platelet microaggregation using quartz crystal microbalance with dissipation: comparison with light aggregometry. *Int J Nanomed.* (2015) 10:5107–19. doi: 10.2147/IJN.S84305
 100. Deng ZJ, Liang M, Monteiro M, Toth I, Minchin RF. Nanoparticle-induced unfolding of fibrinogen promotes Mac-1 receptor activation and inflammation. *Nat Nanotechnol.* (2011) 6:39–44. doi: 10.1038/nnano.2010.250
 101. Easo SL, Mohanan PV. *In vitro* hematological and *in vivo* immunotoxicity assessment of dextran stabilized iron oxide nanoparticles. *Colloids Surf B.* (2015) 134:122–30. doi: 10.1016/j.colsurfb.2015.06.046
 102. Barry L, Holmes JD, Otway DJ, Copley MP, Kazakova O, Morris MA. Unusual magnetism in templated NiS nanoparticles. *J Phys.* (2010) 22:076001. doi: 10.1088/0953-8984/22/7/076001
 103. Sun C, Ma M, Yang J, Zhang Y, Chen P, Huang W, et al. Phase-controlled synthesis of α -NiS nanoparticles confined in carbon nanorods for high performance supercapacitors. *Sci Rep.* (2014) 4:7054. doi: 10.1038/srep07054
 104. Alonso F, Riente P, Yus M. Nickel nanoparticles in hydrogen transfer reactions. *Acc Chem Res.* (2011) 44:379–91. doi: 10.1021/ar1001582
 105. Ahamed M, Alhadlaq HA. Nickel nanoparticle-induced dose-dependent cyto-genotoxicity in human breast carcinoma MCF-7 cells. *Onco Targets Ther.* (2014) 7:269–80. doi: 10.2147/OTT.S58044
 106. Di Bucchianico S, Gliga AR, Åkerlund E, Skoglund S, Wallinder IO, Fadeel B, et al. Calcium-dependent cyto- and genotoxicity of nickel metal and nickel oxide nanoparticles in human lung cells. *Part Fibre Toxicol.* (2018) 15:32. doi: 10.1186/s12989-018-0268-y
 107. Wahab R, Kaushik NK, Kaushik N, Choi EH, Umar A, Dwivedi S, et al. ZnO nanoparticles induces cell death in malignant human T98G gliomas, KB and non-malignant HEK cells. *J Biomed Nanotechnol.* (2013) 9:1181–9. doi: 10.1166/jbn.2013.1652
 108. Wahab R, Siddiqui MA, Saquib Q, Dwivedi S, Ahmad J, Musarrat J, et al. ZnO nanoparticles induced oxidative stress and apoptosis in HepG2 and MCF-7 cancer cells and their antibacterial activity. *Colloids Surf B Biointerf.* (2014) 117:267–76. doi: 10.1016/j.colsurfb.2014.02.038
 109. Xu X, Chen D, Yi Z, Jiang M, Wang L, Zhou Z, et al. Antimicrobial mechanism based on H₂O₂ generation at oxygen vacancies in ZnO crystals. *Langmuir.* (2013) 29:5573–80. doi: 10.1021/la400378t
 110. Mishra YK, Adelung R, Rohl C, Shukla D, Spors F, Tiwari V. Virostatic potential of micro-nano filopodia-like ZnO structures against herpes simplex virus-1. *Antiviral Res.* (2011) 92:305–12. doi: 10.1016/j.antiviral.2011.08.017
 111. Antoine TE, Mishra YK, Trigilio J, Tiwari V, Adelung R, Shukla D. Prophylactic, therapeutic and neutralizing effects of zinc oxide tetrapod structures against herpes simplex virus type-2 infection. *Antiviral Res.* (2012) 96:363–75. doi: 10.1016/j.antiviral.2012.09.020
 112. Antoine TE, Hadigal SR, Yakoub AM, Mishra YK, Bhattacharya P, Haddad C, et al. Intravaginal zinc oxide tetrapod nanoparticles as novel immunoprotective agents against genital herpes. *J Immunol.* (2016) 196:4566–75. doi: 10.4049/jimmunol.1502373
 113. Ahmad J, Wahab R, Siddiqui MA, Musarrat J, Al-Khedhairi AA. Zinc oxide quantum dots: a potential candidate to detain liver cancer cells. *Bioprocess Biosyst Eng.* (2015) 38:155–63. doi: 10.1007/s00449-014-1254-x
 114. Wahab R, Khan F, Mishra YK, Musarrat J, Al-Khedhairi AA. Antibacterial studies and statistical design set data of quasi zinc oxide nanostructures. *RSC Adv.* (2016) 6:32328–39. doi: 10.1039/C6RA05297E
 115. Wahab R, Kaushik N, Khan F, Kaushik NK, Choi EH, Musarrat J, et al. Self-styled ZnO nanostructures promotes the cancer cell damage and suppresses the epithelial phenotype of glioblastoma. *Sci Rep.* (2016) 6:19950. doi: 10.1038/srep19950
 116. Major TC, Brant DO, Burney CP, Amoako KA, Annich GM, Meyerhoff ME, et al. The hemocompatibility of a nitric oxide generating polymer that catalyzes S-nitrosothiol decomposition

- in an extracorporeal circulation model. *Biomaterials*. (2011) 32:5957–69. doi: 10.1016/j.biomaterials.2011.03.036
117. Warheit DB, Sayes CM, Reed KL, Swain KA. Health effects related to nanoparticle exposures: environmental, health and safety considerations for assessing hazards and risks. *Pharmacol Ther*. (2008) 120:35–42. doi: 10.1016/j.pharmthera.2008.07.001
 118. Hussain SM, Hess KL, Gearhart JM, Geiss KT, Schlager JJ. *In vitro* toxicity of nanoparticles in BRL 3A rat liver cells. *Toxicol In Vitro*. (2005) 19:975–83. doi: 10.1016/j.tiv.2005.06.034
 119. Sayes CM, Wahi R, Kurian PA, Liu Y, West JL, Ausman KD, et al. Correlating nanoscale titania structure with toxicity: a cytotoxicity and inflammatory response study with human dermal fibroblasts and human lung epithelial cells. *Toxicol Sci*. (2006) 92:174–85. doi: 10.1093/toxsci/kfj197
 120. Bihari P, Holzer M, Praetner M, Fent J, Lerchenberger M, Reichel CA, et al. Single-walled carbon nanotubes activate platelets and accelerate thrombus formation in the microcirculation. *Toxicology*. (2010) 269:148–54. doi: 10.1016/j.tox.2009.08.011
 121. Bobo D, Robinson KJ, Islam J, Thurecht KJ, Corrie SR. Nanoparticle-based medicines: a review of FDA-approved materials and clinical trials to date. *Pharm Res*. (2016) 33:2373–87. doi: 10.1007/s11095-016-1958-5
 122. Tajdidzadeh M, Azmi BZ, Yunus WMM, Talib ZA, Sadrolhosseini AR, Karimzadeh K, et al. Synthesis of silver nanoparticles dispersed in various aqueous media using laser ablation. *Sci World J*. (2014) 2014:324921. doi: 10.1155/2014/324921
 123. Albanese A, Tang PS, Chan CW. The effect of nanoparticle size, shape, and surface chemistry on biological systems. *Annu Rev Biomed Eng*. (2012) 14:1–16. doi: 10.1146/annurev-bioeng-071811-150124
 124. Hulla J, Sahu S, Hayes A. Nanotechnology: history and future. *Hum Exp Toxicol*. (2015) 34:1318–21. doi: 10.1177/0960327115603588
 125. Khalili Fard J, Jafari S, Eghbal MA. A review of molecular mechanisms involved in toxicity of nanoparticles. *Adv Pharm Bull*. (2015) 5:447–54. doi: 10.15171/apb.2015.061

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Hante, Medina and Santos-Martinez. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Platelet Phenotype and Function in the Setting of Pediatric Extracorporeal Membrane Oxygenation (ECMO): A Systematic Review

Hui Ping Yaw^{1,2*}, Suelyn Van Den Helm¹, Graeme MacLaren^{1,2,3,4}, Matthew Linden⁵, Paul Monagle^{1,2,6} and Vera Ignjatovic^{1,2}

¹ Department of Haematology Research, Murdoch Children's Research Institute, Melbourne, VIC, Australia, ² Department of Paediatrics, The University of Melbourne, Melbourne, VIC, Australia, ³ Paediatric Intensive Care Unit, The Royal Children's Hospital, Melbourne, VIC, Australia, ⁴ Cardiothoracic Intensive Care Unit, National University Health System, Singapore, Singapore, ⁵ School of Biomedical Sciences, The University of Western Australia, Perth, WA, Australia, ⁶ Department of Clinical Haematology, The Royal Children's Hospital, Melbourne, VIC, Australia

OPEN ACCESS

Edited by:

Rory R. Koenen,
Maastricht University, Netherlands

Reviewed by:

Patricia B. Maguire,
University College Dublin, Ireland
Philipp Diehl,
University Heart Center
Freiburg, Germany
Heidi Dalton,
Inova Health System, United States

*Correspondence:

Hui Ping Yaw
hpyaw1006@gmail.com

Specialty section:

This article was submitted to
Atherosclerosis and Vascular
Medicine,
a section of the journal
Frontiers in Cardiovascular Medicine

Received: 20 June 2019

Accepted: 29 August 2019

Published: 18 September 2019

Citation:

Yaw HP, Van Den Helm S,
MacLaren G, Linden M, Monagle P
and Ignjatovic V (2019) Platelet
Phenotype and Function in the Setting
of Pediatric Extracorporeal Membrane
Oxygenation (ECMO): A
Systematic Review.
Front. Cardiovasc. Med. 6:137.
doi: 10.3389/fcvm.2019.00137

Background: Despite increasing technical improvement and extracorporeal membrane oxygenation (ECMO)-related knowledge over the past three decades, morbidity and mortality associated with bleeding and clotting complications remain high in pediatric patients undergoing ECMO. Platelets, a key element of the coagulation system, have been proposed to be the main cause of coagulopathy in the setting of ECMO. This systematic review aims to summarize and discuss the existing knowledge of platelet phenotype and function in the pediatric ECMO population.

Methods: A systematic review was conducted for the Embase, Medline, and PubMed databases following the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines.

Results: The detailed study selection process yielded a total of 765 studies and only 3 studies that fulfilled the selection criteria were included in this review. Techniques used to assess platelet function in the three existing studies included platelet aggregometry, flow cytometry, and thromboelastography-platelet mapping. The finding that is common to the three studies is reduced platelet function in pediatric patients during ECMO either compared to before the initiation of ECMO or in non-survivors compared to survivors. Two studies demonstrated reduced platelet aggregation that are irreversible by platelet transfusion during ECMO. Two studies reported bleeding events and mortality in children on ECMO and none of the studies investigated thrombotic events.

Conclusions: This systematic review demonstrates the extremely limited information available for platelet phenotype and function in the pediatric ECMO population. Evidence from the existing literature suggests reduced platelet aggregation and increased platelet activation in children during ECMO. However, this needs to be interpreted with care due to the limitations associated with the techniques used for platelet function testing.

Furthermore, the association between platelet dysfunction and clinical outcomes in the pediatric ECMO population remains elusive. Multiple research gaps have been identified when it comes to the knowledge of platelet phenotype and function of children on ECMO, highlighting the need for robust, well-designed studies in this setting.

Keywords: platelet, extracorporeal membrane oxygenation (ECMO), pediatric, bleeding, clotting, phenotype and function

INTRODUCTION

Extracorporeal membrane oxygenation (ECMO) is a modified form of cardiopulmonary bypass that aims to provide short to medium-length cardiac and/or respiratory support to patients. Despite the improvement in technologies and clinical practice over the years, bleeding, and/or thrombosis complications remain a major cause of morbidity and mortality in children on ECMO, with an overall survival rate of 54.9% (1). Using definitions of bleeding and thrombosis from the Extracorporeal Life Support Organization (ELSO), a study by Dalton et al. on 514 patients showed that bleeding is common in children on ECMO, with a rate of 70.2%, including intracranial hemorrhage in 16% of cases (1). Thrombotic events occurred in 37.5% of the patients, with 31.1% of patients requiring circuit component changes due to thrombosis (1).

The artificial surface and shear stress originating from the ECMO system have been proposed to be the main cause of coagulopathy seen in ECMO patients, via mechanisms such as activation of the hemostatic system. Modification of platelet function, a key element of the clotting system, has been suggested to be the main determinant of ECMO-induced bleeding and/or thrombosis in both the adult and pediatric ECMO population. Particularly, shear stress-induced platelet activation and loss of important platelet receptors have been reported in multiple studies for adults on ECMO and the resultant platelet dysfunction has been associated with increased thrombotic propensity and reduced hemostatic capacity (2–5).

Differences in platelet function and proteome between healthy adults and healthy children have been evaluated in multiple studies (6–8). Existing research in the ECMO population focuses on adults. However, the results may not be applicable to children due to the physiological differences between adults and children and the complex nature of ECMO. In addition, differences in response toward unfractionated heparin (UFH) (the main anticoagulant used in the setting of ECMO) between adults and children have also been documented (9, 10) and may be associated with the increased risk of anticoagulation-related complications in the pediatric ECMO cohort. Furthermore, differences in the clinical outcome between the adult and children ECMO patients (11) may also indicate differences in the pathophysiological development of coagulopathy between these two patient cohorts. Currently, there is very limited knowledge specific to platelet phenotype and function in children on ECMO (12–14) and there is no systematic review that summarizes the existing evidence for this population. On the other hand, existing studies relevant to platelet function and acquired von Willebrand syndrome were discussed previously for the adult

ECMO population (15, 16). Hence, this systematic review aims to assemble and elaborate on existing studies focusing on platelet phenotype and function in children on ECMO.

METHODS

Search Strategy

This systematic review was conducted based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (17). The Embase, Medline and PubMed databases were searched systematically on August 1, 2019 to identify studies in human and limited to the English language only. The following search terms were used for each of the database:

Embase: (*extracorporeal oxygenation OR *membrane oxygenator OR extracorporeal-membrane-oxygenation OR extra-corporeal-membrane-oxygenation OR extracorporeal-life-support OR extra-corporeal-life-support OR extracorporeal-cardiopulmonary-resuscitation OR extra-corporeal-cardiopulmonary-resuscitation OR ecmo OR ecls OR ecpr) AND (thrombocyte OR thrombocyte activation OR antithrombocytic agent OR platelet count OR blood clotting parameters OR thrombocytopenia OR thrombocytopenia OR thrombocytosis OR thrombocyte transfusion OR fluoroimmunoassay OR *flow cytometry OR flow-cytometry OR aggregometry or platelet* OR thrombocyt* OR *thrombocyte function OR *thrombocyte dysfunction OR thrombocyte-count) AND (newborn* OR baby OR babies OR neonat* OR infan* OR toddler* OR pre-schooler* OR preschooler* OR kindergarten OR boy OR boys OR girl OR girls OR child OR children OR childhood OR adolescen* OR pediatric* OR paediatric* OR youth* OR teen OR teens OR teenage* OR school-aged* OR school-child* OR school-girl* OR school-boy* OR schoolgirl* OR schoolboy*).

Medline: (extracorporeal membrane oxygenation OR extracorporeal circulation OR oxygenators, membrane) AND (blood platelets OR platelet activation OR platelet aggregation inhibitors OR platelet count OR platelet function tests OR plateletpheresis OR thrombocytopenia OR thrombocytopenia OR thrombocytosis OR platelet transfusion OR fluoroimmunonoassay OR flow cytometry OR flow-cytometry or aggregometry or platelet* or thrombocyt*) AND (newborn* OR baby OR babies OR neonat* OR infan* OR toddler* OR pre-schooler* OR preschooler* OR kindergarten OR boy OR boys OR girl OR girls OR child OR children OR childhood OR adolescen* OR pediatric* OR paediatric* OR youth* OR teen OR teens OR teenage*

OR school-aged* OR school-child* OR school-girl* OR school-boy* OR schoolgirl* OR schoolboy*).

PubMed: #1 (Extracorporeal-oxygenation OR Extracorporeal-oxygenation OR Extracorporeal-membrane-oxygenation OR Extra-corporeal-membrane-oxygenation OR extracorporeal-life-support OR extra-corporeal-life-support OR extracorporeal-cardiopulmonary-resuscitation OR extra-corporeal-cardiopulmonary-resuscitation OR ECMO OR ECLS OR ECPR OR Extracorporeal-circulation OR Extra-corporeal-circulation OR membrane-oxygenator*) AND (Platelet* OR thrombocyt* OR Platelet-count OR Platelet-activation* OR thrombocyte-activation* OR Platelet-function* OR blood-clotting-parameter* OR Thrombocytopaenia* OR Thrombocytopenia* OR Thrombo-cytopenia* OR Thrombo-cytopenia* OR Platelet-transfusion* OR thrombocyte-transfusion* OR Flow-cytometry OR fluoroimmunoassay* OR fluoro -immunoassay* OR Platelet-aggregation OR antithrombotic-agent* OR anti-thrombotic-agent* OR Plateletpheresis OR Thrombopheresis OR thrombocytophoresis OR aggregometry) AND (NOT(NLM OR publisher[sb] OR inprocess[sb] OR pubmednotmedline[sb] OR indatereview[sb] OR pubstatusaheadofprint)) NOT ((animal OR animals OR rat OR rats OR mouse OR mice OR rodent* OR murine OR sheep) NOT (human OR humans OR newborn* OR baby OR babies OR neonat* OR infan* OR toddler* OR pre-schooler* OR preschooler* OR kindergarten OR boy OR boys OR girl OR girls OR child OR children OR childhood OR adolescen* OR pediatric* OR paediatric* OR youth* OR teen OR teens OR teenage* OR school-aged* OR school-child* OR school-girl* OR school-boy* OR schoolgirl* OR schoolboy*)). #2 (Case Reports[ptyp]) OR (Review[ptyp]). #3 #1 NOT #2.

Study Selection

Inclusion criteria: (I) pediatric patients (0–18 years), (II) primary research, (III) platelet function assessed, (IV) English language, and (V) human study.

Exclusion criteria: (I) adult patients (>18 years), (II) review, case report, guideline, editorial correspondence, or conference abstract, (III) only platelet count measured, and (IV) animal study.

RESULTS

A total of 765 studies were identified with this systematic review search strategy. The majority of the studies ($n = 757$) that did not fulfill the inclusion criteria were excluded, including case reports that did not focus on assessing platelet phenotype and function. Eight studies were included for full-text screening and five of them were excluded for either patients aged >18 years or had assessed only platelet count in the study. A total of three studies matched the inclusion criteria and were summarized in this review. The steps used following the PRISMA guidelines are outlined in **Figure 1**. The summary for each of the included studies is included in **Table 1**.

DISCUSSION

This systematic review demonstrates the absolute lack of studies that have focused on platelet phenotype and function in pediatric patients on ECMO. Such a lack of information in this population could reflect challenges in ethical approval and limited availability of blood sample volume for pediatric research. The main techniques used in the three existing studies included aggregometry, flow cytometry, and thromboelastography-platelet mapping. The common findings are reduced platelet function during ECMO. Particularly, evidence showed that the acquired platelet dysfunction during ECMO is not resolved by platelet transfusion. The relationship of plasma problems such as acquired von Willebrand's disease to the platelet dysfunction is unknown. In addition, the association between platelet dysfunction and bleeding and/or thrombosis commonly seen in children on ECMO has not been investigated to date.

Two out of the three studies utilized platelet aggregometry to investigate platelet function using agonists such as collagen, ADP and ristocetin (12, 13). Both studies reported reduced platelet aggregation in pediatric patients during ECMO despite the platelet transfusions given to the patients during ECMO. Robinson et al. reported reduced platelet aggregation in response to ADP and ristocetin but not for collagen within 15 min upon initiation of ECMO (12) whereas Cheung et al. have shown a decrease in platelet aggregation in response to collagen in a time-dependent manner starting from within 24 h upon cannulation for ECMO (13). The difference in collagen-induced platelet aggregation between the two studies may be related to the difference in concentration of collagen (5 vs. 10 $\mu\text{g/mL}$) used.

Despite being the gold standard in platelet function testing for many years, the applicability of aggregometry to the pediatric patients is often limited by the requirement of a large volume of blood. Specifically, platelet aggregometry usually requires 0.5–2 mL of blood which is a significant volume of blood to be withdrawn on multiple occasions from children who are critically ill and are sampled very frequently for a variety of clinical assays. Dalton et al. showed in children on ECMO that laboratory blood sampling is the primary cause of significant blood loss and is the main reason of transfusion in 42.2% of the studied population (1). Furthermore, multiple studies have suggested a strong association between platelet aggregometry and platelet count (18–20), which is problematic given that thrombocytopaenia is commonly found in children on ECMO. A study by Fernia et al. showed that light transmission aggregometry can be affected by platelet counts of $<150 \times 10^9 /\text{L}$ (20). None of the existing studies have reported whether any cut-off value was set for the platelet count for patients to be included in the analysis of platelet function using platelet aggregometry. Hence, results from the existing studies need to be interpreted with care.

In recent years, whole blood flow cytometry analysis of platelet function has gained attention in the pediatric setting for the requirement of only a minimal amount of blood and enabling analysis of platelets in their physiological environment, in the presence of the other blood cells with minimal manipulation. However, multiple technical challenges such as the requirement for trained-personnel and expensive reagents have limited its

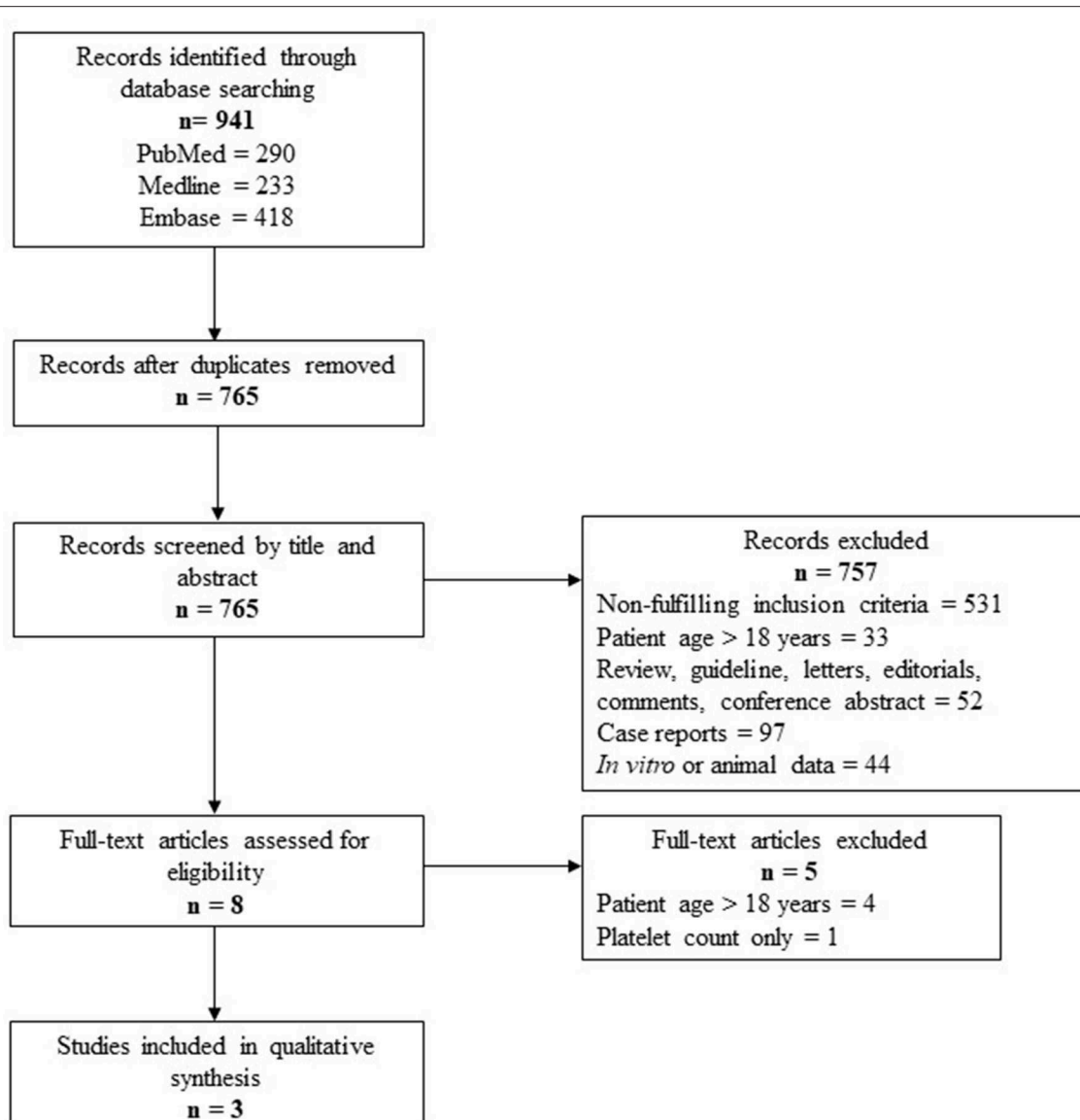


FIGURE 1 | Summary of the study selection process for the systematic review.

usage in a wider population. Only one study utilized flow cytometry to evaluate the expression of GPIb and IIIa, platelet markers important for adhesion and aggregation (12). There was no difference in the expression of these two receptors during ECMO compared to before initiation of ECMO. However, this study is now over 25 years old and flow cytometry techniques have improved considerably. In addition, these results must be interpreted with care as platelets are highly subjected to pre-analytical activation and have a limited processing window. Optimization of the flow cytometry methods for evaluation of platelet phenotype and function is important and this was not indicated as part of the study by Robinson et al. (12). Flow cytometric analysis of surface receptors on circulating platelets in adults on mechanical circulatory support (MCS) showed that ECMO patients had significantly lower expression of GPIbα

and GPVI (platelet adhesion receptors) compared to healthy individuals (21). Whether the same changes occur in children of different ages remains to be determined.

In addition to platelet aggregometry and flow cytometry, thromboelastography-platelet mapping (TEG®-PM) has gained popularity as a platelet function assay in recent years and few centers have incorporated this system for hemostasis monitoring in children on ECMO (22). By using TEG®-PM, Saini et al. reported severe platelet dysfunction in more than 75% of the patient cohort (14). Using Multiplate® analyser with ADP and ristocetin, Nair et al. demonstrated 50–72% incidence of platelet dysfunction in the adult ECMO population (23). Furthermore, platelet count and TEG®-PM were shown to be a better predictor of severe bleeding and mortality compared to the activated clotting time in children on ECMO (14). A study by Weitzel

TABLE 1 | The summary of the studies included in the systematic review for platelet phenotype and function in pediatric patients on ECMO.

References	Sample size	Age	Study design	Mode of ECMO	Indication	Duration of ECMO	Time points	Clinical events (number of events)	Therapeutic agents with reported anti-platelet effects	Monitoring of platelet function (method)	Results	
											Platelet count	Platelet function
Robinson et al. (12)	<i>n</i> = 10	Reported: Mean (range) = 3 (1–8) days Calculated: Range = 7 days	Prospective	Not reported	Respiratory failure (<i>n</i> = 10)	Reported: Range = 4–16 days Calculated: Range = 12 days	Baseline 15 min 60 min after initiation of ECMO 15 min 60 min after platelet transfusion	Bleeding <i>n</i> = 0 Thrombosis Not reported Mortality Not reported	Not reported	Flow cytometry (Surface-bound GPIb and IIIa) Platelet aggregation (Whole blood aggregometer using collagen, ADP and ristocetin as agonists) Bioluminescent assay (Luciferase detection of platelet ATP release in response to collagen and thrombin)	Significant reduction 60 min after ECMO initiation compared to baseline Decreased to pre-transfusion value by 60 min after transfusion	Significant reduction 15 min after ECMO initiation compared to baseline No change in GPIb and IIIa expression Platelet aggregation was not improved by platelet transfusion but resolved within 8 h after decannulation from ECMO
Cheung et al. (13)	<i>n</i> = 10	Reported: Mean ± SEM = 25 ± 4 h postnatal age Calculated: Range = 48 h of postnatal age	Prospective	Not reported	Meconium aspiration syndrome (<i>n</i> = 5) Congenital diaphragmatic hernia (<i>n</i> = 5)	Reported: Mean ± SEM = 91 ± 4 h Calculated: Range = 157 h	Baseline 1 h 2 h 4 h 12 h 24 h after initiation of ECMO 1 h before decannulation 24 h post-decannulation	Bleeding <i>n</i> = 1 (10%) Thrombosis Not reported In-hospital mortality <i>n</i> = 2 (20%)	Not reported	Platelet aggregation (Whole blood aggregometer using collagen as agonist) ELISA (Soluble P-selectin) Zymography (Gelatinolytic activities of plasma matrix metalloproteinases, MMP-2, and MMP-9)	Significant reduction 1 h after ECMO initiation and 24 h after decannulation; no significant difference 4, 12, and 24 h after ECMO initiation and 1 h before decannulation compared to baseline	Significant reduction 1, 2, 4, 12, and 24 h after ECMO initiation; no significant difference 1 h before and 24 h after decannulation compared to baseline Significant increase in soluble P-selectin 4, 12, and 24 h after ECMO initiation; no significant difference 1 and 2 h after ECMO initiation and 24 h after decannulation compared to baseline
Saini et al. (14)	<i>n</i> = 24	Reported: Median (IQR) = 9 (1–70) months Calculated: N/A	Retrospective	VA-ECMO <i>n</i> = 18 (75%) VV-ECMO <i>n</i> = 6 (25%)	Acute respiratory distress syndrome (<i>n</i> = 4) Pulmonary hypertension (<i>n</i> = 4) Congenital diaphragmatic hernia (<i>n</i> = 2) Myocarditis/dilated cardiomyopathy (<i>n</i> = 3) Post-operative (<i>n</i> = 11)	Reported: Median (IQR) = 8 (6–10) days Calculated: N/A	24 h prior to a severe bleeding event Prior to ECMO decannulation	Bleeding <i>n</i> = 10 (42%) severe Thrombosis Not reported In-hospital mortality <i>n</i> = 13 (54%)	Inhaled nitric oxide, milrinone, and H ₂ blockers	Thromboelastography (Platelet-mapping using ADP and AA)	No significant difference between patients who had severe bleeding and those who had not Significant reduction in non-survivors compared to survivors	Significant reduction in patients who had severe bleeding compared to those who had not Significant reduction in non-survivors compared to survivors

AA, arachidonic acid; ADP, adenosine diphosphate; ATP, adenosine triphosphate; ECMO, extracorporeal membrane oxygenation; GPIb, glycoprotein Ib; ELISA, enzyme-linked immunosorbent assay; GPIIIa, glycoprotein IIIa; IQR, inter-quartile range; N/A, not available; PLC, platelet count; SEM, standard error of the mean; VA-ECMO, venoarterial ECMO; VV-venovenous ECMO.

et al. on the effects of cardiopulmonary bypass on cardiac surgery patients [mean (standard deviation) age: 58 (17)] also found reduced platelet aggregation in response to collagen, ADP and AA in the patient cohort and reported 83% sensitivity and 68% specificity for TEG®-PM to predict post-operative bleeding (24).

Thrombocytopenia is the main trigger of platelet administration in ECMO patients and has been shown to be an important predictor of hemorrhage (25–27). Nevertheless, a study by Reed and Rutledge found no correlation between platelet level and hemorrhage or thrombosis in a cohort of deceased pediatric ECMO patients (28). On average, children on ECMO received 1.3 platelet transfusions per day to maintain platelet level of $>100 \times 10^9/L$ (29). However, frequent platelet transfusions in ECMO patients with excessive bleeding who had normal platelet counts may indicate occurrence of platelet dysfunction. Hence, in addition to the platelet count, it will also be important to monitor platelet function, which has been proposed to be the main cause of coagulopathy in ECMO patients.

In recent years, interactions between platelets and leukocytes have gained increased attention for their important roles in the cross-talk between the hemostatic and inflammatory systems. Currently, there are no studies that have investigated platelet-leukocyte interactions in children or adults on ECMO. Initiation of ECMO is known to induce inflammation via different mechanisms (30, 31) and elevated monocyte-platelet levels have been found in various thromboinflammatory conditions such as cardiovascular diseases (32, 33). Hence, future research focusing on investigating platelet-leukocyte interactions will be important for better understanding of how the interaction between the hemostatic and inflammatory systems may be

associated with the development of clinical events in the pediatric ECMO population.

CONCLUSIONS

This systematic review revealed the existing research gaps in platelet function for children on ECMO. Existing studies are limited by small sample sizes and the types of platelet function tests performed. Pediatric ECMO patients represent a complex patient cohort with diverse etiologies and complications, resulting in pathological bleeding, and clotting. Hence, it will be crucial for future assessment of platelet function to investigate platelet phenotype and function from multiple aspects tailored to the extensive roles of platelets that are not only limited to coagulation but also as immune and inflammatory cells. In particular, future research using the multi-advantageous whole blood flow cytometry should focus on optimized and robust experimental design to ensure standardization and validity of the method used. Furthermore, it will be important to take into account the multiple patient-related factors such as interventions and procedures leading up to commencement of ECMO, age, and duration on ECMO for analysis in relation to the complex nature of this patient cohort.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This study was funded by National Health and Medical Research Council (NHMRC), Grant Number: APP1129317.

REFERENCES

- Dalton HJ, Reeder R, Garcia-Fillon P, Holubkov R, Berg RA, Zuppa A, et al. Factors associated with bleeding and thrombosis in children receiving extracorporeal membrane oxygenation. *Am J Respir Crit Care Med*. (2017) 196:762–71. doi: 10.1164/rccm.2016091945OC
- Yoshimoto Y, Hasebe T, Takahashi K, Amari M, Nagashima S, Kamijo A, et al. Ultrastructural characterization of surface-induced platelet activation on artificial materials by transmission electron microscopy. *Microsc Res Tech*. (2013) 76:342–9. doi: 10.1002/jemt.22172
- Chen Z, Mondal NK, Ding J, Koenig SC, Slaughter MS, Wu ZJ. Paradoxical effect of non-physiological shear stress on platelets and von Willebrand factor. *Artif Organs*. (2016) 40:659–68. doi: 10.1111/aor.12606
- Chen Z, Mondal NK, Zheng S, Koenig SC, Slaughter MS, Griffith BP, et al. High shear induces platelet dysfunction leading to enhanced thrombotic propensity and diminished hemostatic capacity. *Platelets*. (2019) 30:112–9. doi: 10.1080/09537104.2017.1384542
- Heilmann C, Geisen U, Beyersdorf F, Nakamura L, Benk C, Trummer G, et al. Acquired von Willebrand syndrome in patients with extracorporeal life support (ECLS). *Intensive Care Med*. (2012) 38:62–8. doi: 10.1007/s00134-011-2370-6
- Yip, C, Ignjatovic, V, Attard, C, Monagle, P, Linden, MD. First report of elevated monocyte-platelet aggregates in healthy children. *PLoS ONE*. (2013) 8:e67416. doi: 10.1371/journal.pone.0067416
- Yip, C, Linden, MD, Attard, C, Monagle, P and Ignjatovic, V. Platelets from children are hyper-responsive to activation by thrombin receptor activator peptide and adenosine diphosphate compared to platelets from adults. *Br J Haematol*. (2015) 168:526–32. doi: 10.1111/bjh.13153
- Cini, C, Yip, C, Karlaftis, V, Monagle, P, Linden, M, Ignjatovic, V. Differences in the resting platelet proteome and platelet releasate between healthy children and adults. *J Proteomics*. (2015) 123:78–88. doi: 10.1016/j.jprot.2015.04.003
- Ignjatovic, V, Straka, E, Summerhayes, R, Monagle, P. Age-specific differences in binding of heparin to plasma proteins. *J Throm Haemos*. (2010) 8:1290–4. doi: 10.1111/j.1538-7836.2010.03847.x
- Newall, F, Ignjatovic, V, Johnston, L, Summerhayes, R, Lane, G, Cranswick, N, et al. Clinical use of unfractionated heparin therapy in children: time for change? *Br J Haematol*. (2010) 150:674–8. doi: 10.1111/j.1365-2141.2010.08302.x
- Extracorporeal Life Support Organization. *Extracorporeal Life Support Registry Report*. (2019).
- Robinson TM, Kickler TS, Walker LK, Ness P, Bell W. Effect of extracorporeal membrane oxygenation on platelets in newborn. *Crit Care Med*. (1993) 21:1029–34.
- Cheung PY, Sawicki G, Salas E, Etches PC, Schulz R, Radomski MW. The mechanisms of platelet dysfunction during extracorporeal membrane oxygenation in critically ill neonates. *Crit Care Med*. (2000) 28:2584–90. doi: 10.1097/00003246-200007000-00067
- Saini A, Hartmen ME, Gage BF, Said A, Gazit AZ, Boston US, et al. Incidence of platelet dysfunction by thromboelastography-platelet mapping in children supported with ECMO: a pilot retrospective study. *Front Pediatr*. (2016) 3:116. doi: 10.3389/fped.2015.00116

15. Malfetherthner MV, Pimenta LP, Bahr VV, Millar JE, Obonyo NG, Suen JY, et al. Acquired von Willebrand syndrome in respiratory extracorporeal life support: a systematic review of the literature. *Crit Care Resusc.* (2017) 19:45–52.
16. Balle CM, Jeppesen AN, Christensen S, Hvas AM. Platelet function during extracorporeal membrane oxygenation in adult patients: a systematic review. *Front Cardiovasc Med.* (2018) 5:157. doi: 10.3389/fcvm.2018.00157
17. Moher D, Shamseer L, Clarke M, Ghersi D, Liberati A, Petticrew M, et al. Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015 statement. *Syst Rev.* (2015) 4:1. doi: 10.1186/2046-4053-4-1
18. Wurtz M, Hvas AM, Kristensen SD, Grove EL. Platelet aggregation is dependent on platelet count in patients with coronary artery disease. *Thromb Res.* (2012) 129:56–61. doi: 10.1016/j.thromres.2011.08.019
19. Stissing T, Dridi MP, Ostrowski SR, Bochen L, Johansson PI. The influence of low platelet count on whole blood aggregometry assessed by Multiplate. *Clin Appl Thromb Hemost.* (2011) 17:E211–7. doi: 10.1177/1076029610397183
20. Fernia EA, Scavone M, Lecchi A, Cattaneo M. Effect of platelet count on platelet aggregation measured with impedance aggregometry (MultiplateTM analyzer) and with light transmission aggregometry. *J Throm Haemost.* (2013) 11:2193–6. doi: 10.1111/jth.12432
21. Lukito P, Wong A, Jing J, Arthur JF, Marasco SF, Murphy DA, et al. Mechanical circulatory support is associated with loss of platelet receptors glycoprotein Iba1 and glycoprotein VI. *J Throm Haemost.* (2016) 14:2253–60. doi: 10.1111/jth.13497
22. Bembea MM, Schwartz JM, Shah N, Colantuoni E, Lehmann CU, Kickler T, et al. Anticoagulation monitoring during pediatric extracorporeal membrane oxygenation. *ASAIO J.* (2013) 59:63–8. doi: 10.1097/MAT.0b013e318279854a
23. Nair P, Hoechter DJ, Buscher H, Venkatesh K, Whittam S, Joseph J, et al. Prospective observational study of hemostatic alterations during adult extracorporeal membrane oxygenation (ECMO) using point-of-care thromboelastometry and platelet aggregometry. *J Cardiothorac Vasc Anesth.* (2015) 29:288–96. doi: 10.1053/j.jvca.2014.06.006
24. Weitzel NS, Weitzel LB, Epperson LE, Karimpour-Ford A, Tran ZV, Seres T. Platelet mapping as part of modified thromboelastography (TEG[®]) in patients undergoing cardiac surgery and cardiopulmonary bypass. *Anaesthesia.* (2012) 67:1158–65. doi: 10.1111/j.1365-2044.2012.07231.x
25. Hirthler MA, Blackwell E, Abbe D, Doe-Chapman R, LeClair Smith C, Goldthorn J, et al. Coagulation parameter instability as an early predictor of intracranial hemorrhage during extracorporeal membrane oxygenation. *J Pediatr Surg.* (1992) 27:40–3.
26. Sell LL, Cullen ML, Whittlesey GC, Yedlin ST, Philippart AI, Bedard MP, et al. Hemorrhagic complications during extracorporeal membrane oxygenation: prevention and treatment. *J Pediatr Surg.* (1986) 21:1087–91.
27. Dela Cruz TV, Stewart DL, Winston SJ, Weatherman KS, Phelps JL, Mendoza JC. Risk factors for intracranial hemorrhage in the extracorporeal membrane oxygenation patients. *J Perinatol.* (1997) 17:18–23.
28. Reed RC, Rutledge JC. Laboratory and clinical predictors of thrombosis and hemorrhage in 29 pediatric extracorporeal membrane oxygenation nonsurvivors. *Pediatr Dev Pathol.* (2010) 13:385–92. doi: 10.2350/09-09-0704-OA.1
29. Chevuru SC, Sola MC, Theriaque DW, Hutson AD, Leung WC, Rerez JA, et al. Multiple analysis of platelet transfusion usage among neonates on extracorporeal membrane oxygenation. *Pediatrics.* (2002) 109:e89. doi: 10.1542/peds.109.6.e89
30. Plotz FB, van Oeveren W, Bartlett RH, Wildevuur CR. Blood activation during neonatal extracorporeal life support. *J Thorac Cardiovasc Surg.* (1993) 105:823–32.
31. Millar JE, Fanning JP, McDonald CI, McAuley DF, Fraser JF. The inflammatory response to extracorporeal membrane oxygenation (ECMO): a review of the pathophysiology. *Crit Care.* (2016) 20:387. doi: 10.1186/s13054-016-1570-4
32. Furman MI, Barnard MR, Krueger LA, Fox ML, Shilale EA, et al. Circulating monocyte-platelet aggregates are an early marker of acute myocardial infarction. *J Am Coll Cardiol.* (2001) 38:1002–6. doi: 10.1016/s0735-1097(01)01485-1
33. Nagareddy P, Smyth S. Inflammation and thrombosis in cardiovascular disease. *Curr Opin Hematol.* (2013) 20:457–63. doi: 10.1097/MOH.0b013e328364219d

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Yaw, Van Den Helm, MacLaren, Linden, Monagle and Ignjatovic. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Shear-Dependent Platelet Aggregation: Mechanisms and Therapeutic Opportunities

Akshita Rana, Erik Westein, Be'eri Niego and Christoph E. Hagemeyer*

Nanobiotechnology Laboratory, Australian Centre for Blood Diseases, Central Clinical School, Monash University, Melbourne, VIC, Australia

OPEN ACCESS

Edited by:

Paul Jurasz,
University of Alberta, Canada

Reviewed by:

Teresa Padro,
Sant Pau Institute for Biomedical
Research, Spain
Matthew Dean Linden,
University of
Western Australia, Australia

*Correspondence:

Christoph E. Hagemeyer
christoph.hagemeyer@monash.edu

Specialty section:

This article was submitted to
Atherosclerosis and Vascular
Medicine,
a section of the journal
Frontiers in Cardiovascular Medicine

Received: 05 June 2019

Accepted: 03 September 2019

Published: 20 September 2019

Citation:

Rana A, Westein E, Niego B and
Hagemeyer CE (2019)
Shear-Dependent Platelet
Aggregation: Mechanisms and
Therapeutic Opportunities.
Front. Cardiovasc. Med. 6:141.
doi: 10.3389/fcvm.2019.00141

Cardiovascular diseases (CVD) are the number one cause of morbidity and death worldwide. As estimated by the WHO, the global death rate from CVD is 31% wherein, a staggering 85% results from stroke and myocardial infarction. Platelets, one of the key components of thrombi, have been well-investigated over decades for their pivotal role in thrombus development in healthy as well as diseased blood vessels. In hemostasis, when a vascular injury occurs, circulating platelets are arrested at the site of damage, where they are activated and aggregate to form hemostatic thrombi, thus preventing further bleeding. However, in thrombosis, pathological activation of platelets occurs, leading to uncontrolled growth of a thrombus, which in turn can occlude the blood vessel or embolize, causing downstream ischemic events. The molecular processes causing pathological thrombus development are in large similar to the processes controlling physiological thrombus formation. The biggest challenge of anti-thrombotics and anti-platelet therapeutics has been to decouple the pathological platelet response from the physiological one. Currently, marketed anti-platelet drugs are associated with major bleeding complications for this exact reason; they are not effective in targeting pathological thrombi without interfering with normal hemostasis. Recent studies have emphasized the importance of shear forces generated from blood flow, that primarily drive platelet activation and aggregation in thrombosis. Local shear stresses in obstructed blood vessels can be higher by up to two orders of magnitude as compared to healthy vessels. Leveraging abnormal shear forces in the thrombus microenvironment may allow to differentiate between thrombosis and hemostasis and develop shear-selective anti-platelet therapies. In this review, we discuss the influence of shear forces on thrombosis and the underlying mechanisms of shear-induced platelet activation. Later, we summarize the therapeutic approaches to target shear-sensitive platelet activation and pathological thrombus growth, with a particular focus on the shear-sensitive protein von Willebrand Factor (VWF). Inhibition of shear-specific platelet aggregation and targeted drug delivery may prove to be much safer and efficacious approaches over current state-of-the-art antithrombotic drugs in the treatment of cardiovascular diseases.

Keywords: platelets, shear, aggregation, thrombosis, VWF

INTRODUCTION

Since Bizzozzero first identified the remarkable contribution of platelets in both hemostasis and thrombosis (1), substantial progress has been made in understanding, diagnosing and treating several platelet-related disorders. With technological advances in platelet research, immense insights were generated into the mechanisms underlying “hemostatic” and “thrombotic” activities of these intriguing blood cells.

Platelets, or thrombocytes (2–4 μm in greatest diameter), are anucleate cells produced by megakaryocytes, that circulate in the human bloodstream for 7–10 days before being eliminated by the liver and spleen (2, 3). Primarily, platelets sense and respond to vascular injury by preventing blood loss following vessel damage and tissue trauma. But in addition to this essential hemostatic function, platelets also play a key pathological role in cardiovascular diseases.

Several decades of research into platelets and their varying pro-thrombotic roles at sites of vascular injury have established the synergistic interplay between two distinct, yet complimentary systems in the platelet activation process, being (i) biochemical factors (adhesive molecules and extra-cellular matrix proteins, as well as soluble agonists) and (ii) biomechanical factors, such as hemodynamic shear forces (4, 5).

While the biochemical factors mediating clot formation have been extensively investigated, the vital role of the local fluid mechanical microenvironment that facilitates platelet-ligand interactions, thus regulating the release of biochemical cues, must not be overlooked in the development process of effective anti-thrombotic strategies. In particular, the mechano-sensitive plasma protein von Willebrand factor (VWF) and its binding with platelet receptor glycoprotein Ib α (GPIb α) play a crucial role in hemostasis, yet is also key to pathological thrombus initiation and propagation. Here, we discuss the mechanosensitive interaction of VWF with GPIb α , its dependency on shear forces generated by blood flow and possible therapeutic approaches exploiting this protein axis. Furthermore, novel shear-sensitive drug delivery platforms, designed to specifically target occluded vessels, are also discussed in this review.

THE TRADITIONAL, BIOCHEMICAL CASCADE OF THROMBOSIS

Upon vascular injury, platelets adhere to exposed sub-endothelial matrix proteins, mainly VWF and collagen, followed by their activation, aggregation and finally formation of a “platelet plug” at the injury site. Under normal physiological conditions, the pro-thrombotic hemostatic response is neutralized by counter anti-thrombotic and anti-coagulatory mechanisms that confine the clot formation specifically to the injury site. However, at diseased vascular sites (such as atherosclerotic lesions) this balance is disrupted due to the highly thrombogenic contents and the stenotic geometry of the lesions, which cause an increase in local shear rates. The exacerbated biochemical and biomechanical responses combined can lead to abnormal

thrombus propagation, turning a life-saving defense mechanism into a life threatening one.

In the traditional concept of platelet aggregation, soluble-agonists such as ADP and thromboxane A₂ (TxA₂) were assumed to play the central role in driving thrombus development. As per this model, the process of thrombogenesis and propagation comprised of three sequential steps, including platelets adhesion, activation, and subsequent aggregation at the site of vascular injury.

Platelet Adhesion

The initial arrest of platelets at the site of vascular damage is primarily facilitated by shear-dependent interaction of platelet receptor GPIb α with the A1 domain of VWF (6, 7). The GPIb α -A1 binding is also key for platelet aggregation during thrombosis, which will be discussed in detail later. Other transmembrane platelet receptors mediating platelet adhesion include GPVI (8) and integrin $\alpha_2\beta_1$ (9), that bind directly to collagen (10).

Platelet Activation

The adhesion of platelets to sub-endothelial matrix proteins leads to platelet intracellular signaling, which activates the cell. Briefly, platelet activation results in conformational change (from the typical discoid shape to an elongated form) (11) and degranulation (i.e., release of the platelet cytoplasmic granules content) (12). α -granules release VWF and coagulation factors, such as fibrinogen, factor V, factor XIII, and P-selectin (also expressed on the platelet surface following activation); Dense granules release platelets agonists, such as serotonin and adenosine diphosphate (ADP) (13). This process results in sustained, oscillatory cytosolic Ca^{2+} flux, production of TxA₂, exposure of phosphatidylserine (PS), expression of P-selectin, and microvesiculation, allowing coagulation factors to bind the activated platelet. The activation step also involves additional platelet recruitment via an outside-in signaling, induced by a paracrine action of the soluble agonists released from activated platelets themselves (e.g., ADP, TxA₂) or by activated coagulation factors, such as thrombin. While the purinergic receptor P2Y₁₂ is the platelet receptor for ADP, protease-activated receptors (PAR) 1 and 4 bind and potentially react to thrombin (12, 14, 15).

Platelet Aggregation

Following platelet adhesion and activation, the integrin $\alpha_{\text{IIb}}\beta_3$ (also known as GPIIb/IIIa) undergoes conformational change through inside-out signaling and binds fibrinogen (16, 17). This receptor-ligand interaction results in the formation of platelet aggregates, ultimately forming a platelet plug that can prevent bleeding in case of minor injuries. However, for a damage of a greater magnitude, primary hemostasis is followed by secondary hemostasis, in which fibrin production stabilizes the clot to prevent hemorrhage (18). Since only initially-adhered platelets are in direct contact with thrombogenic surfaces, post-activation signals originating from platelets themselves as well as soluble agonists play a central role in triggering ongoing thrombus growth.

It has long been established that thrombus formation occurs via an interplay between two distinct, yet complementary

platelet aggregation mechanisms: soluble agonist-dependent (biochemical) and rheology-dependent (biomechanical) pathways. However, major anti-thrombotic drugs target the biochemical axis of platelet aggregation without considering the biomechanical aspect. As a result, current anti-platelet drugs not only have limited clinical efficacy in treating cardiovascular diseases, but also carry significant side effects, that can be life-threatening at times. Thus, there is a pressing need to develop novel drugs that target growing thrombi specifically under pathological shear forces.

Limitations of Current Anti-platelet Drugs

Anti-platelet drugs have proven to be the keystone of anti-thrombotic therapy, as demonstrated by multiple, large-scale clinical trials and meta-analyses (19, 20). Despite being a mainstay in preventing atypical platelet activation/aggregation in cardiovascular events, anti-platelet drugs face several limitations in the clinical settings. These include heterogeneity in interpatient response with considerable drug resistance (21–24), delayed onset of action (25, 26), low safety profile (27, 28) and systemic administration nature, resulting in limited effective concentration at the site of interest (29, 30). However, the most crucial side effect of antiplatelet drugs is a bleeding risk, that can have serious implications for the patient, including death (31–34). Conventional anti-platelet drugs blocking GPIIb/IIIa (abciximab, eptifibatide, tirofiban) or targeting TxA₂ (dazoxiben, ifetroban, dipyridamole) and ADP-P2Y₁₂ pathways (ticagrelor, cangrelor) have been associated with enhanced hemorrhagic episodes due to undesired impairment of hemostasis in addition to the desired prevention of arterial thrombosis (35, 36). Further, prolonged use of some cyclooxygenase-2 (COX-2) inhibitors, such as valdecoxib, parecoxib, and celecoxib, has been found to increase the recurrence of serious cardiovascular events in patients (37, 38).

Dual Antiplatelet Therapy and Triple Therapy

The Clopidogrel in Unstable Angina to Prevent Recurrent Events (CURE) trial demonstrated the advantages of dual antiplatelet therapy (DAPT), including aspirin and P2Y₁₂ receptor antagonist (clopidogrel, prasugrel, or ticagrelor) in acute coronary syndrome (ACS) patients over aspirin monotherapy (39, 40). Despite vast evidence supporting the usage of DAPT over aspirin alone in ACS or post-percutaneous coronary intervention (PCI), clinicians have not reached a consensus on the optimum duration of the therapy in these patients (41). Short-term DAPT is typically less effective in attenuating thrombotic events, but has lower bleeding risk. In contrast, long-term DAPT may reduce ischemic risk but increase the occurrence of major bleeding events, with a higher all-cause mortality rate (42, 43).

Triple therapy, including aspirin, P2Y₁₂ antagonist and oral anticoagulant has shown a 3-fold increase in bleeding events, as compared to oral anticoagulant alone (44, 45). Generally, intensification of antithrombotic regimens for a maximal clinical benefit in a broader patient population comes at a major cost of significant bleeding events that may be proven fatal at

times. Refining the anti-thrombotic effects of a drug without exacerbating the bleeding risks remains a major challenge to date.

In conclusion, current antiplatelet therapeutics are unable to meet the major clinical need to selectively prevent pathological thrombosis without interfering with the physiological process of hemostasis. As mentioned above, one of the significant distinctions between the two processes is the substantial difference in local shear rates, with exceedingly high shear levels observed in pathological thrombi at sites of vessel occlusion or atherosclerotic plaque rupture (46, 47). No existing, clinically-used antiplatelet drug is known to specifically respond to this biomechanical force at the site of pathological thrombus formation.

BIOMECHANICAL ASPECT OF THROMBOSIS

Shear Forces Resulting From Blood Rheology

Blood, circulating in the vessels via pressure differences, exerts forces on its components as well as the vascular walls (48). Blood flow is laminar, with maximal velocity at the vessel lumen center and zero at the vessel wall. The velocity of circulating blood changes differentially between its discrete fluid layers, thereby generating a tangential force (force acting along a tangent to the object) between them. This tangential force per unit area between laminae, termed “fluid shear stress” (ζ), is expressed in Pascal (Pa). Alternatively, wall shear rate (γ , expressed in s^{-1}), defined as the rate of change in fluid velocity as a function of distance from the vessel wall, is used to describe hemostasis and thrombosis. In veins and larger arteries experiencing high shear rates, blood exhibits the characteristics of a Newtonian fluid, with the resulting wall shear stress directly proportional to the local shear rate (49).

The biomechanical forces generated by the virtue of blood flow play a vital role in uniting or separating the essential hematological components engaged in clot formation. Under normal hematocrit conditions ($\sim 40\%$) (50), erythrocytes mainly circulate along the central axis of the blood vessel due to axial migration. Consequently, platelets travel in close proximity to the vessel walls, a phenomenon that facilitates their binding to adhesive ligands at the reactive endothelial layer in damaged vascular sites (49, 51–53).

Under physiological conditions, typical wall shear rates of $300\text{--}800\ s^{-1}$ occur in the large arteries, $15\text{--}200\ s^{-1}$ in veins and $450\text{--}1,600\ s^{-1}$ in micro arterioles (54). However, vessel stenosis due to atherosclerotic lesions or pre-existing thrombi can increase shear rates well beyond $10,000\ s^{-1}$ resulting in a localized pro-thrombotic microenvironment (48, 54, 55).

Platelet Adhesion and Tethering to VWF Under Shear Conditions

The arrest of flowing platelets occurs on immobilized VWF via interactions between the A1 domain of VWF and the platelet receptor GPIIb α . These bonds have a rapid on-rate but short lifetimes, hence only supporting transient platelets adhesion and

classical stop and go motion (56). Membrane tethers, consisting of smooth cylinders of lipid bilayer, then extend from the platelet surface and are pulled by shear forces. Membrane tethers are vital in prolonging platelet adhesion times, allowing for small platelet aggregates to form, which in turn maintain high local concentration of activating signals by eradicating any “washout effects” of soluble agonists by blood flow.

Tethers act as adhesion contacts between platelets and platelet-matrix, and subsequently facilitate bonding between the $\alpha_{IIb}\beta_3$ platelet receptors and the Arg-Gly-Asp (RGD) motif of the VWF C1 domain (57). In normal physiology, under low-shear regimes ($600\text{--}900\text{ s}^{-1}$), the aggregation is dominated by $\alpha_{IIb}\beta_3$ -dependent interactions, while under intermediate shear rates ($1,000$ to $10,000\text{ s}^{-1}$) both GPIb α and $\alpha_{IIb}\beta_3$ play an important role in platelet aggregation. However, VWF-dependent interactions become increasingly dominating as shear rates increase ($>10,000\text{ s}^{-1}$) (4, 58, 59). Consequently, VWF and GPIb α play a vital role in hemostasis but an even more pivotal role in the onset of thrombosis, thus contributing in several diseases.

VWF Structure, Native Confirmation, and Pathophysiological Significance

Von Willebrand Factor is a large, blood-borne multimeric glycoprotein, crucial for hemostasis and thrombosis. It is secreted by both endothelial cells (where it is stored in Weibel-Palade bodies) and megakaryocytes (that also pack VWF in α -granules in platelets) as a globular multimer, consisting of identical subunits of 250 kDa, connected via disulfide bonds to form ultra-large VWF (ULVWF), weighing up to 20,000 kDa. Each subunit of ULVWF harbors multiple functional domains, each with a specific function. The major functionalities of plasma VWF are exhibited by three adjacent A domains, namely A1, A2, and A3. While A1 is primarily responsible for binding with the platelet receptor GPIb α , A3 acts as the immobilization site for VWF on the collagen matrix (60, 61). A2 is responsible for size regulation of ULVWF in blood physiology, as described below. Under shear forces, self-association of plasma VWF occurs also on the surface of platelets (62) and endothelial cells (63), thereby creating fibrillar structures that further facilitate platelet adhesion. As mentioned earlier, VWF is physiologically and pathologically important protein. It is responsible for several hemorrhagic disorders, including von Willebrand disease (VWD) and Bernard Soulier syndrome. VWD is the most common, hereditary bleeding disorder, resulting from qualitative or quantitative deficiencies in VWF, presiding a diverse range of mild to severe hemorrhagic episodes. VWD can be categorized into three types: VWD type 1, a quantitative defect; VWD type 2, a qualitative defect and VWD type 3, the most severe defect characterized by complete absence of VWF. VWD type 2 can be further divided into four sub-types, including type 2A (dominant loss-of-function mutations), type 2B (dominant gain-of-function mutations), 2M (impaired binding to GPIb α) and type 2N, or Normandy defect (impaired binding to factor VIII) (64). Moreover, acute coronary syndrome (ACS), acute myocardial infarction, stroke and ischemic events have been

associated with increased levels of plasma VWF in several population-based studies (65, 66). Other studies have suggested that the single nucleotide polymorphisms (SNPs) on the gene coding for VWF may lead to coronary heart diseases in advanced atherosclerotic and diabetic patients (67, 68). Histopathological studies have also identified the pathogenic role of VWF in venous thromboembolism (VTE) by revealing VWF-rich thrombi in patients who died from the disease (69).

Shear-Induced Activation and Conformational Change of VWF

Following secretion from Weibel-Palade bodies in the endothelium, ULVWF multimers (several $100\mu\text{m}$ long) are inherently hyperactive as they carry numerous ligand binding sites and are more receptive to hemodynamic forces due to their size (70).

The shear force is comprised of rotational and elongational flow components (**Figure 1A**) (71, 72). Under low shear forces, VWF exists in “coiled” or globular conformation due to monomeric self-association (73). In this native conformation, binding sites in the A1 domain are believed to be buried and thus, inaccessible to platelets or other biomolecules (74, 75). Under flow-induced shear stress, VWF exhibits conformational variation, including end-over-end tumbling and periodic elongation and compaction (76, 77). Indeed, above a critical shear rate, the intrinsic interactions between the monomeric units are overcome by the hydrodynamic drag, resulting in stretching of VWF, thereby exposing the cryptic A1 epitopes (77, 78). While the critical shear rate to elongate surface-immobilized VWF is about $3,000\text{ s}^{-1}$, circulating plasma VWF requires $\sim 5,000\text{ s}^{-1}$ (79, 80). Interestingly, the required force for VWF elongation increases with increasing shear rates (77).

At sites of vessel constriction, fluid acceleration and subsequent deceleration create local force gradients. As a result, VWF circulating nearby experiences an elongational pull in the direction of flow due to a faster-moving proximal end and a slower-moving distal end (**Figure 1B**). Importantly, studies have shown that the unfolding process of VWF can be achieved at significantly reduced flow rates if they produce a gradient of shear forces (i.e., when flow is accelerating or decelerating), compared to a flow with high, but constant shear rate (81, 82). The is because of the large size of VWF where the leading end of the molecule will experience a different strain rate compared to the trailing end, hence substantially lower forces are required to unfold the protein (76).

Shear-Sensitive VWF A2 Domain and Mechanoenzymatic Cleavage

ULVWF concatemers (long molecules containing multiple copies of the same protein sequence, linked end-to-end in series; **Figure 3**) remain tethered to the endothelial cell surface in stretched conformation with exposed A2 domain that completely unfolds due to missing long-range disulfide linkages, unlike A1 and A3 domains (84). On complete refolding of the A2 domain, a peptide bond, Tyr1605–Met1606, located in the centrally situated β_4 sequence in the β -sheet (at the center of the folded

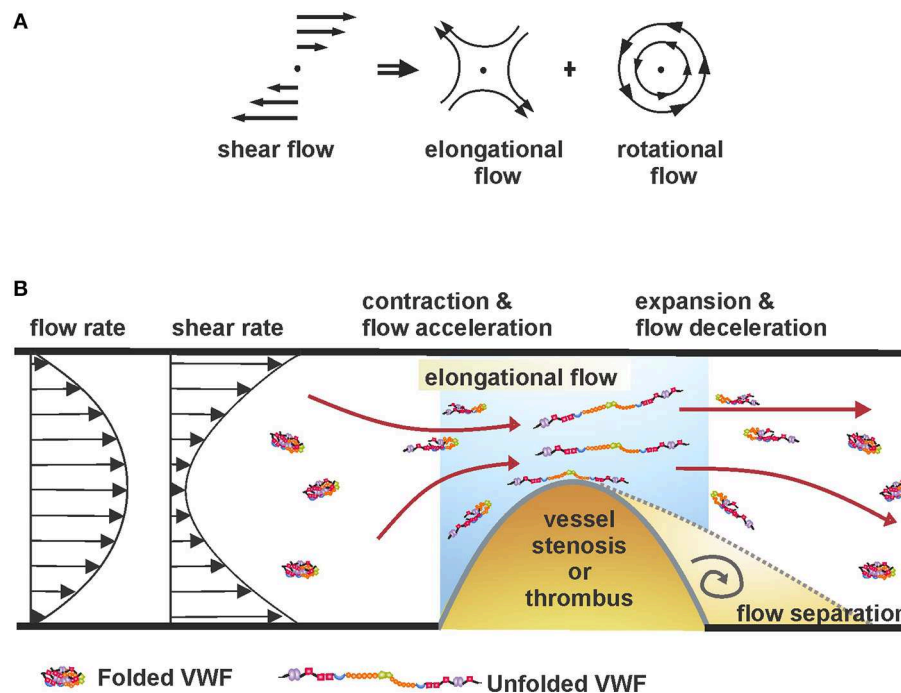


FIGURE 1 | (A) Shear flow, as a superimposition of rotational and elongational flow components (71, 72). **(B)** Unfolding of coiled VWF under elongational flow or shear rate gradients at sites of vessel stenosis.

A2 domain), is recognized by ADAMTS-13, a metalloprotease enzyme (81). ADAMTS-13 cleaves ULVWF at this scissile bond and releases the smaller and less hemostatically active VWF strings into the blood stream. This regulatory mechanism prevents VWF hyperactivation under physiological shear rates. Severe deficiency of ADAMTS-13 activity caused by inhibiting autoantibodies results in ultra-large multimers of VWF in blood. These ULVWF cause systemic microvascular thrombosis, ultimately leading to a blood disorder called thrombotic thrombocytopenic purpura (TTP) (85). Single molecule studies found that proteolysis of VWF is regulated by a Ca^{2+} binding $\alpha 3\text{-}\beta 4$ loop in the A2 domain. Therefore, within the physiological calcium concentration range the refolding kinetics of A2 can increase five times without affecting the unfolding event (86).

Shear-Sensitive VWF A1 Domain and Platelet Arrest

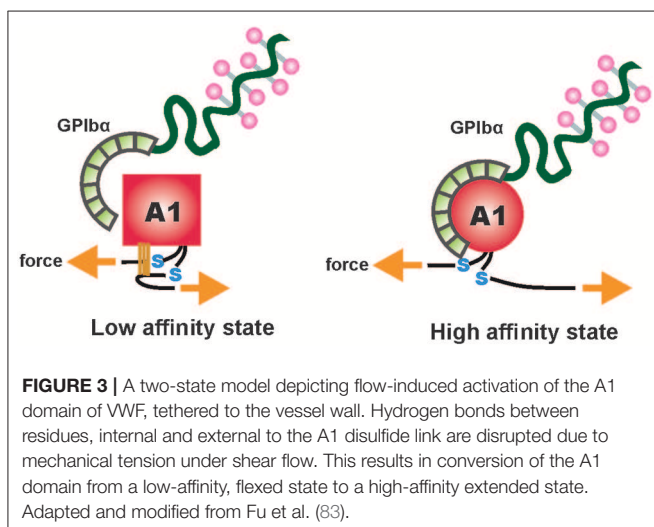
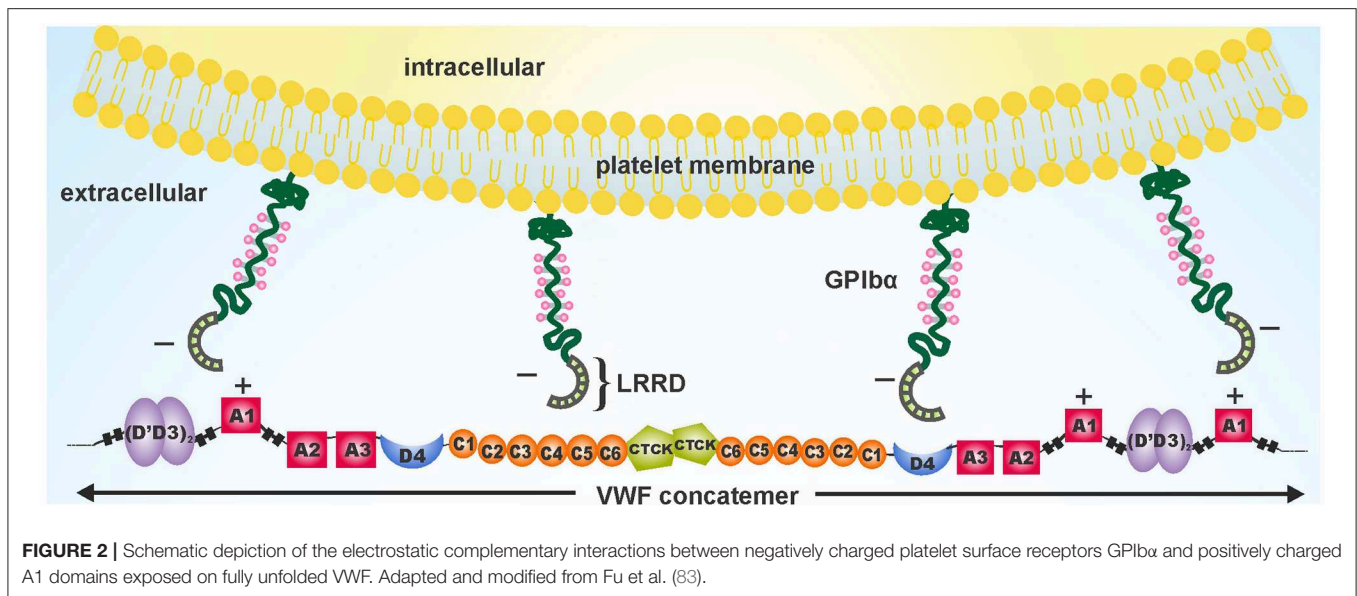
The VWF A1 domain plays a remarkable role in initiating thrombus formation under pathological shear rates. Under low shear conditions or absence of any exogenous VWF modulators, such as ristocetin and botrocetin, no interaction occurs between VWF and platelet surface receptors. However, above threshold shear levels (800 s^{-1} in humans), the exposed A1 domain of unfolded VWF—immobilized onto exposed collagen—binds GPIIb α receptors in (GP)Ib-IX complex on platelets cruising within the “latching distance” of the vascular wall, thus initiating platelet adhesion to VWF and aggregate formation (57, 59, 87–89).

A1 domain is the most positively charged domain of VWF due to the presence of Cys509-Cys695 disulfide bond, a major positively charged region surrounded by two discontinuous anion flanking sequences, which contains seven sialylated glycosylation sites (74). Studies using artificial peptides indicated that residues Asp514-Glu542 within this disulphide loop in the A1 domain could be involved in GPIIb α binding (90). Ristocetin, an antibiotic from *Nocardia lurida*, activates VWF and facilitates A1-GPIIb α interaction in a similar fashion as elevated shear. Ristocetin binds to proline-rich sequence, Glu700 to Asp709 and the C-terminal to the Cys509-Cys695 disulphide bond in the A1 domain. In some studies, anti-VWF A1 (5D2, CR1, and 6G1) and anti-GPIIb α monoclonal antibodies (AK2 and AN51), mapping ristocetin-bound A1 and GPIIb α epitopes, selectively inhibited both ristocetin-induced and shear-induced platelet aggregation (74, 91).

Finally, the A1 domain also acts as the main binding site to non-fibrillar collagen type VI (92). Like the A3 domain, which serves as the main binding site for collagens I and III, the A1 domain can bind both types, although with less propensity. In fact, under high shear forces, the unraveled A1 domain can act as surrogate for the A3 domain in recruiting platelets to collagen.

Force-Induced A1 Activation

Recent study described a two-step conformational transformation mechanism of A1 activation after VWF senses mechanical force (83). The first step involves elongation of VWF, immobilized to the vessel wall, from a globular form to a stretched form, followed by a tension-dependent local transition



to a high affinity state. Elongation results in breaking of weaker hydrogen bonds between distal monomers in the tethered VWF concatemer. The molecule then experiences a tensile force upstream, along the backbone toward the tether point. This exposes an almost linear array of A1 domains that become easily accessible to platelets. When this tensile strength reaches ~ 21 piconewton (pN) in the upstream regions, A1 transforms into a second state exhibiting high affinity for GPIb α , that depends upon electrostatic interactions [Figure 2; (83)].

Platelet GPIb α and A1 Binding

At the onset of hemostasis and thrombosis, platelets tether or translocate onto the VWF substrate immobilized on the damaged vessel site. The initial contact between circulating platelets and VWF is made via binding interactions between the A1 domain of VWF and the N terminal domain of GPIb α

receptor (56, 57). In physiology, the levels of ligand-receptor interactions are regulated, as insufficient interactions may affect hemostasis by causing bleeding while excessive interactions may lead to thrombosis.

GPIb α Receptor

GPIb-IX-V receptor complex is abundantly expressed with about 25,000 copies of GPIb-IX complex and 12,000 GPV copies on resting platelets, making it suitable for platelet adhesion and aggregation (93). The complex consists of GPIb α , GPIb β , GPIX, and GPV sub-units in the stoichiometric ratio of 2:4:2:1 (94). GPIb complex comprises of GPIb α and GPIb β , connected via disulfide bond that tightly link with GPIX to form GPIb-IX complex. GPV does not participate in VWF binding or signaling (95). GPIb α consists of a N-terminal leucine-rich repeat domain, a macro glycopeptide region followed by a long stalk, a C-terminal transmembrane helix and a short cytoplasmic domain (96). Polymorphisms in GPIb α have been linked with a greater risk of cardiovascular manifestations, such as stroke and myocardial infarction in young individuals (97, 98).

GPIb α -VWF A1 Binding Kinetics

Crystal structures of the VWF A1-GPIb α complex, both of wild-type or a gain-of-function A1 and GPIb α mutations, exhibited two distinct binding interfaces (99). The leucine rich repeat domain (LRRD) of GPIb α [basal isoelectric point (pI): 5.87] is a negatively charged, concave- or horseshoe-shaped region, that binds with the positively charged VWF A1 domain (basal pI: 9.4) via electrostatic interactions [Figure 3; (99)]. The electrostatic complementarity of the binding interfaces enhances the association rate of A1-GPIb α complex (100).

Shear flow modulates the dissociation rates of the A1-GPIb α bond. Earlier studies hypothesized that at forces < 22 pN, A1-GPIb α exhibits a “catch” bond behavior, in which bond lifetime prolongs as shear forces increase (translating as tensile forces).

Above 22 pN, the bond acts as a “slip” bond, where increasing shear results in shortened bond lifetime (101, 102).

Later studies using Receptor and Ligand In a Single Molecule (ReaLISM) constructs described the A1-GPIb α bond as a mechanically stabilized “flex bond.” The bond exists in two “slip” bond states, one flexed, low-affinity state associated with lower force and the other extended, high-affinity state engaging ~ 10 pN with around 20-fold longer bond lifetime and greater ability to withstand strong hemodynamic forces (103). Tensile force stabilizes the high-affinity state, thus strengthening the bond under pathological shear rates. Similar phenomenon was observed for ristocetin, a VWF modulator that alters the conformational state of the A1-GPIb α bond in a sheared-like configuration. Both ristocetin and tensile force selectively stabilize the extended state, corresponding to shear-induced state (74, 91, 103). Formation of mechanically stabilized flex-bonds may account for the resistance of A1-GPIb α complex under extreme pathological shear rates, resulting in platelet adhesion and activation-independent platelet aggregation at these extreme shear conditions (80). Interestingly, the VWF A1 domain with type 2B mutations forms “slip” bonds with platelet GPIb α , resulting in prolonged bond duration even at low shear rates (101).

GPIb α Mechanosensing and Mechanotransduction

Recent findings employing biomechanical techniques at the single molecule level have provided insight into how platelets mechanosense shear forces and subsequently convert these mechanical cues into biochemical signals to promote thrombus growth and stability. A structured, quasi-stable Mechano-Sensitive Domain (MSD), consisting of around 60 residues, was identified in the juxtamembrane stalk region of GPIb α (104). VWF A1-mediated mechanical pulling on the GPIb α N-terminal domain results in unfolding of the MSD in GPIb-IX complex. Furthermore, this shear-mediated pulling of tethered platelet onto immobilized VWF generates tensile force along GPIb α that not only unfolds the relatively unstable MSD, but also lowers the overall force applied on A1-GPIb α complex, in turn stabilizing the tethering of platelet. MSD unfolding occurs at similar force range (5–20 pN) as that required for the A1-GPIb α complex to transition into high-affinity flex-bond state (101, 103). Due to direct contact of the MSD with GPIb β and GPIX subunits, unfolding of the mechanosensitive domain may induce conformational changes in these subunits, thereby facilitating intracellular signal transmission (104).

In mutational studies with human/canine chimeras of GPIb α , co-crystal structure of the A1-GPIb α complex showed minimal contact between the predominantly negatively charged LRR2-4 (Leu60-Glu128) sequence and VWF A1 under static conditions. Interestingly however, this sequence was not only important for ristocetin-induced binding but also increasingly fundamental for platelet adhesion at increasing shear rates. This finding further supports the idea of force-mediated transition from low to a stabilized, high-affinity state of ligand-receptor flex-bond wherein, the LRR2-4 of GPIb α establishes tight contact with

the A1 domain (105, 106). Yet, the conformational dynamics of the LRRD and its role in signal transduction within platelets is not well-understood.

Recently, using molecular dynamic simulations and biomembrane force probe, unfolding of LRRs in the N-terminal domain of GPIb α was observed under force application, initiating at LRR2-4 and propagating to neighboring LRR sequences. LRR folding prolongs the A1-GPIb α bond lifetime by increasing the force required for its disconnection from 10 to 25 pN (i.e., a “catch” bond behavior) and reduces the bond lifetime thereafter (a “slip” bond behavior) as described earlier in section GPIb α -VWF A1 Binding Kinetics (101, 107).

Thus, far, the GPIb α mechanosensing was recapitulated by the conformational dynamics of MSD and LRRD. However, mechano-transduction or how the mechanical cues were transformed into biochemical signals remained elusive. Ju et al. demonstrated that mechanotransduction of force into signals was done by cooperative interplay between the mechanosensing domains MSD and LRRD (108). The study showed that unfolding of LRRD enhances the unfolding of MSD by force transmission through mucin-like macroglycopeptide located between the two domains. MSD unfolding event may aid further unfolding of LRRD, thus prolonging the A1-GPIb α bond lifetime. This cooperativity pattern between the mechanoreceptor domains is maximal around the optimal force of 25 pN [108].

Single platelet calcium imaging identified three types of Ca²⁺ fluxes: Null-type, baseline with background noise; α -type, latent phase following a high spike with a rapid decay; and β -type, being fluctuating or slowly increasing Ca²⁺ signals to an intermediate level followed by slow decay to the baseline. GPIb α -A1 binding results in transient α -type Ca²⁺ flux, promoting reversible arrest of translocating platelets on immobilized VWF (92, 109). Time-lapse correction analysis revealed that while LRRD unfolding enhances the Ca²⁺ flux, MSD unfolding event was essential to generate α -type Ca²⁺ signal. In resting platelets, a signaling molecule—14-3-3 ζ —is bound to both GPIb α and GPIb β (110). Upon A1-GPIb α binding, the head domain of GPIb β binds with the unfolded MSD while detaching its cytoplasmic tail from GPIb α -associated 14-3-3 ζ . 14-3-3 ζ is proposed to transduce the mechanical signal of MSD unfolding into intracellular biochemical signaling across the platelet membrane. This leads to the downstream activation of adapter molecules, ultimately resulting in cytoplasmic calcium flux [108]. In addition, GPIb α -A1 interactions lead to other intracellular signaling events, including integrin $\alpha_{IIb}\beta_3$ activation, ADP, thrombin and TxA2 production and P13K activation.

Integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa) Activation

GPIb α mechano-signaling results in a transient calcium flux that thrusts the majority of integrins to an intermediate activation state. This intermediate state subsequently facilitates the outside-in signaling of $\alpha_{IIb}\beta_3$ integrins for further transformation into an active state. These intermediate and active integrin states have longer bond lifetimes and higher ligand affinity that stabilize the platelet aggregates (111). The high-affinity state of integrin $\alpha_{IIb}\beta_3$ enables its binding with fibrinogen and VWF to strengthen the adhesive bonds among platelets and stabilize aggregation.

Shear Dependent Platelet Aggregation: Evolving Concepts From Constant Shear to Gradients of Shear

Distinct aggregation mechanisms operate based on different shear ranges (5, 112). $\alpha_{IIb}\beta_3$ -fibrinogen-dominated platelet aggregation occurs mainly under low shear rates ($<1,000\text{ s}^{-1}$) in veins and larger arteries, with soluble agonists maintaining the activated state of integrins, thus stabilizing the bonds. Platelet-platelet interactions become increasingly dependent on VWF and its binding with both $\alpha_{IIb}\beta_3$ and GPIIb α receptors at increasing shear range ($1,000\text{--}10,000\text{ s}^{-1}$), as observed in moderate arterial stenosis and arterioles. Notably, at pathological shear rates ($>10,000\text{ s}^{-1}$), resulting from acute vessel constriction, initial aggregation exclusively relies upon VWF-GPIIb α bonding, without the prerequisite of platelet activation or $\alpha_{IIb}\beta_3$ interaction (5, 80). Above this shear threshold, soluble VWF binds with GPIIb α receptors on adherent platelets, resulting in dominating platelet-platelet interactions that induce “large rolling aggregates” onto immobilized VWF. Enhanced inter-platelet interactions mediated by soluble VWF result in a growing transient aggregate under extreme shear conditions (80). A dramatic increase in shear may also destabilize vulnerable thrombi formed post-plaque rupture, generating emboli that can flow to distal organs, causing partial or complete vessel blockage downstream of plaque origin (113).

The mechanisms of platelet aggregation described up until this point operate under simplified laminar shear conditions, with constant shear and no turbulence in consideration. However, in pathology, blood rheology can alter dramatically, both spatially and temporally, in the local microenvironments generated by vascular lesions, acute stenosis or pre-existing thrombi. Thrombus propagation within the blood vessel is a complex and dynamic process that can incur flow perturbations, resulting in vortex and eddy formation, shear gradients, recirculation zones, flow separation and reciprocation (114, 115). These disturbed flow parameters have substantially stronger prothrombotic effects as compared to constant shear flows of the same magnitude.

Platelet Aggregation Mechanisms Under Complex Shear Gradient: Novel Concepts

Elegant studies both *in vitro* and *in vivo* have emphasized the indispensable role of shear gradient-dependent functionality of VWF in recruiting platelets for thrombus propagation. As mentioned above, thrombus development under high shear conditions is an intricate process that is principally driven by hemodynamic forces. Vessel stenosis can create a microenvironment wherein the forces experienced by blood components vary vastly as compared to any other non-obstructed sections of the vasculature.

Pathological shear rates briefly exceeding $10,000\text{ s}^{-1}$ can be generated locally by occlusive thrombi at the sites of atherosclerotic plaque ruptures, causing an exaggerated aggregation response by platelets, thus precipitating myocardial infarction or ischemic stroke (24, 116). A landmark study by Nesbitt et al. (117) elucidated the principle role of shear

microgradients, resulting from vascular constriction, in promoting stabilized, discoid platelet aggregation and thrombus progression post-stenosis. Shear gradient regions consist of three zones: a shear acceleration zone at the proximal end of stenosis, a peak shear zone at the apex of stenosis and a shear deceleration zone at the distal end of stenosis. While the high shear region enhances platelet activation (118, 119), the low shear region post stenosis provides sufficient time and conducive flow conditions for platelet hyper-aggregation and thrombus progression [Figure 4; (121–123)]. Computational Fluid Dynamics (CFD) modeling of stenosed vessels showed that for an input shear rate of $1,800\text{ s}^{-1}$ upstream of the stenosis, a shear rate $20,000\text{ s}^{-1}$ is generated at the apex with rapid deceleration to about 800 s^{-1} post-stenosis, inducing exacerbated platelet aggregation. Interestingly, no thrombus has developed under similar conditions with a constant high shear rate of $20,000\text{ s}^{-1}$ (117). As it turns out, platelets experiencing sudden and steep shear acceleration form transient aggregates without prior activation or shape change, by tethering onto immobilized VWF with the boost of soluble VWF. Labile interactions between VWF and GPIIb α result in rapid translocation of platelets into downstream region with much lower shear rates. Marked reduction in shear forces in the deceleration zone results in $\alpha_{IIb}\beta_3$ engagement corresponding to localized Ca^{2+} spikes. Ca^{2+} -dependent signaling causes restructuring of filamentous platelet tethers into bulbous morphology, which ultimately stabilizes discoid platelet aggregates. On the thrombus surface, platelets remain loosely adhere via GPIIb α without any activation. On the contrary, platelets consolidate in the stable thrombus core via $\alpha_{IIb}\beta_3$ interactions following weak activation (117).

Westein et al. further elucidated the process of shear gradient-dependent thrombus formation in atherosclerosis by characterizing the central role of VWF in promoting platelet aggregation post-stenosis in both *in vivo* stenosis models of damaged murine carotid arteries and *in vitro* microfluidic perfusion assays emulating arterial flow (120). Shear microgradients or elongational flow unfold VWF at forces two orders of magnitude lower compared to constant shear, enhancing platelet recruitment substantially even at low venous flow rates (82). Adhesion of a few circulating platelets by elongated VWF immobilized at the stenosis apex can create a snowball effect with hyper-aggregation of platelets, leading to full vessel occlusion in minutes. Significantly, at shear stress exceeding 100 dynes/cm^2 , wherein VWF takes charge of platelet recruitment, aspirin, the gold standard of antiplatelet therapy, is not able to inhibit platelet aggregation, as shown in multiple studies (124–126). Selective COX-2 inhibition even enhanced platelet aggregation under elevated arterial shear stress by reducing the basal production of prostacyclin (prostaglandin I₂; PGI₂), a potent inhibitor of platelet aggregation especially at high shear stress (127, 128).

Overall, it is clear that the shear environment at sites of thrombus formation has a profound effect on various aspects of platelet behavior. In particular, high shear forces induced by the architecture of large thrombi create a feed-forward mechanism that can precipitate occlusive thrombus formation. As such, a better understanding of the shear-dependent aspects of thrombus

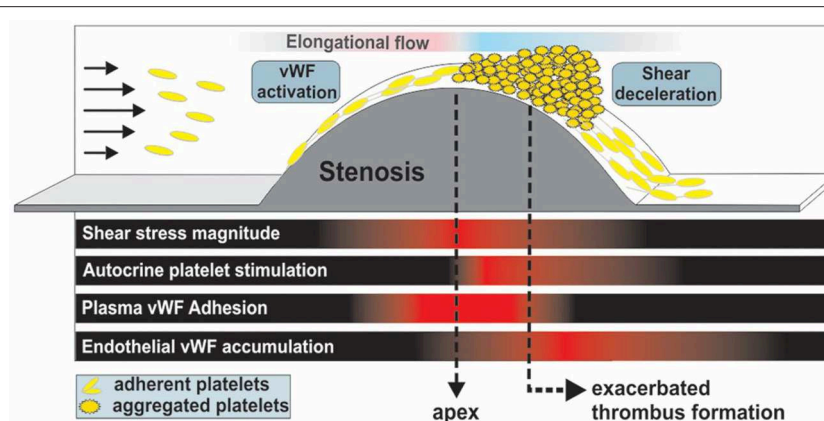


FIGURE 4 | Thrombus formation and propagation is aggravated post-stenosis (simulating atherosclerotic-like geometries) in a VWF-dependent fashion due to shear deceleration, autocrine platelet stimulation and elevated VWF deposition at the stenosis outlet. Adapted with permission from Westein et al. (120).

formation will aid in the design and development of future anti-thrombotic therapies.

THERAPEUTICS TARGETING SHEAR-DEPENDENT THROMBUS FORMATION

As discussed in the previous sections, shear-dependent VWF unfolding is crucial for initial platelet adhesion, followed by their aggregation and potential formation of a pathological thrombus. Failure of current antithrombotics to effectively prevent pathological thrombosis without bleeding side-effects has been a major obstacle in combating cardiovascular diseases.

Therefore, targeting pathological shear rates that are uniquely present at sites of thrombus development using “smart” therapeutics can be highly effective in preventing total vessel occlusion, significantly improving the efficacy and safety profile of anti-thrombotic therapy.

Two approaches of preventing occlusive thrombi by exploiting pathological shear conditions are discussed hereafter. One approach would be to inhibit shear-driven interaction of the VWF A1 domain and GPIIb α . The second approach involves shear-sensitive vehicles that specifically target occlusive thrombi due to the abnormally-high shear rates surrounding this environment.

Therapeutics Targeting Shear-Driven VWF A1-GPIIb α Interaction

Considering the diverse pathophysiological impact of the VWF-GPIIb α interaction, this ligand-receptor pair has been an attractive target in the evolutionary field of antithrombotic therapy. Undeniably, VWF's foremost interaction with the circulating platelet is shear-mediated; therefore, blocking this interaction at pathological shear rates is anticipated to inhibit hyper-aggregation of platelets in the diseased blood vessel, leaving the healthy vessels unaffected. Assorted agents have been designed and tested to target VWF-GPIIb α binding under shear

conditions, as depicted in **Figure 5**. The results from preclinical and clinical studies are outlined in **Table 1**.

AJvW-2

AJvW-2 is a murine, monoclonal IgG₁ directed against the A1 domain of human VWF. It inhibits botrocetin- (IC_{50} $1.8 \pm 0.3 \mu\text{g ml}^{-1}$), ristocetin- (IC_{50} $0.7 \pm 0.1 \mu\text{g ml}^{-1}$), and high shear stress [$10.8 \text{ Newton meter (Nm)}^{-2}$]-induced aggregation, without affecting low shear stress (1.2 Nm^{-2})-induced aggregation of human platelets *in vitro*. The antibody prevented thrombus formation *in vivo* in photochemically-induced arterial thrombosis in carotid artery of guinea pigs, without prolonging the bleeding time (129). The efficacy of AJvW-2 on shear-specific inhibition of VWF-GPIIb α interaction was tested in ACS patients (12 with unstable angina, 20 with acute myocardial infarction) with enhanced Shear-Induced Platelet Aggregation (SIPA). Using cone and plate viscometer, SIPA was measured for the patients' blood and the antibody was found to inhibit at $10 \mu\text{g ml}^{-1}$ high SIPA, without affecting aggregation at low shear stress (130). AJvW-2's prevention of thrombus generation and platelet adhesion within the carotid artery was also observed in guinea pigs, following a balloon catheter injury. Platelet aggregation was significantly inhibited for 2 days and neointima formation was circumvented 14 days following injury (131). The shear-specific blockade of VWF-GPIIb α interaction by this monoclonal antibody provided it with an advantage of lower bleeding risk over GPIIb/IIIa antagonists.

AJW200

AJW200 is the humanized form of the anti-VWF monoclonal antibody, AJvW-2 (above), synthesized to overcome the immunogenicity and rapid clearance of AJvW-2 in humans. *In vitro* studies using cone and plate viscometer showed the selective suppression of thrombin generation, adhesion and aggregation of human platelets under high shear conditions, but not under low shear conditions. Moreover, the pharmacokinetic (PK) and pharmacodynamic (PD) profiles of AJW200 were investigated *ex vivo* in cynomolgus monkeys after intravenous

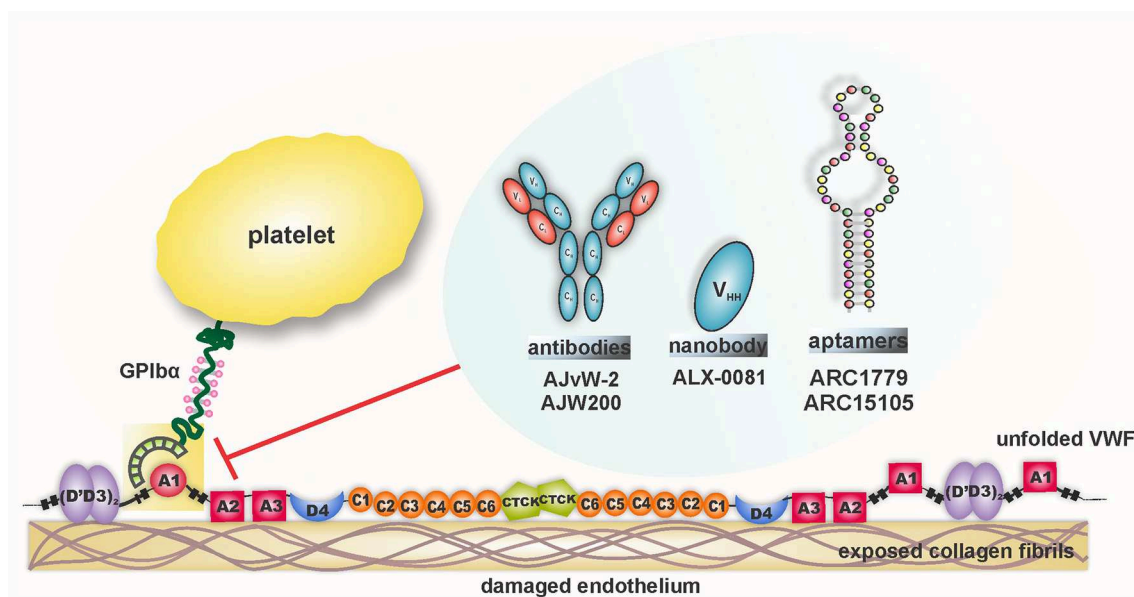


FIGURE 5 | Therapeutics targeting and inhibition of shear-driven VWF A1-GPIIb interaction.

administration. Dose-dependent inhibition of ristocetin-induced platelet aggregation was observed without extensively prolonged bleeding times as compared to abciximab (132). In the Folt's model of coronary arterial thrombosis in beagle dogs, significant inhibition of botrocetin-induced platelet aggregation and cyclic flow reductions were observed, but with marked bleeding time prolongation (133). Clinically, dose-dependent inhibition of platelet ristocetin cofactor (RiCof) activity was observed after AJW200 infusion along with an increased Platelet Function Assay (PFA)-100 closure time, with skin bleeding time augmentation (134). While the initial results with AJW200 seemed encouraging, data from the latter has not been published and no further clinical investigations with AJW200 have been reported.

ARC1779

ARC1779 is a synthetically manufactured aptamer (a short, single-stranded oligonucleotides) against the A1 domain of VWF, that strongly binds with its target (~ 2 nM dissociation constant) to prevent VWF-mediated platelet aggregation.

ARC1779 inhibited botrocetin-induced ($IC_{90} \sim 300$ nM) and high shear-induced ($IC_{95} \sim 400$ nM) aggregation of human platelets *in vitro*. In a study using electrically-induced arterial thrombosis in cynomolgus monkeys, ARC1779 inhibited occlusive thrombus formation with mild bleeding time extension (135). Overall, ARC1779 exhibited comparable antithrombotic efficacy as abciximab with lesser bleeding time prolongations. In a prospective, partial cross-over phase I/II clinical trial, safety and efficacy of ARC1779 were investigated in patients with congenital TTP (137). The results suggested that ARC1779 dose-dependently inhibited VWF-triggered platelet plug formation and stabilized the platelet count in TTP patients during infusion, with no associated bleeding events. However, the subcutaneous

delivery of the aptamer yielded plasma concentrations that were insufficient to correct all the clinical or laboratory features of TTP (137). Later, a randomized, double-blind, placebo-controlled clinical study was performed in 36 patients undergoing carotid endarterectomy (CEA) to evaluate the immediate effect of ARC1779 on cerebral emboli post CEA (138). Embolic signals (ES), detected by transcranial Doppler ultrasound (TCD), were used to determine the effect of the drug. Rapid reduction of ES signals in terms of mean signal and frequency was seen by ARC1779. However, bleeding and anemic episodes were also reported in this study (138).

ARC15105

ARC15015 is the second generation, chemically modified, anti-VWF aptamer that was designed to target shear-dependent platelet aggregation in the clinical setting of myocardial infarction. Pegylated ARC15015 was developed to substantially enhance the suppression capacity of VWF activity, with increased bioavailability and half-life. ARC15105 completely inhibited ristocetin-induced platelet aggregation in whole blood and caused over 90% inhibition of platelet adhesion in denuded porcine aortic segments under high shear conditions. In PK and PD analysis in cynomolgus monkeys, the aptamer inhibited more than 90% of VWF activity without any spontaneous bleeds throughout the study (139).

ALX-0081/0681

ALX-0081 or Caplacizumab (INN) is the only drug candidate targeting the VWF A1 domain that has been approved by the FDA to treat adult aTTP patients (136). ALX-0081 is the first in class, humanized, bivalent nanobody that specifically binds to the GPIIb binding site on VWF. Nanobodies are derived from the heavy-chain variable domains (VHH) of naturally occurring

TABLE 1 | Outline of approaches that inhibit VWF-GPIIb/IIIa interaction specifically under high shear rates.

Name	Molecule type	Injury model/organism/administration	Experimental outcome	Development phase	References
AJvW-2	Murine monoclonal antibody	–	Inhibition of Ristocetin and Botrocetin-induced aggregation of human and guinea pig platelets; dose dependent inhibition of H-SIPA in human and guinea pig platelets, L-SIPA unaffected	<i>In-vitro</i>	(129)
		Photochemically –induced thrombosis/guinea pigs/intravenous	Thrombus prevention without prolonged bleeding times	<i>In-vivo</i>	(129)
		ACS patients with UAP/AMI	Inhibition of H-SIPA of acute ACS patients	<i>In-vitro</i>	(130)
		Balloon injury/guinea pigs/intravenous	Dose dependent inhibition of thrombus and neointima formation	<i>Ex-vivo</i>	(131)
AJW200	Humanized monoclonal antibody	–	Inhibition of H-SIPA, adhesion, thrombin generation in human platelets	<i>In-vitro</i>	(132)
		cynomolgus monkeys/intravenous	Sustained inhibition of RIPA without extensive prolongation of the bleeding time in cynomolgus monkeys	<i>Ex-vivo</i>	(132)
		Balloon injury/Japanese white rabbits/intravenous	Inhibition of botrocetin-induced aggregation after first injury; reduction of cell proliferation thrombus and neointimal growth following second injury	<i>Ex-vivo</i>	(133)
		Folts injury model/Beagle dogs/intravenous	Thrombus inhibition in the stenosed coronary arteries without prolonged bleeding times	<i>Ex-vivo</i>	(133)
		Humans (healthy)/intravenous	Dose-dependent inhibition of RiCof activity, prolongation of PFA-100 closure time, no prolonging bleeding times, no significant adverse effects, no evidence of immunogenicity	Phase I	(134)
ARC1779	Aptamer	–	Inhibition of botrocetin and high shear-induced aggregation in human platelets	<i>In-vitro</i>	(135)
		Denuded porcine aorta segments	Reduction of platelet adhesion on collagen coated plates and thrombi formation in injured porcine arteries	<i>Ex-vivo</i>	(135)
		Electrical injury arterial thrombosis model/cynomolgus monkeys/intravenous	Dose-dependent inhibition of occlusive thrombus formation in cynomolgus monkeys with mild bleeding time prolongation	<i>In-vivo</i>	(135)
		Humans (healthy)/intravenous	Dose and concentration dependent VWF and platelet activity inhibition without major bleeding events or side effects	Phase I	(136)
		Humans (patients with congenital TTP)/intravenous	Dose-dependent inhibition of VWF-related platelet function, stabilized platelet count in congenital TTP patients, Test doses insufficient to suppress all the clinical/laboratory features of TTP	Phase I/II	(137)
		Humans (Patients undergoing CEA)/intravenous	Reduction in cerebral embolic events in patients post CEA, Increased anemic and bleeding events	Phase II	(138)
ARC15105	Aptamer	Denuded porcine aorta segments	Better aggregate inhibition than ARC1779 under arterial shear conditions in perfusion chamber, >90% platelet activity reduction in segmented porcine arteries	<i>In-vitro/ex-vivo</i>	(139)
		cynomolgus monkeys/intravenous, subcutaneous	>90% VWF activity reduction in cynomolgus monkeys	<i>In-vivo</i>	(139)
ALX-0081/ ALX-0681 (INN: Caplacizumab/ Cablivi)	Bivalent nanobody	–	Selective inhibition of platelet adhesion to collagen at high shear rates	<i>In-vitro</i>	(140)
		–	Complete inhibition of platelet adhesion on collagen in blood from PCI patients on standard antithrombotics	<i>Ex-vivo</i>	(140)
		Modified Folts injury model/baboons/intravenous	Lower effective dose and bleeding risk, superior therapeutic window in baboons as compared to commercial antithrombotics	<i>In-vivo</i>	(140)
		Humans (Patients undergoing PCI)/intravenous	Dose-dependent inhibition of platelet adhesion and aggregation in CAD patients prior to PCI, prolongation of col/ADP time in PFA-100	<i>In-vitro</i>	(141)
		mAb-3H9 (ADAMTS-13 inhibiting Ab) induced aTTP/Baboons/subcutaneous	Complete neutralization of VWF activity without severe bleeding risk in thrombocytopenia	<i>In-vivo/ex-vivo</i>	(142)
		Humans (healthy)/intravenous	Total RIPA inhibition at high dose in healthy subjects, no bleeding risk and immunogenic response reported, drug well-tolerated and safe	Phase I	(140)
		Humans (stable angina patients undergoing PCI)/intravenous	Transient but clinically insignificant decrease in FVIII and VWF levels in patients, no increased bleeding risk or immunogenic response on dose increase even in addition to standard therapy, rapid clearance of unbound antibody	Phase Ib	(140)

(Continued)

TABLE 1 | Continued

Name	Molecule type	Injury model/organism/administration	Experimental outcome	Development phase	References
		Humans (aTTP patients)/intravenous, subcutaneous	More rapid resolution of TTP episodes, faster platelet-count normalization, higher frequency of complete remission and increased bleeding tendency as compared to placebo	Phase II (TITAN, NCT01151423)	(143)
		Humans (aTTP patients)/intravenous, subcutaneous	Faster resolution of TTP episode with shorter time to platelet count response, clinically significant reduction in aTTP recurrence, aTTP related deaths and major thromboembolic events as compared to placebo; higher bleeding-related TEAEs than placebo (Ongoing study)	Phase III (HERCULES, NCT02553317)	(144)
				Phase IIIb (Post-Hercules, NCT02878603)	—

CEA, carotid endarterectomy; PCI, Percutaneous Coronary Intervention; TTP, Thrombotic thrombocytopenic purpura; aTTP, Acquired Thrombotic thrombocytopenic purpura; H-SIPA, High shear-induced platelet aggregation; L-SIPA, Low shear-induced platelet aggregation; Ab, antibody; CAD, Coronary artery disease; RIPA, Ristocetin-induced platelet aggregation; RiCof, Ristocetin cofactor; ACS, Acute coronary syndrome; TEAEs, Treatment-emergent adverse events; UAP, Unstable angina; AMI, Acute myocardial infarction.

camelid heavy chain antibodies. The bi-valency of ALX-0081 equips the nanobody with greater avidity to VWF. *In vitro* perfusion chamber studies, using blood from healthy individuals and PCI patients receiving standard-of-care treatment (aspirin, clopidogrel, and unfractionated heparin), showed the selective inhibition of platelet adhesion on collagen type III by the nanobody under high, arterial shear conditions (shear rates $> 1,500 \text{ s}^{-1}$), without any effect under low shear conditions. Plasma levels of 0.3 to 0.5 $\mu\text{g/ml}$ were required for total inhibition of cyclic flow reductions (CFRs) by ALX-0081, corresponding to an effective concentration of 0.8 $\mu\text{g/ml}$ in ACS patients and 0.4 $\mu\text{g/ml}$ in healthy individuals in *ex vivo* experiments (145). The therapeutic window (the difference between the dose required for CFR and the dose resulting in enhanced bleeding rates) of ALX-0081 was found to be superior over abciximab and clopidogrel in baboons. Ristocetin-induced aggregation was inhibited by ALX-0081 in healthy individuals (at 0.4 $\mu\text{g/ml}$) and in Coronary Artery Disease (CAD) patients (at 0.8 $\mu\text{g/ml}$) with higher plasma VWF antigen levels. The nanobody also suppressed platelet adhesion to collagen (Col) under high shear rates and prolonged Col/ADP closure time in PFA-100, both in a dose-dependent fashion (141).

In the setting of TTP, the anti-VWF nanobody rapidly reversed the marked decline in platelet count, along with normalization of lactate dehydrogenase (LDH) levels (which correlates with VWF activity). Most importantly, no drug-related excessive bleeding risks were observed, regardless of reduced VWF activity and platelet count (142). In an open-label extension study, comprising stage C heart failure patients, an i.v. bolus injection of the drug at 6 mg was administered in 22 Stable Angina (SA) patients. Similar bleeding episodes were observed in the drug-treated and placebo-treated groups, thus proving that ALX-0081 did not result in a greater bleeding risk in these subjects, even in combination with standard antithrombotic therapy. Stable inhibition of VWF-mediated platelet aggregation was found in patients until 30 h post-multiple administration. Nineteen patients exhibited normal VWF-dependent aggregation of platelets within 48–168 h post first bolus injection (140). Later, in a Phase II trial (TITAN), 75 acquired TTP patients received caplacizumab (10 mg) and 39 received placebo intravenously,

from 6 h to 15 min before initiation of plasma exchange. Thereafter, caplacizumab was administered subcutaneously in 10 mg doses daily within half an hour of the exchange. While the time of response (confirmed normalization of platelet count) was the primary end-point of the study, exacerbations and relapses formed major secondary end-points. The daily administration of the drug continued for 30 days following plasma exchange, reaching the maximum duration of 90 days. Impressively, the median response time with the drug decreased by 39% compared to placebo (median days 2.97 for caplacizumab vs. 4.79 for placebo, $p = 0.005$), along with an event rate ratio of 2.2 (95% CI 1.28–3.78, $p = 0.005$) as compared to the placebo. While 11 patients in the placebo group had exacerbation, only three of the caplacizumab-treated patients showed the same. Within a month of cessation of the study drug, 8 patients had a relapse with baseline ADAMTS13 activity persistently below 10% in seven of them. This could mean that the underlying autoimmune disorder was not resolved completely (146). Mild to moderate, bleeding-related adverse events were reported at a higher frequency for the caplacizumab-treated group (54% cases) as compared to the placebo group (38% cases). However, the bleeding risk was not clinically-significant and the events did not require medical attention.

The results from this trial proved significant in building the case for caplacizumab in fast and sustained resolution of aTTP and paved the way for a phase III clinical trial.

This phase III study—the HERCULES trial—was performed on 145 patients (73 receiving placebo, 72 receiving caplacizumab) already receiving daily plasma exchange and corticosteroids. Duration to platelet count response formed the primary endpoint. Seventy-four percentage reduction ($p < 0.00001$) in the occurrence of composite primary endpoints was observed for patients on caplacizumab, with the incidence rate reducing to 12.7 vs. 49.3% for placebo-treated patients. Bleeding-related Treatment Emergent Adverse Events (TEAE) were 45.6% ($n = 33$) for caplacizumab in comparison to 23.3% ($n = 17$) for the placebo group. Following these remarkable results from the HERCULES trial, caplacizumab received its first global approval last year (2018) in the European Union for the treatment of adult

aTTP patients, followed by approval from the US Food and Drug Administration (FDA) (136, 144).

In summary, VWF A1-GPIIb α binding remains pre-eminent in targeting coronary heart diseases, owing to the elevated levels of VWF specifically in these conditions. Hence, impeding the capacity of VWF to recruit platelets at the injured arterial wall is a crucial factor in designing new anti-platelet regimens. Despite the substantial efforts to specifically target the VWF-GPIIb α axis as described above, and notwithstanding the promising, preliminary preclinical and clinical results, no other inhibitors except ALX-0081 have received clinical approval so far.

Local shear-gradients have recently been recognized as the critical driving force behind pathological platelet aggregation, ultimately resulting in insidious thrombus formation. However, their paramount significance in thrombosis has been underplayed from the perspective of their exploitation for novel anti-thrombotic drug designs. Targeting the thrombosis-specific shear microgradients may prove to be the cornerstone in the field of antithrombotic therapeutics. While various direct anti-VWF agents are being developed (above), another approach is utilizing “smart” shear-responsive vehicles to locally deliver current anti-platelet drugs to sites of pathological thrombus formation, as we specify next.

Shear-Sensitive Drug Delivery Platforms Targeting Occlusive Thrombi

Cardiovascular manifestations resulting from exacerbated thrombus formation are fundamentally distinct from physiological thrombus formation because of the abnormally high and complex blood shear forces associated with the latter. Exploitation of this spatially confined high shear environment could be achieved by delivering anti-thrombotic drugs using “smart,” shear-sensitive vehicles. Targeted delivery of current drugs, leveraging these unique shear forces, could improve the therapeutic out-turn of traditional anti-thrombotic drugs by resolving their associated bleeding limitations. Different shear-sensitive platforms have been outlined in **Table 2** and are depicted in **Figure 6**. Those therapeutics, which have been reviewed elsewhere (147, 152–154), will be discussed briefly in this section.

Shear-Activated Nanoparticle Aggregates

Korin and colleagues engineered micron-sized (1–5 μ m), shear-activated nanotherapeutics (SA-NTs) or microscale aggregates from poly(lactic-co-glycolic acid), composed of smaller shear-activated nanoparticles (SA-NPs, $\sim$ 200 nm) to target constricted, diseased blood vessels. The SA-NTs maintain a size similar to platelets, to circulate peripherally along the vessel wall, encountering minimal flow velocity and maximal shear rate (49).

The micro-sized aggregates are unique in their ability to breakdown only under pathologically-high shear rates, remaining intact under physiological shear. Consolidated via weak Van der Waals interactions among the constitutive nanoparticles, the aggregates disintegrate in the presence of overpowering shear forces at the pathological thrombus site. Following shear-triggered disintegration of the aggregates, disseminated nanoparticles (facing much lower hydrodynamic

drag than the larger microaggregates) accumulate downstream of the stenosis, thereby releasing the cargo.

In the mesenteric ferric chloride-induced injury model, the intravenous administration of tissue-type plasminogen activator (t-PA)-coated SA-NPs resulted in rapid clot dissolution (within 5 min). Bolus injection of SA-NTs coated with 50 ng t-PA almost doubled the time to vessel occlusion vs. free, soluble t-PA at the same concentration. In a typically-fatal pulmonary embolism model in the mouse, almost 86% survival rate was observed in mice injected with t-PA-coated SA-NPs. Significantly reduced effective concentration of t-PA was indicated by the SA-NTs, that normalized pulmonary arterial pressure at a 100-times lower drug concentration than the control conditions (free drug). Targeted delivery of t-PA via SA-NPs, specifically to stenotic sites, can result in higher localized drug concentrations at stenotic sites without increased systemic concentrations of free drug, leading to lower hemorrhagic complications. So far, favorable results obtained from various *in vitro*, *ex vivo*, and *in vivo* models have illustrated the pre-clinical advantages of employing these engineered SA-NPs in achieving targeted delivery of the thrombolytic drug in a safe and effective manner. However, further investigations are essential to determine whether this targeting approach could be translated to the clinical setting. Furthermore, other clinically-approved antiplatelet and antithrombotic agents could also be loaded onto SA-NTs to prevent thrombosis.

Shear-Sensitive Liposomes or Vesicles

Liposomes are spherical vesicles (100–250 nm in diameter), consisting of an aqueous core surrounded by a phospholipid bilayer. Over the years, liposomal nanocapsules (NCs) have provided enormous opportunities in targeted drug delivery by their unique ability to load and stabilize a plethora of hydrophilic and hydrophobic drugs (155–158). In general, liposomes possess spherical morphology due to its minimum energy and maximum stability (159). This morphology imparts robustness to the vesicles against mechanical disruption. Bernard et al. demonstrated shear-induced membrane permeability in unilamellar, egg phosphatidyl choline (EPC) vesicles (50–400 nm) using a non-ionic surfactant, Brij 76. The study provided an experimental proof that on addition of detergents, membrane leakage can be induced by shear. Greater shear-driven membrane leakage was observed for large, unilamellar vesicles (LUVs) as compared to the smaller ones, in the presence of Brij 76 (160). Holme et al. fabricated non-spherical, lenticular vesicles (Pad-PC-Pad) exhibiting a relatively larger bending moduli and curvature around the equator, that could lead to transient pore formation under elevated shear stress. The lenticular morphology of these liposomes could increase their sensitivity toward higher shear levels. The liposomes were able to release the loaded fluorescent dye, carboxyfluorescein, under mechanical shaking even without the surfactant. However, no leakage of the liposomes was observed in the absence of mechanically disruptive forces. An extracorporeal circulation model was designed to mimic physiological and pathological (75% lumen restriction) arterial flow conditions with the shear stresses of 2 Pa and about 40 Pa, respectively. A clear, shear-specific fluorescent dye release

TABLE 2 | Outline of shear-sensitive drug delivery platforms targeting occluded blood vessels.

Delivery platform	Carrier composition	Carrier morphology	Biologically active agent/loading method	Targeted site	Experimental setup	Experimental model	Experimental outcome	References
Nanoparticle aggregates								
SA-NTs	PLGA	3.8 ± 1.6 μm aggregates composed of 180 ± 70 nm NPs	tPA/coating r-TPA	Occluded mesentery artery	<i>In vivo</i>	Mouse arterial thrombus model	Total dissolution of thrombus within 5 min of local administration	(147)
		4 μm aggregates composed of ~200 nm NPs		Occluded pulmonary artery	<i>In vivo</i>	Mouse PE model	Reinstatement of arterial flow with ~86% survival rate in mice post administration	(147)
SA-NTs (combined with TEB)	PLGA			Occluded carotid artery	<i>In vivo</i>	Rabbit carotid vessel occlusion model	Higher rate of complete recanalization of ELVO with r-TPA coated SA-NTs with TEB, significantly less vascular trauma than stent-retriever TM	(148)
Liposomes								
Lenticular vesicles	Pad-PC-Pad	Lenticular, 100 nm	[5(6)-FAM]	–	<i>In-vitro</i> (extracorporeal pump flow setup)	–	Selective payload (dye) release in stenosed arterial model at high shear	(149)
Nanocapsules	Egg-PC	~200 nm	Eptifibatide/encapsulation	Occluded carotid artery	<i>In-vivo</i>	Mouse carotid vessel occlusion model	Reduction of pulmonary thrombus load without prolonged bleeding times in mice	(150)

SA-NTs, shear-activated nanotherapeutics; SA-NPs, shear-activated nanoparticles; NPs, nanoparticles; PLGA, poly(lactic-co-glycolic acid); tPA, tissue plasminogen activator; PE, pulmonary embolism; TEB, temporary endovascular bypass; r-TPA, recombinant tissue plasminogen activator; ELVO, emergent large vessel occlusion; TM, thromboectomy; FAM, carboxyfluorescein (model-dye); Pad-PC-Pad, 1,3-dipalmitamidopropan-2-yl 2-(trimethylammonio)ethyl phosphate; PC, phosphatidylcholine.

was observed for the Pad-PC-Pad liposomes, with $45.5 \pm 11\%$ payload release in the healthy arteries and $70 \pm 2.3\%$ release in the constricted arteries after one passage only. In comparison, the fluorophore release by the egg PC liposomal formulations was below 10% after one passage. These promising *in vitro* results for the Pad-PC-Pad liposomes highlight their potential in the area of targeted, shear-specific delivery to the occluded vessels in MI patients (149).

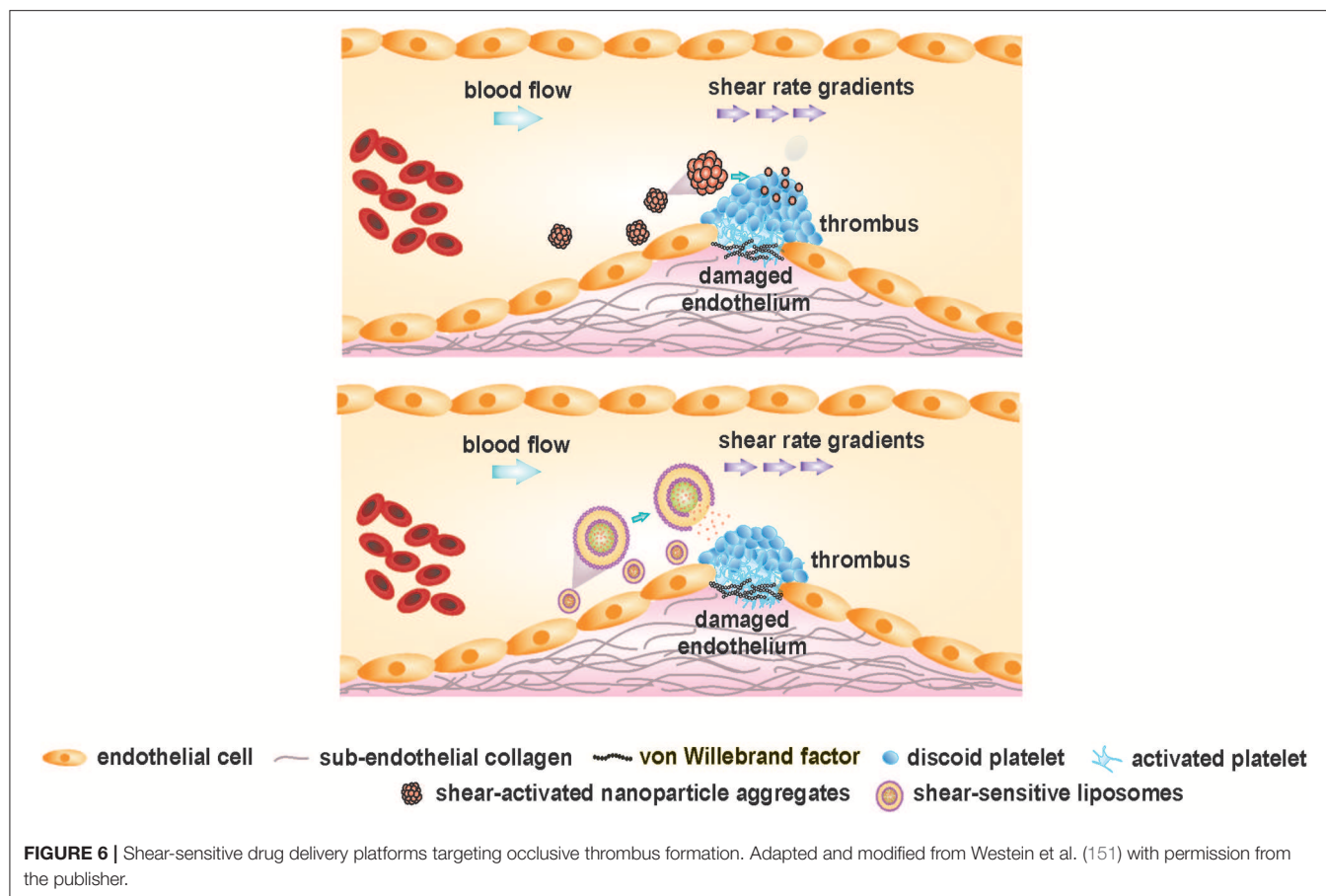
Recently, Molloy et al. (150) formulated shear-sensitive egg phosphatidylcholine (PC) liposomes that targeted the clinical antiplatelet drug, eptifibatide, to the local thrombus site. *In vitro* blood perfusion assays were done in stenotic microchannels emulating stenotic shear conditions. The liposomes released eptifibatide at stenotic sites with governing shear rates of $\sim 8,000 \text{ s}^{-1}$, inhibiting platelet aggregation specifically in the narrowed region without effects on thrombus volume in regions of lower shear ($\sim 1,000 \text{ s}^{-1}$). Modulation of the shear-sensitive drug release properties of the nanocapsules was demonstrated by incorporating Brij 76 in the formulations, reducing the liposomal shear-threshold from over $1,500 \text{ s}^{-1}$ to between 500 and $1,000 \text{ s}^{-1}$. When applied *in vivo* in a carotid artery occlusion mouse model, the shear-sensitive, eptifibatide-loaded liposomes significantly reduced thrombus load as compared with free eptifibatide, without any prolongation of bleeding times. This safety profile has most likely been achieved by the marked reduction in systemic eptifibatide concentrations, enabled by the targeted drug-release approach.

Surface functionalization of shear-sensitive liposomes with targeting moieties could actively direct the liposomes toward thrombi *in vivo*, further lowering the required dosage. Site-specific delivery of anti-platelet drugs could increase the local drug concentration at the stenosed vessel region without posing major bleeding risks.

In summary, liposomes can serve as an ideal carrier of an unprecedented range of antithrombotic agents, given the versatility of their physical and chemical structures and tunable functionality of the lipid surfaces to achieve desired physiological stimuli responsiveness. Tailoring their mechanical properties to induce sensitivity to pathological shear forces may result in efficacious, targeted drug delivery platform. Proof-of-concept studies have already shown promising results of liposomal drug delivery to constricted vessels with an increased local drug concentration. Drug dosage can be further reduced by chemical or biological modifications of liposomes via targeting moieties that direct the payload to the stenotic site, thereby attenuating thrombus progression (161–163).

CONCLUSION AND CURRENT CLINICAL CHALLENGES

VWF is an attractive, shear-sensitive target for drug delivery due to its altered biophysical properties under the pathological



hemodynamic microenvironment, leading to hyper-aggregation of platelets in thrombotic events. Selective targeting of the VWF binding sites with platelet receptors could prove safer and more effective than systemic inhibition of platelet function via current antiplatelet drugs. Several agents targeting VWF under high shear conditions have been developed, yet despite initial success in some reports many challenges still require resolution for future clinical use. For example, *in vitro* assays and *in vivo* models emulating shear microenvironments in blood vessels adopt an over-simplified approach, while the physiological blood rheology and microvasculature vastly differ within and among organisms. This may account for the all too often failure to reproduce encouraging preclinical findings in clinical trials, seen with a number of compounds to-date.

Current blood coagulation tools, including tail bleeding time, skin puncture test, activated partial thromboplastin time (APTT), prothrombin time (PT) or platelet aggregometry do not incorporate biomechanical aspects of thrombosis, i.e., shear stress (164–166). The inability of the standard testing tools to embed the effects of local shear rates and shear rate gradients limits their assessment accuracy in the clinical settings. Rheological devices, such as parallel plate flow chambers and microfluidic blood perfusion channels coated with extracellular matrix proteins, can effectively emulate the pathological shear conditions in

stenotic vessels. These devices provide versatile platforms with customizable flow and coating conditions, using minuscule blood volumes, to optimally indicate the net coagulative state of a given sample when all major pathways are in place (117, 120, 167, 168).

Several considerations should be taken into account when designing a novel shear-sensitive therapy, including the choice of a suitable, biologically-active compound(s), the need to tailor the shear sensitivity of a given template for maximum drug release without amplifying any negative effects, and the requirement to tune the circulation times (“half-life”) according to the drug’s therapeutic window. This can be a challenging task, as most nanoparticles are immediately uptaken and rapidly cleared by the reticuloendothelial system (RES). Opsonization and immunogenicity can be other obstacles for *in vivo* utilization of these nano-agents (169). Therefore, optimization of particle hydrodynamic diameter, surface morphology and functionality can prolong their circulation times in blood to attain the desired therapeutic effect. PEGylation can also result in stealth nanoparticles with longer half-lives (169). Toxicity is yet another problem associated with nanoparticles, that can be significantly reduced or completely avoided by opting for natural compounds instead of synthetic ones (170). Storage stability as well as scale-up feasibility of nanoparticles can pose additional challenges in their production. Selecting a simple, cost-effective and easily reproducible production

strategy can offer some remedy for these issues (171). Despite these challenges, nanoparticles are increasingly used in clinical applications (172).

FUTURE PERSPECTIVES

For decades, the focus of anti-platelet drug development has been on platelet activation pathways induced by soluble agonists and ligand binding (173, 174). In recent years, the concept of complex shear forces, which contribute to pathological thrombus formation, has gained traction in multiple studies using sophisticated mouse models (175, 176) as well as *in vitro* blood perfusion flow assays (4, 117, 120) and novel single-molecule techniques (7, 108, 177, 178). Nevertheless, so far these studies have not resulted in any translation into shear selective therapies.

The role of shear, and in particular gradients of shear, in driving VWF-GPIb α -dependent thrombus formation in cardiovascular diseases has become apparent. The difference in propensity of VWF-GPIb α interaction under constant, physiological shear rates and pathological shear gradients can be crucial in distinguishing between the life-saving process of hemostasis and the life-threatening process of thrombosis. Selective interference with the VWF-GPIb α axis under shear gradients may not only increase the efficacy of future anti-thrombotic agent, but potentially resolve the most dangerous limitation of current therapeutics, being bleeding complications.

Several strategies that selectively target VWF A1-GPIb α binding under high shear rates have been developed, including antibodies (AJvW-2, AJW200), aptamers (ARC1779,

ARC15105), and nanobodies (ALX-0081). In a related strategy, shear-sensitive “smart” nanoparticles have been produced to target vessel occlusions, including nanoparticle aggregates and liposomes. Although further studies are required to validate the efficacy and safety of these therapeutic nanotechnologies, preliminary results have been highly promising.

Taken together, nanoparticles exhibit unique physical, chemical, structural, and biological properties, which can be harnessed to revolutionize the field of thrombosis. Nanoparticles versatility truly offers immense possibilities for surface modification and functionalization to suit the desired application. Nano formulations indeed provide great opportunities for targeted drug delivery due to their high drug payload capacity, reduced antigenicity and prolonged plasma half-life (179–182). Lower effective dosage and higher localization of the drug at the affected sites may prove critical in addressing the age-old bleeding issues. Although the full potential of shear-sensitive antithrombotic/antiplatelet therapy is yet to be clinically realized, shear-responsive strategies are destined to transform the future of thrombosis.

AUTHOR CONTRIBUTIONS

AR has drafted the first version of the paper and all other authors (CH, BN, and EW) have reviewed and revised the review.

FUNDING

This work was support by the National Health and Medical Research Council of Australia.

REFERENCES

- Bizzozero G. Su di un nuovo elemento morfologico del sangue dei mammiferi e della sua importanza nella trombosi e nella coagulazione. *L'Osservatore*. (1881) 17:785–7.
- Paulus JM. Platelet size in man. *Blood*. (1975) 46:321–36.
- Quach ME, Chen W, Li R. Mechanisms of platelet clearance and translation to improve platelet storage. *Blood*. (2018) 131:1512–21. doi: 10.1182/blood-2017-08-743229
- Maxwell MJ, Westein E, Nesbitt WS, Giuliano S, Dopheide SM, Jackson SP. Identification of a 2-stage platelet aggregation process mediating shear-dependent thrombus formation. *Blood*. (2007) 109:566–76. doi: 10.1182/blood-2006-07-028282
- Jackson SP, Nesbitt WS, Westein E. Dynamics of platelet thrombus formation. *J Thromb Haemost*. (2009) 7(Suppl. 1):17–20. doi: 10.1111/j.1538-7836.2009.03401.x
- Kang I, Raghavachari M, Hofmann CM, Marchant RE. Surface-dependent expression in the platelet GPIb binding domain within human von Willebrand factor studied by atomic force microscopy. *Thromb Res*. (2009) 119:731–40. doi: 10.1016/j.thromres.2006.06.010
- Ju L, Chen Y, Zhou F, Lu H, Cruz MA, Zhu C. Von Willebrand factor-A1 domain binds platelet glycoprotein Ib α in multiple states with distinctive force-dependent dissociation kinetics. *Thromb Res*. (2015) 136:606–12. doi: 10.1016/j.thromres.2015.06.019
- Jung SM, Moroi M. Platelet glycoprotein VI. *Adv Exp Med Biol*. (2008) 640:53–63. doi: 10.1007/978-0-387-09789-3_5
- Staat WD, Walsh JJ, Pexton T, Santoro SA. The alpha 2 beta 1 integrin cell surface collagen receptor binds to the alpha 1 (I)-CB3 peptide of collagen. *J Biol Chem*. (1990) 265:4778–81.
- Farndale RW, Sixma JJ, Barnes MJ, De Groot PG. The role of collagen in thrombosis and hemostasis. *J Thromb Haemost*. (2004) 2:561–73. doi: 10.1111/j.1538-7836.2004.00665.x
- Moskalensky AE, Litvinenko AL. The platelet shape change: biophysical basis and physiological consequences. *Platelets*. (2019) 30:543–8. doi: 10.1080/09537104.2018.1514109
- Golebiewska EM, Poole AW. Platelet secretion: from haemostasis to wound healing and beyond. *Blood Rev*. (2015) 29:153–62. doi: 10.1016/j.blre.2014.10.003
- Blair P, Flaumenhaft R. Platelet alpha-granules: basic biology and clinical correlates. *Blood Rev*. (2009) 23:177–89. doi: 10.1016/j.blre.2009.04.001
- Macfarlane DE, Walsh PN, Mills DC, Holmsen H, Day HJ. The role of thrombin in ADP-induced platelet aggregation and release: a critical evaluation. *Br J Haematol*. (1975) 30:457–63. doi: 10.1111/j.1365-2141.1975.tb01860.x
- Harrison P, Martin Cramer E. Platelet α -granules. *Blood Rev*. (1993) 7:52–62. doi: 10.1016/0268-960X(93)90024-X
- Ma YQ, Qin J, Plow EF. Platelet integrin alpha(IIb)beta(3): activation mechanisms. *J Thromb Haemost*. (2007) 5:1345–52. doi: 10.1111/j.1538-7836.2007.02537.x
- Vickers JD. Binding of polymerizing fibrin to integrin alpha IIb beta3 on chymotrypsin-treated rabbit platelets decreases phosphatidylinositol 4,5-bisphosphate and increases cytoskeletal actin. *Platelets*. (1999) 10:228–37. doi: 10.1080/09537109976077

18. Gale AJ. Continuing education course #2: current understanding of hemostasis. *Toxicol Pathol.* (2011) 39:273–80. doi: 10.1177/0192623310389474
19. Adams HP Jr. Secondary prevention of atherothrombotic events after ischemic stroke. *Mayo Clin Proc.* (2009) 84:43–51. doi: 10.4065/84.1.43
20. Papp J, Kenyeres P, Toth K. Clinical importance of antiplatelet drugs in cardiovascular diseases. *Clin Hemorheol Microcirc.* (2013) 53:81–96. doi: 10.3233/CH-2012-1578
21. Angiolillo DJ. Variability in responsiveness to oral antiplatelet therapy. *Am J Cardiol.* (2009) 103:27a–34a. doi: 10.1016/j.amjcard.2008.11.020
22. Kuliczowski W, Witkowski A, Polonski L, Watala C, Filipiak K, Budaj A, et al. Interindividual variability in the response to oral antiplatelet drugs: a position paper of the Working Group on antiplatelet drugs resistance appointed by the Section of Cardiovascular Interventions of the Polish Cardiac Society, endorsed by the Working Group on Thrombosis of the European Society of Cardiology. *Eur Heart J.* (2009) 30:426–35. doi: 10.1093/eurheartj/ehn562
23. Sofi F, Marcucci R, Gori AM, Giusti B, Abbate R, Gensini GF. Clopidogrel non-responsiveness and risk of cardiovascular morbidity. An updated meta-analysis. *Thromb Haemost.* (2010) 103:841–8. doi: 10.1160/TH09-06-0418
24. Di Minno MND, Guida A, Camera M, Colli S, Di Minno G, Tremoli E. Overcoming limitations of current antiplatelet drugs: a concerted effort for more profitable strategies of intervention. *Ann Med.* (2011) 43:531–44. doi: 10.3109/07853890.2011.582137
25. Verheugt FWA. In search of a better platelet ADP-receptor blocker. *Neth Heart J.* (2009) 17:188–9. doi: 10.1007/BF03086244
26. Ferro A. Newer antiplatelet agents in acute coronary syndrome. *BMJ.* (2016) 352:h7025. doi: 10.1136/bmj.h7025
27. Paradiso-Hardy FL, Angelo CM, Lancôt KL, Cohen EA. Hematologic dyscrasia associated with ticlopidine therapy: evidence for causality. *Can Med Assoc J.* (2000) 163:1441–8.
28. Eikelboom JW, Hirsh J, Weitz JI, Johnston M, Yi Q, Yusuf S. Aspirin-resistant thromboxane biosynthesis and the risk of myocardial infarction, stroke, or cardiovascular death in patients at high risk for cardiovascular events. *Circulation.* (2002) 105:1650–5. doi: 10.1161/01.CIR.0000013777.21160.07
29. Pedersen AK, Fitzgerald GA. Dose-related kinetics of aspirin. Presystemic acetylation of platelet cyclooxygenase. *N Engl J Med.* (1984) 311:1206–11. doi: 10.1056/NEJM198411083111902
30. Kastrati A, Schomig A, Schomig E. Are we making efficient use of clopidogrel? *Eur Heart J.* (2004) 25:454–6. doi: 10.1016/j.ehj.2004.02.005
31. Husted S. Benefits and risks with antiplatelet therapy: how great a problem is bleeding? *Eur Heart J Suppl.* (2008) 10:119–24. doi: 10.1093/eurheartj/sun036
32. Antithrombotic Trialists C, Baigent C, Blackwell L, Collins R, Emberson J, Godwin J, et al. Aspirin in the primary and secondary prevention of vascular disease: collaborative meta-analysis of individual participant data from randomised trials. *Lancet.* (2009) 373:1849–60. doi: 10.1016/S0140-6736(09)60503-1
33. Farre AJ, Tamargo J, Mateos-Caceres PJ, Azcona L, Macaya C. Old and new molecular mechanisms associated with platelet resistance to antithrombotics. *Pharm Res.* (2010) 27:2365–73. doi: 10.1007/s11095-010-0209-4
34. Tsai Thomas T, Ho PM, Xu S, Powers JD, Carroll Nikki M, Shetterly Susan M, et al. Increased risk of bleeding in patients on clopidogrel therapy after drug-eluting stents implantation. *Circulation.* (2010) 3:230–5. doi: 10.1161/CIRCINTERVENTIONS.109.919001
35. Patrono C, Collier B, Fitzgerald GA, Hirsh J, Roth G. Platelet-active drugs: the relationships among dose, effectiveness, and side effects: the seventh ACCP Conference on antithrombotic and thrombolytic therapy. *Chest.* (2004) 126:234s–64s. doi: 10.1378/chest.126.3_suppl.234S
36. Germing A, Bojara W, Lawo T, Ewers A, Grewe P, Mügge A, et al. Bleeding complications associated with glycoprotein IIb/IIIa inhibitors in patients 80 years of age and older undergoing percutaneous coronary intervention. *Exp Clin Cardiol.* (2010) 15:e57–60.
37. Nussmeier NA, Whelton AA, Brown MT, Langford RM, Hoeft A, Parlow JL, et al. Complications of the COX-2 inhibitors parecoxib and valdecoxib after cardiac surgery. *N Engl J Med.* (2005) 352:1081–91. doi: 10.1056/NEJMoa050330
38. Solomon SD, McMurray JJV, Pfeffer MA, Wittes J, Fowler R, Finn P, et al. Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention. *N Engl J Med.* (2005) 352:1071–80. doi: 10.1056/NEJMoa050405
39. Sabatine MS, Cannon CP, Gibson CM, López-Sendón JL, Montalescot G, Theroux P, et al. Addition of clopidogrel to aspirin and fibrinolytic therapy for myocardial infarction with ST-segment elevation. *N Engl J Med.* (2005) 352:1179–89. doi: 10.1056/NEJMoa050522
40. Yusuf S, Zhao F, Mehta SR, Chrolavicius S, Tognoni G, Fox KK, et al. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. *N Engl J Med.* (2001) 345:494–502. doi: 10.1056/NEJMoa010746
41. Levine GN, Bates ER, Bittl JA, Brindis RG, Fihn SD, Fleisher LA, et al. 2016 ACC/AHA guideline focused update on duration of dual antiplatelet therapy in patients with coronary artery disease: a report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. *J Thorac Cardiovasc Surg.* (2016) 152:1243–75. doi: 10.1016/j.jtcvs.2016.07.044
42. Bittl JA, Baber U, Bradley SM, Wijesundera DN. Duration of dual antiplatelet therapy: a systematic review for the 2016 ACC/AHA guideline focused update on duration of dual antiplatelet therapy in patients with coronary artery disease: a report of the American college of cardiology/American heart association task force on clinical practice Guidelines. *J Am Coll Cardiol.* (2016) 68:1116–39. doi: 10.1016/j.jacc.2016.03.512
43. Wilson SJ, Newby DE, Dawson D, Irving J, Berry C. Duration of dual antiplatelet therapy in acute coronary syndrome. *Heart.* (2017) 103:573. doi: 10.1136/heartjnl-2016-309871
44. Hansen ML, Sorensen R, Clausen MT, Fog-Petersen ML, Raunso J, Gadsboll N, et al. Risk of bleeding with single, dual, or triple therapy with warfarin, aspirin, and clopidogrel in patients with atrial fibrillation. *Arch Intern Med.* (2010) 170:1433–41. doi: 10.1001/archinternmed.2010.271
45. Dans AL, Connolly SJ, Wallentin L, Yang S, Nakamya J, Brueckmann M, et al. Concomitant use of antiplatelet therapy with dabigatran or warfarin in the Randomized Evaluation of Long-Term Anticoagulation Therapy (RE-LY) trial. *Circulation.* (2013) 127:634–40. doi: 10.1161/CIRCULATIONAHA.112.115386
46. Strony J, Beaudoin A, Brands D, Adelman B. Analysis of shear stress and hemodynamic factors in a model of coronary artery stenosis and thrombosis. *Am J Physiol.* (1993) 265:H1787–96. doi: 10.1152/ajpheart.1993.265.5.H1787
47. Sakariassen KS, Orning L, Turitto VT. The impact of blood shear rate on arterial thrombus formation. *Future Sci OA.* (2015) 1:FSO30. doi: 10.4155/fso.15.28
48. Kroll MH, Hellums JD, McIntire LV, Schafer AI, Moake JL. Platelets and shear stress. *Blood.* (1996) 88:1525–41.
49. Goldsmith HL, Turitto VT. Rheological aspects of thrombosis and haemostasis: basic principles and applications. ICHT-report-subcommittee on rheology of the international committee on thrombosis and haemostasis. *Thromb Haemost.* (1986) 55:415–35. doi: 10.1055/s-0038-1661576
50. Livio M, Gotti E, Marchesi D, Mecca G, Remuzzi G, De Gaetano G. Uraemic bleeding: role of anaemia and beneficial effect of red cell transfusions. *Lancet.* (1982) 2:1013–5. doi: 10.1016/S0140-6736(82)90050-2
51. Lipowsky HH, Kovalcheck S, Zweifach BW. The distribution of blood rheological parameters in the microvasculature of cat mesentery. *Circ Res.* (1978) 43:738–49. doi: 10.1161/01.RES.43.5.738
52. Goldsmith HL, Cokelet GR, Gaetgens P, Robin Fahraeus: evolution of his concepts in cardiovascular physiology. *Am J Physiol.* (1989) 257:H1005–15. doi: 10.1152/ajpheart.1989.257.3.H1005
53. Basmadjian D. The effect of flow and mass transport in thrombogenesis. *Ann Biomed Eng.* (1990) 18:685–709. doi: 10.1007/BF02368455
54. Gogia S, Neelamegham S. Role of fluid shear stress in regulating VWF structure, function and related blood disorders. *Biorheology.* (2015) 52:319–35. doi: 10.3233/BIR-15061
55. Sakariassen KS. Thrombus formation on apex of arterial stenoses: the need for a fluid high shear stenosis diagnostic device. *Future Cardiol.* (2007) 3:193–201. doi: 10.2217/14796678.3.2.193

56. Dopheide SM, Maxwell MJ, Jackson SP. Shear-dependent tether formation during platelet translocation on von Willebrand factor. *Blood*. (2002) 99:159. doi: 10.1182/blood.V99.1.159
57. Savage B, Saldívar E, Ruggeri ZM. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. *Cell*. (1996) 84:289–97. doi: 10.1016/S0092-8674(00)80983-6
58. Ikeda Y, Handa M, Kawano K, Kamata T, Murata M, Araki Y, et al. The role of von Willebrand factor and fibrinogen in platelet aggregation under varying shear stress. *J Clin Invest*. (1991) 87:1234–40. doi: 10.1172/JCI115124
59. Kulkarni S, Dopheide SM, Yap CL, Ravanat C, Freund M, Mangin P, et al. A revised model of platelet aggregation. *J Clin Invest*. (2000) 105:783–91. doi: 10.1172/JCI7569
60. Titani K, Kumar S, Takio K, Ericsson LH, Wade RD, Ashida K, et al. Amino acid sequence of human von Willebrand factor. *Biochemistry*. (1986) 25:3171–84. doi: 10.1021/bi00359a015
61. Ruggeri ZM. Structure of von Willebrand factor and its function in platelet adhesion and thrombus formation. *Best Pract Res Clin Haematol*. (2001) 14:257–79. doi: 10.1053/beha.2001.0133
62. Shankaran H, Alexandridis P, Neelamegham S. Aspects of hydrodynamic shear regulating shear-induced platelet activation and self-association of von Willebrand factor in suspension. *Blood*. (2003) 101:2637–45. doi: 10.1182/blood-2002-05-1550
63. Li Y, Choi H, Zhou Z, Nolasco L, Pownall HJ, Voorberg J, et al. Covalent regulation of ULVWF string formation and elongation on endothelial cells under flow conditions. *J Thromb Haemost*. (2008) 6:1135–43. doi: 10.1111/j.1538-7836.2008.02991.x
64. Swami A, Kaur V. von Willebrand disease: a concise review and update for the practicing physician. *Clin Appl Thromb Hemost*. (2017) 23:900–10. doi: 10.1177/1076029616675969
65. Spiel Alexander O, Gilbert James C, Jilma B. Von Willebrand factor in cardiovascular disease. *Circulation*. (2008) 117:1449–59. doi: 10.1161/CIRCULATIONAHA.107.722827
66. Rivera J, Lozano ML, Navarro-Núñez L, Vicente V. Platelet receptors and signaling in the dynamics of thrombus formation. *Haematologica*. (2009) 94:700–11. doi: 10.3324/haematol.2008.003178
67. Frankel David S, Meigs James B, Massaro Joseph M, Wilson Peter WF, O'donnell Christopher J, D'agostino Ralph B, et al. Von Willebrand factor, type 2 diabetes mellitus, and risk of cardiovascular disease. *Circulation*. (2008) 118:2533–9. doi: 10.1161/CIRCULATIONAHA.108.792986
68. Van Loon JE, Kavousi M, Leebeek FW, Felix JE, Hofman A, Witteman JC, et al. von Willebrand factor plasma levels, genetic variations and coronary heart disease in an older population. *J Thromb Haemost*. (2012) 10:1262–9. doi: 10.1111/j.1538-7836.2012.04771.x
69. Takahashi M, Yamashita A, Moriguchi-Goto S, Marutsuka K, Sato Y, Yamamoto H, et al. Critical role of von Willebrand factor and platelet interaction in venous thromboembolism. *Histol Histopathol*. (2009) 24:1391–8. doi: 10.14670/HH-24.1391
70. Sadler JE. Biochemistry and genetics of von Willebrand factor. *Annu Rev Biochem*. (1998) 67:395–424. doi: 10.1146/annurev.biochem.67.1.395
71. De Gennes PG. Coil-stretch transition of dilute flexible polymers under ultrahigh velocity gradients. *J Chem Phys*. (1974) 60:5030–42. doi: 10.1063/1.1681018
72. Smith DE, Babcock HP, Chu S. Single-polymer dynamics in steady shear flow. *Science*. (1999) 283:1724. doi: 10.1126/science.283.5408.1724
73. Ulrichts H, Vanhoorelbeke K, Girma JP, Lenting PJ, Vauterin S, Deckmyn H. The von Willebrand factor self-association is modulated by a multiple domain interaction. *J Thromb Haemost*. (2005) 3:552–61. doi: 10.1111/j.1538-7836.2005.01209.x
74. De Luca M, Facey DA, Favalaro EJ, Hertzberg MS, Whisstock JC, McNally T, et al. Structure and function of the von Willebrand factor A1 domain: analysis with monoclonal antibodies reveals distinct binding sites involved in recognition of the platelet membrane glycoprotein Ib-IX-V complex and ristocetin-dependent activation. *Blood*. (2000) 95:164.
75. Di Stasio E, De Cristofaro R. The effect of shear stress on protein conformation: physical forces operating on biochemical systems: the case of von Willebrand factor. *Biophys Chem*. (2010) 153:1–8. doi: 10.1016/j.bpc.2010.07.002
76. Doyle PS, Ladoux B, Viovy J-L. Dynamics of a tethered polymer in shear flow. *Phys Rev Lett*. (2000) 84:4769–72. doi: 10.1103/PhysRevLett.84.4769
77. Alexander-Katz A, Schneider MF, Schneider SW, Wixforth A, Netz RR. Shear-flow-induced unfolding of polymeric globules. *Phys Rev Lett*. (2006) 97:138101. doi: 10.1103/PhysRevLett.97.138101
78. Springer TA. von Willebrand factor, Jedi knight of the bloodstream. *Blood*. (2014) 124:1412–25. doi: 10.1182/blood-2014-05-378638
79. Siedlecki CA, Lestini BJ, Kottke-Marchant KK, Eppell SJ, Wilson DL, Marchant RE. Shear-dependent changes in the three-dimensional structure of human von Willebrand factor. *Blood*. (1996) 88:2939–50.
80. Ruggeri ZM, Orje JN, Habermann R, Federici AB, Reininger AJ. Activation-independent platelet adhesion and aggregation under elevated shear stress. *Blood*. (2006) 108:1903–10. doi: 10.1182/blood-2006-04-011551
81. Zhang X, Halvorsen K, Zhang C-Z, Wong WP, Springer TA. Mechanoenzymatic cleavage of the ultralarge vascular protein von willebrand factor. *Science*. (2009) 324:1330. doi: 10.1126/science.1170905
82. Sing CE, Alexander-Katz A. Elongational flow induces the unfolding of von Willebrand factor at physiological flow rates. *Biophys J*. (2010) 98:L35–7. doi: 10.1016/j.bpj.2010.01.032
83. Fu H, Jiang Y, Yang D, Scheiflinger F, Wong WP, Springer TA. Flow-induced elongation of von Willebrand factor precedes tension-dependent activation. *Nat Commun*. (2017) 8:324. doi: 10.1038/s41467-017-00230-2
84. Ying J, Ling Y, Westfield LA, Sadler JE, Shao J-Y. Unfolding the A2 domain of von Willebrand factor with the optical trap. *Biophys J*. (2010) 98:1685–93. doi: 10.1016/j.bpj.2009.12.4324
85. Joly BS, Coppo P, Veyradier A. Thrombotic thrombocytopenic purpura. *Blood*. (2017) 129:2836–46. doi: 10.1182/blood-2016-10-709857
86. Xu AJ, Springer TA. Calcium stabilizes the von Willebrand factor A2 domain by promoting refolding. *Proc Natl Acad Sci USA*. (2012) 109:3742. doi: 10.1073/pnas.1121261109
87. Reininger AJ, Heijnen HFG, Schumann H, Specht HM, Schramm W, Ruggeri ZM. Mechanism of platelet adhesion to von Willebrand factor and microparticle formation under high shear stress. *Blood*. (2006) 107:3537–45. doi: 10.1182/blood-2005-02-0618
88. Ruggeri ZM, Mendolicchio GL. Interaction of von Willebrand factor with platelets and the vessel wall. *Hamostaseologie*. (2015) 35:211–24. doi: 10.5482/HAMO-14-12-0081
89. Ruggeri ZM. Platelet adhesion under flow. *Microcirculation*. (2009) 16:58–83. doi: 10.1080/10739680802651477
90. Berndt MC, Ward CM, Booth WJ, Castaldi PA, Mazurov AV, Andrews RK. Identification of aspartic acid 514 through glutamic acid 542 as a glycoprotein Ib-IX complex receptor recognition sequence in von Willebrand factor. Mechanism of modulation of von Willebrand factor by ristocetin and botrocetin. *Biochemistry*. (1992) 31:11144–51. doi: 10.1021/bi00160a027
91. Dong J-F, Berndt MC, Schade A, McIntire LV, Andrews RK, López JA. Ristocetin-dependent, but not botrocetin-dependent, binding of von Willebrand factor to the platelet glycoprotein Ib-IX-V complex correlates with shear-dependent interactions. *Blood*. (2001) 97:162. doi: 10.1182/blood.V97.1.162
92. Mazzucato M, Spessotto P, Masotti A, De Appollonia L, Cozzi MR, Yoshioka A, et al. Identification of domains responsible for von Willebrand factor type VI collagen interaction mediating platelet adhesion under high flow. *J Biol Chem*. (1999) 274:3033–41. doi: 10.1074/jbc.274.5.3033
93. Bergmeier W, Piffath CL, Goerge T, Cifuni SM, Ruggeri ZM, Ware J, et al. The role of platelet adhesion receptor GPIIb/IIIa far exceeds that of its main ligand, von Willebrand factor, in arterial thrombosis. *Proc Natl Acad Sci USA*. (2006) 103:16900–5. doi: 10.1073/pnas.0608207103
94. Clemetson KJ, Clemetson JM. Platelet GPIb-V-IX complex. Structure, function, physiology, and pathology. *Semin Thromb Hemost*. (1995) 21:130–6. doi: 10.1055/s-2007-1000387
95. McEwan PA, Yang W, Carr KH, Mo X, Zheng X, Li R, et al. Quaternary organization of GPIb-IX complex and insights into Bernard-Soulier syndrome revealed by the structures of GPIIb/IIIa and a GPIIb/IIIa chimera. *Blood*. (2011) 118:5292–301. doi: 10.1182/blood-2011-05-356253
96. Nakamura F, Pudas R, Heikkinen O, Permi P, Kilpeläinen I, Munday AD, et al. The structure of the GPIb-filamin A complex. *Blood*. (2006) 107:1925–32. doi: 10.1182/blood-2005-10-3964

97. Christiaens L, Macchi L, Duplantier C, Allal J, Brizard A, Barraine R, et al. Platelet polymorphism and coronary artery disease. *Arch Mal Coeur Vaiss.* (2002) 95:173–8.
98. Meisel CA, López J, Stangl K. Role of platelet glycoprotein polymorphisms in cardiovascular diseases. *Naunyn Schmiedebergs Arch Pharmacol.* (2004) 369:38–54. doi: 10.1007/s00210-003-0828-y
99. Huizinga EG, Tsuji S, Romijn RA, Schiphorst ME, De Groot PG, Sixma JJ, et al. Structures of glycoprotein Ib α and its complex with von Willebrand factor A1 domain. *Science.* (2002) 297:1176–9. doi: 10.1126/science.107355
100. Alsallaq R, Zhou HX. Electrostatic rate enhancement and transient complex of protein-protein association. *Proteins.* (2008) 71:320–35. doi: 10.1002/prot.21679
101. Yago T, Lou J, Wu T, Yang J, Miner JJ, Coburn L, et al. Platelet glycoprotein Ib α forms catch bonds with human WT vWF but not with type 2B von Willebrand disease vWF. *J Clin Invest.* (2008) 118:3195–207. doi: 10.1172/JCI35754
102. Ju L, Dong JF, Cruz MA, Zhu C. The N-terminal flanking region of the A1 domain regulates the force-dependent binding of von Willebrand factor to platelet glycoprotein Ib α . *J Biol Chem.* (2013) 288:32289–301. doi: 10.1074/jbc.M113.504001
103. Kim J, Zhang C-Z, Zhang X, Springer TA. A mechanically stabilized receptor–ligand flex-bond important in the vasculature. *Nature.* (2010) 466:992. doi: 10.1038/nature09295
104. Zhang W, Deng W, Zhou L, Xu Y, Yang W, Liang X, et al. Identification of a juxtamembrane mechanosensitive domain in the platelet mechanosensor glycoprotein Ib-IX complex. *Blood.* (2015) 125:562–9. doi: 10.1182/blood-2014-07-589507
105. Shen Y, Dong J, Romo GM, Arceneaux W, Aprico A, Gardiner EE, et al. Functional analysis of the C-terminal flanking sequence of platelet glycoprotein Ib α using canine–human chimeras. *Blood.* (2002) 1:145–50. doi: 10.1182/blood.V99.1.145
106. Shen Y, Cranmer SL, Aprico A, Whisstock JC, Jackson SP, Berndt MC, et al. Leucine-rich repeats 2–4 (Leu60-Glu128) of platelet glycoprotein Ib α regulate shear-dependent cell adhesion to von Willebrand factor. *J Biol Chem.* (2006) 281:26419–23. doi: 10.1074/jbc.M604296200
107. Ju L, Lou J, Chen Y, Li Z, Zhu C. Force-induced unfolding of leucine-rich repeats of glycoprotein Ib α strengthens ligand interaction. *Biophys J.* (2015) 109:1781–4. doi: 10.1016/j.bpj.2015.08.050
108. Ju L, Chen Y, Xue L, Du X, Zhu C. Cooperative unfolding of distinctive mechanoreceptor domains transduces force into signals. *Elife.* (2016) 5:e15447. doi: 10.7554/eLife.15447
109. Nesbitt WS, Kulkarni S, Giuliano S, Goncalves I, Dopheide SM, Yap CL, et al. Distinct glycoprotein Ib/V/IX and integrin α IIb β 3 3-dependent calcium signals cooperatively regulate platelet adhesion under flow. *J Biol Chem.* (2002) 277:2965–72. doi: 10.1074/jbc.M110070200
110. Dai K, Bodnar R, Berndt MC, Du X. A critical role for 14-3-3zeta protein in regulating the VWF binding function of platelet glycoprotein Ib-IX and its therapeutic implications. *Blood.* (2005) 106:1975–81. doi: 10.1182/blood-2005-01-0440
111. Chen Y, Ju LA, Zhou F, Liao J, Xue L, Su QP, et al. An integrin α IIb β 3 intermediate affinity state mediates biomechanical platelet aggregation. *Nat Mater.* (2019) 18:760–9. doi: 10.1038/s41563-019-0323-6
112. Jackson SP. The growing complexity of platelet aggregation. *Blood.* (2007) 109:5087. doi: 10.1182/blood-2006-12-027698
113. Slager CJ, Wentzel JJ, Gijzen FJH, Thury A, Van Der Wal AC, Schaar JA, et al. The role of shear stress in the destabilization of vulnerable plaques and related therapeutic implications. *Nat Clin Pract Cardiovasc Med.* (2005) 2:456. doi: 10.1038/npcardio0298
114. Young DF, Tsai FY. Flow characteristics in models of arterial stenoses. II. Unsteady flow. *J Biomech.* (1973) 6:547–59. doi: 10.1016/0021-9290(73)90012-2
115. Chiu J-J, Chien S. Effects of disturbed flow on vascular endothelium: pathophysiological basis and clinical perspectives. *Physiol Rev.* (2011) 91:327–87. doi: 10.1152/physrev.00047.2009
116. Ruggeri ZM. Platelets in atherothrombosis. *Nat Med.* (2002) 8:1227–34. doi: 10.1038/nm1102-1227
117. Nesbitt WS, Westein E, Tovar-Lopez FJ, Tolouei E, Mitchell A, Fu J, et al. A shear gradient-dependent platelet aggregation mechanism drives thrombus formation. *Nat Med.* (2009) 15:665–73. doi: 10.1038/nm.1955
118. Sheriff J, Bluestein D, Girdhar G, Jesty J. High-shear stress sensitizes platelets to subsequent low-shear conditions. *Ann Biomed Eng.* (2010) 38:1442–50. doi: 10.1007/s10439-010-9936-2
119. Rahman SM, Eichinger CD, Hlady V. Effects of upstream shear forces on priming of platelets for downstream adhesion and activation. *Acta Biomater.* (2018) 73:228–35. doi: 10.1016/j.actbio.2018.04.002
120. Westein E, Van Der Meer AD, Kuijpers MJ, Frimat JP, Van Den Berg A, Heemskerk JW. Atherosclerotic geometries exacerbate pathological thrombus formation poststenosis in a von Willebrand factor-dependent manner. *Proc Natl Acad Sci USA.* (2013) 110:1357–62. doi: 10.1073/pnas.1209905110
121. Mustard JF, Murphy EA, Rowsell HC, Downie HG. Factors influencing thrombus formation *in vivo*. *Am J Med.* (1962) 33:621–47. doi: 10.1016/0002-9343(62)90243-7
122. Wentzel JJ, Chatzizisis YS, Gijzen FJ, Giannoglou GD, Feldman CL, Stone PH. Endothelial shear stress in the evolution of coronary atherosclerotic plaque and vascular remodelling: current understanding and remaining questions. *Cardiovasc Res.* (2012) 96:234–43. doi: 10.1093/cvr/cvs217
123. Ha H, Lee S-J. Hemodynamic features and platelet aggregation in a stenosed microchannel. *Microvasc Res.* (2013) 90:96–105. doi: 10.1016/j.mvr.2013.08.008
124. Rinder CS, Student LA, Bonan JL, Rinder HM, Smith BR. Aspirin does not inhibit adenosine diphosphate-induced platelet α -granule release. *Blood.* (1993) 82:505–12.
125. Barstad RM, Orvim U, Hamers MJ, Tjonnfjord GE, Brosstad FR, Sakariassen KS. Reduced effect of aspirin on thrombus formation at high shear and disturbed laminar blood flow. *Thromb Haemost.* (1996) 75:827–32. doi: 10.1055/s-0038-1650374
126. Maalej N, Folts John D. Increased shear stress overcomes the antithrombotic platelet inhibitory effect of aspirin in stenosed dog coronary arteries. *Circulation.* (1996) 93:1201–5. doi: 10.1161/01.CIR.93.6.1201
127. Weiss HJ, Turitto VT. Prostacyclin (prostaglandin I₂, PGI₂) inhibits platelet adhesion and thrombus formation on subendothelium. *Blood.* (1979) 53:244.
128. Borgdorff P, Tangelder GJ, Paulus WJ. Cyclooxygenase-2 inhibitors enhance shear stress-induced platelet aggregation. *J Am Coll Cardiol.* (2006) 48:817–23. doi: 10.1016/j.jacc.2006.03.053
129. Kageyama S, Yamamoto H, Nagano M, Arisaka H, Kayahara T, Yoshimoto R. Anti-thrombotic effects and bleeding risk of AJvW-2, a monoclonal antibody against human von Willebrand factor. *Br J Pharmacol.* (1997) 122:165–71. doi: 10.1038/sj.bjp.0701354
130. Eto K, Isshiki T, Yamamoto H, Takeshita S, Ochiai M, Yokoyama N, et al. AJvW-2, an anti-vWF monoclonal antibody, inhibits enhanced platelet aggregation induced by high shear stress in platelet-rich plasma from patients with acute coronary syndromes. *Arterioscler Thromb Vasc Biol.* (1999) 19:877–82. doi: 10.1161/01.ATV.19.4.877
131. Kageyama S, Yamamoto H, Yoshimoto R. Anti-human von Willebrand factor monoclonal antibody AJvW-2 prevents thrombus deposition and neointima formation after balloon injury in guinea pigs. *Arterioscler Thromb Vasc Biol.* (2000) 20:2303–8. doi: 10.1161/01.ATV.20.10.2303
132. Kageyama S, Yamamoto H, Nakazawa H, Matsushita J, Kouyama T, Gonsho A, et al. Pharmacokinetics and pharmacodynamics of AJW200, a humanized monoclonal antibody to von Willebrand factor, in monkeys. *Arterioscler Thromb Vasc Biol.* (2002) 22:187–92. doi: 10.1161/hq0102.101520
133. Kageyama S, Matsushita J, Yamamoto H. Effect of a humanized monoclonal antibody to von Willebrand factor in a canine model of coronary arterial thrombosis. *Eur J Pharmacol.* (2002) 443:143–9. doi: 10.1016/S0014-2999(02)01590-X
134. Machin S, Clarke C, Ikemura O, Kageyama S, Mackie JJ, Talbot JA, et al. A humanised monoclonal antibody against VWF A1 domain inhibits VWF: R1Cof activity and platelet adhesion in human volunteers. *J Thromb Haemost.* (2003).
135. Diener JL, Daniel Lagasse HA, Duerschmied D, Merhi Y, Tanguay JF, Hutabarat R, et al. Inhibition of von Willebrand factor-mediated platelet activation and thrombosis by the anti-von Willebrand factor A1-domain aptamer ARC1779. *J Thromb Haemost.* (2009) 7:1155–62. doi: 10.1111/j.1538-7836.2009.03459.x

136. (2019). *FDA Approved Caplacizumab-yhdp*. Available online at: <https://www.fda.gov> (accessed April 1, 2019).
137. Jilma-Stohlawetz P, Gilbert JC, Gorczyca ME, Knöbl P, Jilma B. A dose ranging phase I/II trial of the von Willebrand factor inhibiting aptamer ARC1779 in patients with congenital thrombotic thrombocytopenic purpura. *Thromb Haemost.* (2011) 106:391–7. doi: 10.1160/TH11-02-0069
138. Markus HS, McCollum C, Imray C, Goulder MA, Gilbert J, King A. The von Willebrand inhibitor ARC1779 reduces cerebral embolization after carotid endarterectomy: a randomized trial. *Stroke.* (2011) 42:2149–53. doi: 10.1161/STROKEAHA.111.616649
139. Siller-Matula JM, Merhi Y, Tanguay JF, Duerschmied D, Wagner DD, McGinness KE, et al. ARC15105 is a potent antagonist of von Willebrand factor mediated platelet activation and adhesion. *Arterioscler Thromb Vasc Biol.* (2012) 32:902–9. doi: 10.1161/ATVBAHA.111.237529
140. Bartunek JB, Vercruyse K, Duby C, Wijns W, Heyndrickx G, Holz J-B. Abstract 15084: safety and efficacy of anti-von Willebrand factor nanobody® ALX-0081 in stable angina patients undergoing percutaneous coronary intervention. *Circulation.* (2010) 122:A15084.
141. Loon JEV, Jaegere PPTD, Vliet HHDMV, Maat MPMD, De Groot PG, Simoons ML, et al. The *in vitro* effect of the new antithrombotic drug candidate ALX-0081 on blood samples of patients undergoing percutaneous coronary intervention. *Thromb Haemost.* (2011) 106:165–71. doi: 10.1160/TH10-12-0804
142. Callewaert F, Roodt J, Ulrichs H, Stohr T, Van Rensburg WJ, Lamprecht S, et al. Evaluation of efficacy and safety of the anti-VWF Nanobody ALX-0681 in a preclinical baboon model of acquired thrombotic thrombocytopenic purpura. *Blood.* (2012) 120:3603–10. doi: 10.1182/blood-2012-04-420943
143. Peyvandi F, Scully M, Kremer Hovinga JA, Cataland S, Knobl P, Wu H, et al. Caplacizumab for acquired thrombotic thrombocytopenic purpura. *N Engl J Med.* (2016) 374:511–22. doi: 10.1056/NEJMoa1505533
144. Scully M, Cataland SR, Peyvandi F, Coppo P, Knöbl P, Kremer Hovinga JA, et al. Results of the randomized, double-blind, placebo-controlled, phase 3 hercules study of caplacizumab in patients with acquired thrombotic thrombocytopenic purpura. *Blood.* (2017) 130:LBA-1.
145. Gorog DA, Douglas H, Ahmed N, Lefroy DC, Davies GJ. Coronary angioplasty enhances platelet reactivity through von Willebrand factor release. *Heart.* (2003) 89:329–30. doi: 10.1136/heart.89.3.329
146. Kremer Hovinga JA, Meyer SC. Current management of thrombotic thrombocytopenic purpura. *Curr Opin Hematol.* (2008) 15:445–50. doi: 10.1097/MOH.0b013e328309ec62
147. Korin N, Kanapathipillai M, Matthews BD, Crescente M, Brill A, Mammoto T, et al. Shear-activated nanotherapeutics for drug targeting to obstructed blood vessels. *Science.* (2012) 337:738–42. doi: 10.1126/science.1217815
148. Marosfoi MG, Korin N, Gounis MJ, Uzun O, Vedantham S, Langan ET, et al. Shear-activated nanoparticle aggregates combined with temporary endovascular bypass to treat large vessel occlusion. *Stroke.* (2015) 46:3507–13. doi: 10.1161/STROKEAHA.115.011063
149. Holme MN, Fedotenko IA, Abegg D, Althaus J, Babel L, Favarger F, et al. Shear-stress sensitive lenticular vesicles for targeted drug delivery. *Nat Nanotechnol.* (2012) 7:536–43. doi: 10.1038/nnano.2012.84
150. Molloy CP, Yao Y, Kammoun H, Bonnard T, Hoefer T, Alt K, et al. Shear-sensitive nanocapsule drug release for site-specific inhibition of occlusive thrombus formation. *J Thromb Haemost.* (2017) 15:972–82. doi: 10.1111/jth.13666
151. Westein E, Flierl U, Hagemeyer C, Peter K. Destination known: targeted drug delivery in atherosclerosis and thrombosis. *Drug Dev Res.* (2013) 74. doi: 10.1002/ddr.21103
152. Korin N, Gounis MJ, Wakhloo AK, Ingber DE. Targeted drug delivery to flow-obstructed blood vessels using mechanically activated nanotherapeutics for flow-obstructed blood vessels. *JAMA Neurol.* (2015) 72:119–22. doi: 10.1001/jamaneurol.2014.2886
153. Xia Y, Shi CY, Xiong W, Hou XL, Fang JG, Wang WQ. Shear stress-sensitive carriers for localized drug delivery. *Curr Pharm Des.* (2016) 22:5855–67. doi: 10.2174/1381612822666160628081419
154. Epshtein M, Korin N. Shear targeted drug delivery to stenotic blood vessels. *J Biomech.* (2017) 50:217–21. doi: 10.1016/j.jbiomech.2016.11.015
155. Metselaar JM, Storm G. Liposomes in the treatment of inflammatory disorders. *Expert Opin Drug Deliv.* (2005) 2:465–76. doi: 10.1517/17425247.2.3.465
156. Ding BS, Dziubla T, Shuvaev VV, Muro S, Muzykantov VR. Advanced drug delivery systems that target the vascular endothelium. *Mol Interv.* (2006) 6:98–112. doi: 10.1124/mi.6.2.7
157. Hua S, Wu SY. The use of lipid-based nanocarriers for targeted pain therapies. *Front Pharmacol.* (2013) 4:143. doi: 10.3389/fphar.2013.00143
158. Monteiro N, Martins A, Reis RL, Neves NM. Liposomes in tissue engineering and regenerative medicine. *J R Soc Interface.* (2014) 11:20140459. doi: 10.1098/rsif.2014.0459
159. Sackmann E. Membrane bending energy concept of vesicle- and cell-shapes and shape-transitions. *FEBS Lett.* (1994) 346:3–16. doi: 10.1016/0014-5793(94)00484-6
160. Bernard A-L, Guedeau-Boudeville M-A, Marchi-Artzner V, Gulik-Krzywicki T, Di Meglio J-M, Jullien L. Shear-induced permeation and fusion of lipid vesicles. *J Colloid Interface Sci.* (2005) 287:298–306. doi: 10.1016/j.jcis.2004.12.019
161. Guo X, Wu Z, Guo Z. New method for site-specific modification of liposomes with proteins using sortase A-mediated transpeptidation. *Bioconjug Chem.* (2012) 23:650–5. doi: 10.1021/bc200694t
162. Hagemeyer CE, Alt K, Johnston APR, Such GK, Ta HT, Leung MKM, et al. Particle generation, functionalization and sortase A-mediated modification with targeting of single-chain antibodies for diagnostic and therapeutic use. *Nat Protoc.* (2014) 10:90. doi: 10.1038/nprot.2014.177
163. Wöll S, Bachran C, Schiller S, Schröder M, Conrad L, Swee LK, et al. Sortagable liposomes: evaluation of reaction conditions for single-domain antibody conjugation by Sortase-A and targeting of CD11b+ myeloid cells. *Eur J Pharm Biopharm.* (2018) 133:138–50. doi: 10.1016/j.ejpb.2018.09.017
164. Bajaj SP, Joist JH. New insights into how blood clots: implications for the use of APTT and PT as coagulation screening tests and in monitoring of anticoagulant therapy. *Semin Thromb Hemost.* (1999) 25:407–18. doi: 10.1055/s-2007-994943
165. Chee YL, Greaves M. Role of coagulation testing in predicting bleeding risk. *Hematol J.* (2003) 4:373–8. doi: 10.1038/sj.thj.6200306
166. Ganter MT, Hofer CK. Coagulation monitoring: current techniques and clinical use of viscoelastic point-of-care coagulation devices. *Anesth Analg.* (2008) 106:1366–75. doi: 10.1213/ane.0b013e318168b367
167. Para AN, Ku DN. A low-volume, single pass *in-vitro* system of high shear thrombosis in a stenosis. *Thromb Res.* (2013) 131:418–24. doi: 10.1016/j.thromres.2013.02.018
168. Jain A, Graveline A, Waterhouse A, Vernet A, Flaumenhaft R, Ingber DE. A shear gradient-activated microfluidic device for automated monitoring of whole blood haemostasis and platelet function. *Nat Commun.* (2016) 7:10176. doi: 10.1038/ncomms10176
169. Jin-Wook Y, Elizabeth C, Samir M. Factors that control the circulation time of nanoparticles in blood: challenges, solutions and future prospects. *Curr Pharm Des.* (2010) 16:2298–307. doi: 10.2174/138161210791920496
170. Patra JK, Das G, Fraceto LF, Campos EVR, Rodriguez-Torres MDP, Acosta-Torres LS, et al. Nano based drug delivery systems: recent developments and future prospects. *J Nanobiotechnology.* (2018) 16:71. doi: 10.1186/s12951-018-0392-8
171. Paliwal R, Babu RJ, Palakurthi S. Nanomedicine scale-up technologies: feasibilities and challenges. *AAPS PharmSciTech.* (2014) 15:1527–34. doi: 10.1208/s12249-014-0177-9
172. Wilczewska AZ, Niemirowicz K, Markiewicz KH, Car H. Nanoparticles as drug delivery systems. *Pharmacol Rep.* (2012) 64:1020–37. doi: 10.1016/S1734-1140(12)70901-5
173. Bhatt DL, Topol EJ. Scientific and therapeutic advances in antiplatelet therapy. *Nat Rev Drug Discov.* (2003) 2:15–28. doi: 10.1038/nrd985
174. Antithrombotic Trialists' Collaboration. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. *BMJ.* (2002) 324:71–86. doi: 10.1136/bmj.324.7329.71
175. Sands AT. The master mammal. *Nat Biotechnol.* (2003) 21:31–2. doi: 10.1038/nbt0103-31
176. Diaz JA, Obi AT, Myers DD Jr, Wroblewski SK, Henke PK, Mackman N, et al. Critical review of mouse models of venous thrombosis. *Arterioscler*

- Thromb Vasc Biol.* (2012) 32:556–62. doi: 10.1161/ATVBAHA.111.244608
177. Vogel V. Mechanotransduction involving multimodular proteins: converting force into biochemical signals. *Annu Rev Biophys Biomol Struct.* (2006) 35:459–88. doi: 10.1146/annurev.biophys.35.040405.102013
 178. Chen Y, Ju L, Rushdi M, Ge C, Zhu C. Receptor-mediated cell mechanosensing. *Mol Biol Cell.* (2017) 28:3134–55. doi: 10.1091/mbc.e17-04-0228
 179. Perkins WR, Vaughan DE, Plavin SR, Daley WL, Rauch J, Lee L, et al. Streptokinase entrapment in interdigitation-fusion liposomes improves thrombolysis in an experimental rabbit model. *Thromb Haemost.* (1997) 77:1174–8. doi: 10.1055/s-0038-1656133
 180. Leach JK, O'rear EA, Patterson E, Miao Y, Johnson AE. Accelerated thrombolysis in a rabbit model of carotid artery thrombosis with liposome-encapsulated and microencapsulated streptokinase. *Thromb Haemost.* (2003) 90:64–70. doi: 10.1055/s-0037-1613600
 181. Andresen TL, Jensen SS, Jorgensen K. Advanced strategies in liposomal cancer therapy: problems and prospects of active and tumor specific drug release. *Prog Lipid Res.* (2005) 44:68–97. doi: 10.1016/j.plipres.2004.12.001
 182. Arayne MS, Sultana N, Qureshi F. Review: nanoparticles in delivery of cardiovascular drugs. *Pak J Pharm Sci.* (2007) 20:340–8.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Rana, Westein, Niego and Hagemeyer. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Role of Platelet Mitochondria: Life in a Nucleus-Free Zone

Hannah Melchinger, Kanika Jain, Tarun Tyagi and John Hwa*

Department of Internal Medicine, Yale Cardiovascular Research Center, Yale School of Medicine, New Haven, CT, United States

OPEN ACCESS

Edited by:

Paul Jurasz,
University of Alberta, Canada

Reviewed by:

Eric Boillard,
Laval University, Canada
Cécile Oury,
University of Liège, Belgium

*Correspondence:

John Hwa
john.hwa@yale.edu

Specialty section:

This article was submitted to
Atherosclerosis and Vascular
Medicine,
a section of the journal
Frontiers in Cardiovascular Medicine

Received: 03 September 2019

Accepted: 08 October 2019

Published: 29 October 2019

Citation:

Melchinger H, Jain K, Tyagi T and
Hwa J (2019) Role of Platelet
Mitochondria: Life in a Nucleus-Free
Zone. *Front. Cardiovasc. Med.* 6:153.
doi: 10.3389/fcvm.2019.00153

Platelets are abundant, small, anucleate circulating cells, serving many emerging pathophysiological roles beyond hemostasis; including active critical roles in thrombosis, injury response, and immunoregulation. In the absence of genomic DNA transcriptional regulation (no nucleus), platelets require strategic prepackaging of all the needed RNA and organelles from megakaryocytes, to sense stress (e.g., hyperglycemia), to protect themselves from stress (e.g., mitophagy), and to communicate a stress response to other cells (e.g., granule and microparticle release). Distinct from avian thrombocytes that have a nucleus, the absence of a nucleus allows the mammalian platelet to maintain its small size, permits morphological flexibility, and may improve speed and efficiency of protein expression in response to stress. In the absence of a nucleus, platelet lifespan of 7–10 days, is largely determined by the mitochondria. The packaging of 5–8 mitochondria is critical in aerobic respiration and yielding metabolic substrates needed for function and survival. Mitochondria damage or dysfunction, as observed with several disease processes, results in greatly attenuated platelet survival and increased risk for thrombovascular events. Here we provide insights into the emerging roles of platelets despite the lack of a nucleus, and the key role played by mitochondria in platelet function and survival both in health and disease.

Keywords: platelets, mitochondria, anucleate cells, metabolism, apoptosis

PLATELET DISCOVERY AND ORIGINS

Platelets are small (2–4 μm), short-lived (7–10 days), anucleate circulating cells primarily responsible for the prevention of bleeding and the maintenance of hemostasis (1, 2). A healthy adult has a counts in the range of 150,000–450,000 platelets per microliter of blood, though these counts vary with age and health (3). Platelets were first identified by Schultze in 1865 (4), but its functions in hemostasis and thrombosis weren't elucidated until 1881 by Bizzozero (5, 6). In 1906, Wright established megakaryocytes (MKs) in the bone marrow to be the source of platelets (7), though recent studies have shown that mature megakaryocytes in the lungs can also release platelets into the pulmonary vasculature (8). MKs produce billions of platelets daily through fragmentation, in which small cytoplasmic pieces bud off the megakaryocyte to become platelets (9). During fragmentation, MKs undergo a series of elongations to form proplatelet shafts, or cytoplasmic extensions which serve as assembly lines for platelet formation (10). Platelet-sized swellings then form along the shaft (11); as these platelets develop, they are loaded with the necessary organelles and granules from the MK parent (9). Platelets are equipped with mitochondria, a cytoskeleton,

and a dense tubular system (DTS) (3, 12). Additionally, platelets contain secretory organelles categorized as alpha, dense, and lysosomal granules, which are transported and discharged by a surface-connected open canalicular system (OCS) (13). Dense granules generally contain small molecules such as ADP or serotonin, whereas alpha granules contain hemostatic factors such as fibrinogen, as well as other growth factors and cytokines (14). Upon complete generation, platelets are released from the bone marrow into circulation, where they live for the next 7–10 days (15). Historically disregarded as “cellular dust” (16), platelets have only recently emerged as having more diverse homeostatic processes including wound healing, angiogenesis, immunoregulation, and inflammatory response all key components to a stress response (2, 17–19).

PLATELET FUNCTION BEYOND HEMOSTASIS (FIGURE 1)

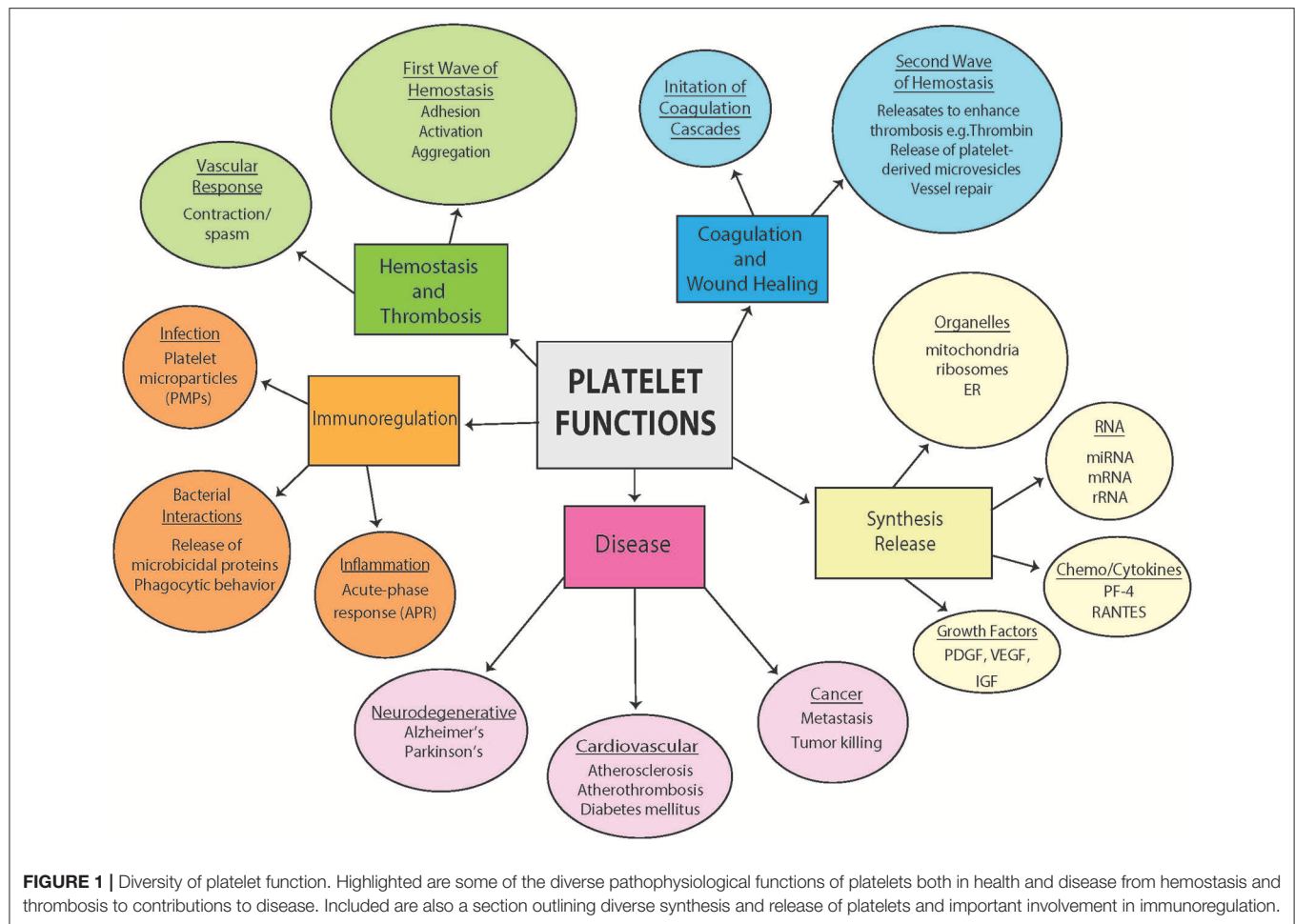
Platelets are primarily responsible for the maintenance of normal hemostasis by the prevention of hemorrhage during vascular injury (20). Hemostasis is achieved by a careful balance of platelet interactions with vascular components, cytokine mediators, fibrinolytic agents, and plasma coagulation mechanisms (21). They assist in initiating a vascular response leading to vasoconstriction, and formation of a hemostatic plug (through adhesion, activation, and aggregation). The blood coagulation cascade is then initiated with expansion of the thrombus and massive release of platelet contents (22). The released factors then also assist in promoting tissue repair and resolving the repair process (23). The role of platelets in thrombosis is essential, and increasingly becoming well-understood. Given the complex content within platelets, researchers have recently begun to investigate platelet function beyond coagulation, and have implicated platelets in several processes including immunoregulation, infection, inflammation, and the pathogenesis of a growing list of diseases (neurodegenerative diseases, cardiovascular disease and cancer) (24–26). In the absence of a nucleus, the role of the platelet mitochondria in these processes has become a focus of intense studies, including how platelet dysfunction is associated with, contribute to, is affected by the disease pathologies (25).

PLATELETS: A NUCLEUS-FREE ZONE

Notably, mammalian platelets do not contain a nucleus (27). Interestingly, non-mammalian vertebrates have nucleated thrombocytes that have limited responses, to thrombin but not to ADP, serotonin or epinephrine (28, 29). As described (9), upon fragmentation, mature mammalian MKs segregate into anucleate platelets; thus, platelets are not endowed with the genomic genetic material generally considered a requirement for complex cellular function (16). Nuclear material (genomic DNA) generally provides functional autonomy; any needed protein can be transcribed from the genomic road map provided

in the nucleus (19). However, the presence of a nucleus in platelets may hinder many of the important roles played by the platelet, at the expense of functional autonomy. To fulfill the many emerging functions, platelets need to be small (able to circulate in small vessels), flexible, highly efficient (produces proteins rapidly and efficiently) and highly sensitive (responds to stressors rapidly).

A human cell nucleus on average is much larger than a platelet (nucleus $\sim 6\mu\text{m}$ in diameter) (30), dictated by DNA content (3 billion base pairs in humans), and cytoplasmic factors (31). The presence of a nucleus even if small and compact, would greatly enhance a platelet's size thus reducing its ability to travel through small vessels and spaces. In addition to small size, platelets need to be flexible, capable of modulating their internal space to undergo extreme morphological changes (19), again allowing platelets to travel through even the smallest vessels in the circulatory system and enter tissues when needed. Further, this enhanced flexibility allows the platelet OCS to fill internal vacancies during activation to increase surface area available for interaction with blood plasma (19, 32). The presence of a nucleus in avian thrombocytes (nucleated platelets) makes them larger than mammalian platelets, causes them to spread less efficiently on collagen, and express much lower levels of the $\alpha_{2b}\beta_3$ integrin required for aggregate formation (28). Similarly, the lack of a nucleus within the red blood cells (RBC), allows them to maintain their distinct bi-concave shape; but additionally, removes the need to maintain nuclear function and genome, allowing the RBC to focus on producing and maintaining hemoglobin. Platelets, like RBCs, do not need to regulate the health of a large complex nucleus, with its transcriptional machinery and chromosomes. Platelets can thus focus on what is needed for their roles in hemostasis and homeostasis with prepackaged, carefully selected RNA and translational machinery (without requiring transcriptional regulation of complex genomic DNA) (33, 34). Each platelet is equipped with an abundance of needed genetic information, and processing machinery required for a highly efficient rapid response (35), within minutes (required for hemostasis), rather than hours or days. Thus, based upon the literature and the lower efficiency of nucleated avian thrombocytes, we believe that the lack of a nucleus allows for improved platelet functional efficiency. More studies are required to support this notion. Interestingly, in the absence of a nuclear source of RNA, platelets are capable of taking up RNA material from external sources through microvesicles (MVs) (36). RNAs can also be donated from platelets to other cells through microvesicle mediated intercellular crosstalk (37–39). The microRNAs within these MVs have proven to be increasingly relevant to understanding the role of platelets in thrombosis, immune response, and various diseases. Not only miRNA but transcription factors and mitochondria are conveyed to such cells as neutrophils, mediated by 12-lipoxygenase and phospholipase A2-IIA (40). Thus, platelets have proven to be “intelligent” even in the absence of a nucleus: a platelet's ability to interact with their environment and efficiently respond to the needs of that environment, has marked them as far more complex than previously thought (41).

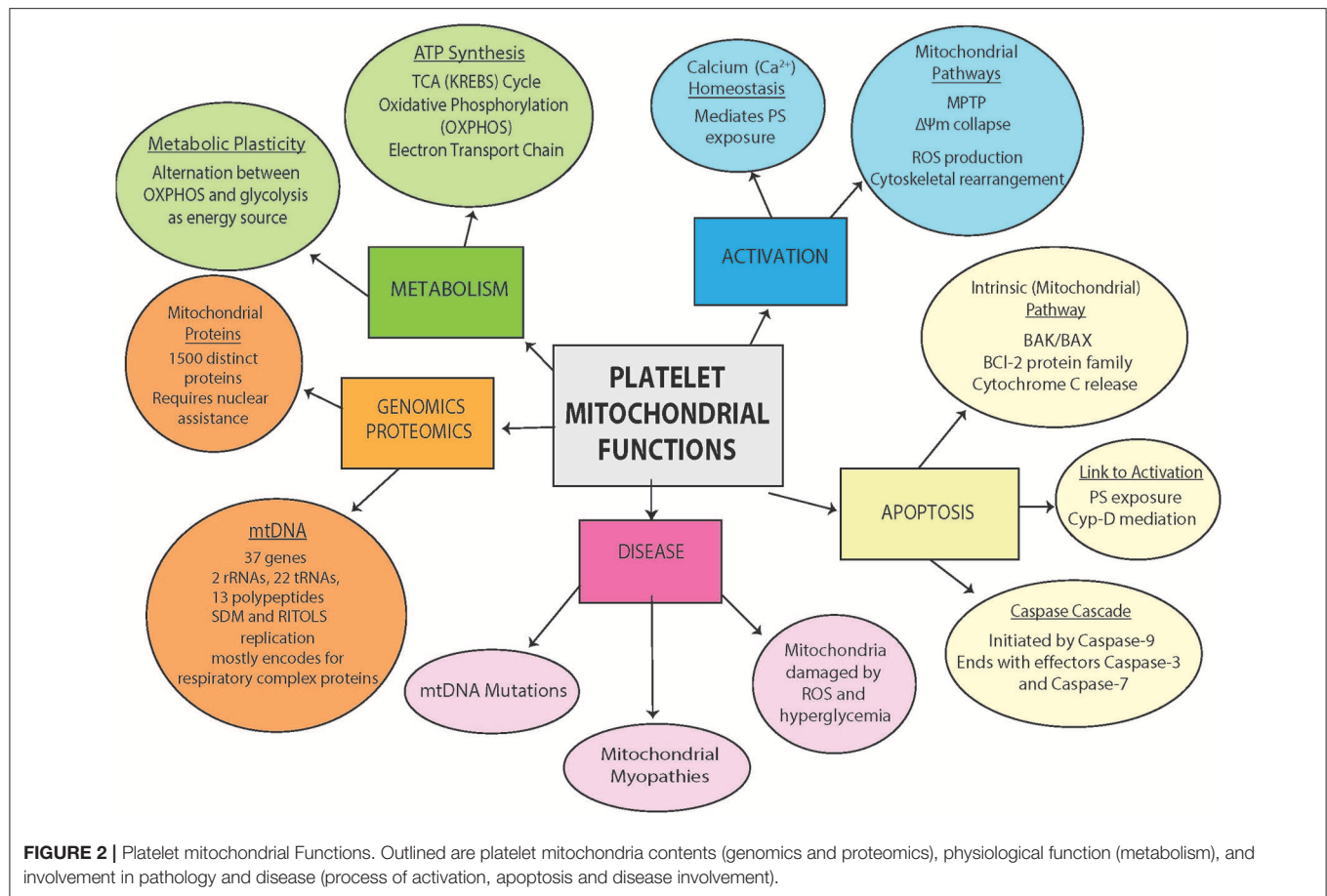


PLATELET MITOCHONDRIA ARE KEY TO FUNCTION AND SURVIVAL OF PLATELETS (FIGURE 2)

To maintain the ability to rapidly respond to stressors or blood vessel damage (thrombosis), a highly efficient source of energy and metabolites are needed to orchestrate the response. Distinct from RBCs that are also devoid of mitochondria, platelets are equipped with mitochondria (42). Interestingly, mitochondria have a number of features in common with a nucleus, both contain DNA, both are surrounded by a double plasma membrane, and both can divide during the cell cycle (30). However, the role of the mitochondria, referred as the “powerhouse of the cell,” is quite different, playing essential roles in energy production and metabolism (43). The mitochondria is home to key energetic processes such as the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS), both of which are involved in the production of adenosine triphosphate (ATP) (44). However, studies have implicated mitochondria in many processes beyond energy production, such as the generation of reactive oxygen species (ROS) (45), Ca^{2+} homeostasis (46), apoptosis regulation (47), and ER-stress response mechanisms (48). Mitochondrial health

and dysfunction also appear to be involved in aging (49), as well as neurodegenerative diseases (e.g., Alzheimer’s (50) and Parkinson’s disease (51)). Healthy platelets contain between 5 and 8 mitochondria, the majority of which must remain uncompromised for the platelet to maintain proper function. In healthy platelets, mitochondria has been demonstrated to serve a variety of purposes as described for nucleated cells, from metabolism, activation, ATP production to the regulation of cell processes and viability (42, 52).

In the absence of nuclear control, platelet health is largely determined by the health of their mitochondria (53). As is apparent in a number of diseases, an excess of damaged platelets can lead to premature apoptosis; therefore, it is essential to keep platelet mitochondria in good health (25). The turnover rate for mitochondria in various nucleated cells ranges from 9 to 24 days (54, 55). Considering, the lifespan of a platelet (7–10 days), the necessity of mitochondria for energy production, and the inability to consistently replenish nuclear encoded mitochondria proteins, the lifespan of the platelet mitochondria likely determines the platelet lifespan. A mitochondrial protein Bcl-xl, master regulator of mitochondrial apoptosis has been reported to determine platelet lifespan (56). Moreover, a recent study implicated TNF-alpha-driven megakaryocyte reprogramming



leading to increased mitochondrial mass and activity as being a major contributor to the observed hyperactivity and thrombosis associated with aging (57). Multiple factors affect mitochondrial health and some of the key components determining mitochondria function and lifespan, especially in context of the anucleate platelets, will now be discussed.

Mitochondria DNA (mtDNA)

A complete mitochondrial genome is needed for both proper mitochondrial as well as platelet function, and the prevention of a number of mitochondrial related diseases such as mitochondrial myopathies caused by mtDNA mutations (58). Mitochondria, along with chloroplasts in plants, are the only organelles besides the nucleus to contain genetic material (59). Human mitochondrial DNA (mtDNA) is double-stranded, circular, and relatively small: at 16.6 kbp, human mtDNA is comprised of 37 genes encoding two rRNAs, 22 tRNAs, and 13 polypeptides, all of which are components of the OXPHOS enzyme complexes (60). Similar to bacterial chromosomal DNA, mtDNA is organized, in multiple copies, in nucleoids within the mitochondria (61). Because they likely originated from prokaryotic ancestors, mitochondria are largely self-sufficient: they are able to maintain, transcribe and translate mtDNA internally and independently (62). Mitochondria have several modes of mtDNA replication which differ significantly from the nuclear mode of replication

including the strand-displacement mode (SDM) (63, 64), ribonucleotide incorporated throughout the lagging strand replication (RITOLS) (65) and coupled leading-lagging strand synthesis (66). mtDNA copy number is considered an indirect measure of mitochondrial function (67) and its quantification in peripheral blood, majorly reflects the mtDNA copy number in leukocytes and platelets (68). The epigenetic regulation of platelet mtDNA is of particular importance with higher methylation of platelet mtDNA being a possible biomarker for cardiovascular disease (CVD) (69, 70).

Mitochondrial Functions

In addition to mtDNA, mitochondria are equipped with a complex array of proteins offering significant insights into mitochondrial activity (71). Mitochondria contain around 1,500 distinct proteins in mammals, compared to around 1,000 in yeast (72). Since the majority of the proteins encoded by mtDNA form components of the respiratory chain complexes, most mitochondrial proteins are encoded by the nucleus and imported into the mitochondria from the cytosol with the help of mitochondrial translocases (73). Each protein is coupled with a distinct import signal which guides it to the appropriate mitochondrial membrane, to which it is then inserted: outer membrane proteins are integrated by the TOM complex, whereas TIM23 is the presequence translocase responsible for

inner membrane protein integration (74). The mechanism by which proteins are incorporated into the mitochondria is posttranslational: unlike ER-protein import, mitochondrial proteins are synthesized in the cytosol as precursor proteins before being translocated into a mitochondrion (75).

Mitochondria and Energy Metabolism in the Platelets

While most human nucleated cells contain hundreds, if not thousands, of active mitochondria, platelets generally contain only 5–8 mitochondria per cell (42). However, metabolically, platelets are quite active: for example, compared to resting mammalian muscle cells, platelets have much higher levels of ATP-turnover (69). This energy demand is met using a metabolic system which combines the efforts of glycolysis and mitochondrial OXPHOS. In platelets, glycolysis provides about 60% of cellular ATP, while OXPHOS provides the remaining 30–40% (25). Out of the platelet's total mitochondrial function, 50% is dedicated to ATP production; the reserve energy is responsible for, among other activities, cellular response to oxidative stress (76). ATP is essential to proper platelet function: several key processes that occur within the platelet, such as the maintenance of calcium homeostasis, require a constant energy supply. Interestingly, platelets have been shown to have a metabolic flexibility that helps them meet this energy demand; activated platelets exhibit a glycolytic phenotype even as they preserve mitochondria function (77). This ability to utilize glycolysis or fatty acid catabolism instead of OXPHOS (mitochondrial ATP production) allows the platelet to adapt to different situations, such as hypoxia or the presence of mitochondrial inhibitory agents (78). Several studies have shown that platelet aggregation along with other metabolic activities are only fully interrupted when mitochondrial OXPHOS and glycolysis are inhibited simultaneously (79). Indeed, double knockout of GLUT1 and GLUT3 (major transporters of platelet glucose) leads to mitochondria reprogramming, reduced thrombosis and reduced platelet activation along with thrombocytopenia (80). This suggests that this metabolic plasticity is the key to enabling platelets to meet their extraordinary energy demand with so few mitochondria.

Mitochondria and Platelet Activation

Platelets are activated during the adhesion events of primary homeostasis, and the initiation of the blood coagulation cascades (81). Until recently, it was assumed that the only role mitochondria played in this process was an energetic one (78). However, new studies have demonstrated the contributions of several mitochondrial functions in platelet activation such as the mitochondrial permeability transition (MPT) (82, 83), increased ROS generation (84–86), and collapse of mitochondrial membrane potential ($\Delta\Psi_m$). Platelet activation is mediated by several agonists: collagen, thrombin, and ADP have all been implicated in the regulation of hemostasis (87). The activity of these agonists is mediated by a common increase in intracellular calcium (88). Mitochondria do little to regulate platelet calcium levels (89), but a simultaneous increase in intramitochondrial calcium levels does mediate phosphatidylserine (PS) exposure, without affecting integrin activation and granule release (86).

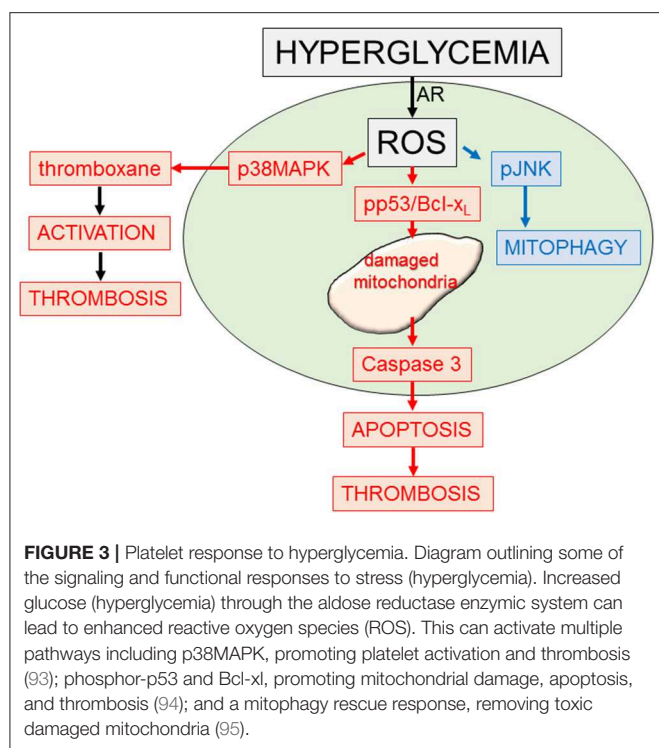
Increased mitochondrial calcium levels also correlate with mitochondrial ROS imbalance and MPT pore activation (90). Strong platelet activation characterized by the drastic increase in mitochondrial and cytosolic calcium also seems to initiate the collapse of the mitochondrial membrane potential ($\Delta\Psi_m$) via a cyclophilin D (CypD)-dependent mechanism (56). Thus, this collapse, mediated by mitochondrial calcium, contributes to further ROS generation and the initiation of the PS exposure essential for platelet adhesion (85). Interestingly, these mitochondrial activation pathways also contribute to the platelet apoptosis framework (84, 91, 92).

Mitochondria and Apoptosis (Figure 3)

Long associated only with nucleated cells, apoptosis is a mechanism of systematic cell deletion which can be induced or inhibited by both normal and abnormal stimuli (96). However, recent studies have identified apoptosis in the anucleate platelet (96, 97). Morphologically, platelet apoptosis is characterized by blebbing, platelet shrinkage, PS exposure, fragmentation into microparticles, and filopod formation (96). Apoptosis follows either an extrinsic or intrinsic pathway, the former stimulated by the activation of cell-surface death receptors, and the latter mediated by mitochondrial coordination of pro- and anti-apoptotic members of the Bcl-2 family (98). The presence of Bcl-2 family proteins within platelets, along with the platelet PS exposure characteristic of apoptosis, suggests that platelets might primarily follow an intrinsic apoptotic pathway (99, 100). Bcl-x_L, the key regulator of platelet survival, is responsible for inhibiting BAK and BAX, two pro-apoptotic proteins which serve to damage the mitochondria: studies in which either Bcl-x_L or BAK/BAX were impaired saw interference with natural platelet lifespan (98, 101, 102). Overexpression of Bcl-2 family pro-survival proteins can increase survival of platelets in the circulation (103). However, deletion of Bcl-2 in mice did not affect thrombopoiesis or platelet life span (104), supporting compensatory responses. When platelets come under stress or reach their natural end, the survival signal is overwhelmed and causes the activation of BAK and BAX, initiating the subsequent release of mitochondrial components such as cytochrome *c* through pores in the mitochondrial membrane (105). The presence of cytochrome *c* in the cytosol triggers the apoptotic caspase cascade, which begins with the initiator caspase-9 and ends with the effectors caspase-3 and caspase-7, which cleave hundreds of intracellular components and effectively destroy several essential cellular processes (106). Interestingly BCL2 family proteins are also involved in platelet formation with the anti-apoptotic family member BCL2L2 being involved in increasing megakaryocyte proplatelet formation in cultures of human cord blood (107).

Mitophagy in Platelets/Mitochondrial Turnover in Platelets (Figure 3)

Highlighting the importance of protecting platelet mitochondria in maintaining platelet health and lifespan, the protective process of induced mitophagy was recently described in platelets (95). This is distinct from basal platelet autophagy needed for an important role in platelet activation (108–110). Mitophagy can



be generated by Parkin-independent or -dependent pathways (111–115). The process in platelets is Parkin-dependent and protects the platelet from oxidative stress and mitochondrial mediated damage (95). In the absence of a nucleus the platelet requires prepacking of the highly ordered and complex process, from initial phagophore formation (nucleation) to subsequent expansion of the membrane by ubiquitin-like conjugating systems, microtubule-associated protein 1 light chain 3 (LC3), and the autophagy protein system (ATGs), ultimately the phagophore completely surrounding its target, followed by fusion with a lysosome, leading to content degradation by lysosomal enzymes (116–118). The energy required to sort and prepackage the mRNA components for this process in anticipation of mitochondria stress/damage suggests an essential role for platelet mitochondria in health and disease. Platelet mitophagy removes toxic damaged mitochondria (as in diabetes mellitus) preventing platelet apoptosis (95, 119). If platelet mitophagy is impaired, increased platelet apoptosis can contribute to enhanced thrombosis (95).

PLATELET MITOCHONDRIA IN DISEASE

Because mitochondria play such an integral role in platelet metabolism, activation, and apoptosis, it is no surprise that mitochondrial dysfunction contribute to dysfunctional platelet activity and apoptosis in several diseases, most notably Alzheimer's and Parkinson's (120), cardiovascular disease (CVD) (121), diabetes mellitus (122), and sepsis (123). Apoptotic platelets induce clotting 50–100 times faster than normal platelets, because phosphatidylserine on the platelet surface acts

as a catalytic site for clotting enzyme assembly and thrombin generation (124, 125). Recent studies have therefore provided invaluable insight into the complex mitochondrial mechanisms that determine platelet function in relation to tissue homeostasis (126). Due to the decreased fidelity of PolyG during replication, the mutation rate of mtDNA is about 100-fold higher than that of nuclear DNA (127). Additionally, the process of mitochondrial DNA segregation occurs randomly and with far less organization than in the nucleus, sometimes leaving daughter cells with similar, but not identical, copies of mtDNA (128). Combined, there is increased risk for mtDNA abnormalities and mutations that can have severe health consequences, hindering ATP generation and increasing oxidative stress (129). Mutations which impact mitochondrial functionality are also relevant in aging (130) and age-related diseases, such as diabetes mellitus (58), Parkinson's (51), and cardiovascular disease (69). Platelet mitochondrial DNA in the circulation may serve as biomarkers for disease (131–133).

Cardiovascular diseases (CVD) including atherosclerosis and thrombosis are the leading causes of death for patients with diabetes (134). Diabetes mellitus, characterized by acute and chronic hyperglycemia have been shown to increase mitochondrial ROS production in platelets leading to activation (85, 93, 135). Platelets have also been identified as leading players in the development of atherosclerotic lesions, contributing, along with monocytes, to the inflammatory environment of atherosclerosis (136, 137). Type 2 diabetes results in alterations of platelet ATP production with an initial increase in platelet mitochondrial ATP content and platelet activity (93, 138), followed by platelet apoptosis and a decrease in ATP production, in the presence of severe persistent oxidative stress (94, 138, 139). Antiplatelet therapies, as well as hyperglycemic control treatments, have become increasingly relevant for the regulation of the high levels of platelet reactivity observed in Type 2 diabetes (140, 141). Preserving platelet mitochondrial function may be an additional means of decreasing the risk of potentially fatal thrombotic events for diabetic patients (142). Platelets may also serve essential functions in immunoregulation (143–146). Alterations in the bioenergetics of platelet mitochondria have been observed in cases of sepsis (147), contributing to drastic, but impermanent, organ failure (148). Thrombocytopenia is associated with increased mortality in septic shock (149, 150). Recently, platelet mitochondria have also been reported to incite an inflammatory response upon activation through release of bioenergetically active mitochondria in free as well as encapsulated form (151). Interestingly, the alphaproteobacterium *Rickettsia prowazekii* may be an evolutionary ancestor of the mitochondria (152).

Both Parkinson's and Alzheimer's are severe neurodegenerative diseases that have been linked to platelets, mitochondrial dysfunction and platelet apoptosis (153, 154). Platelets, are considered to be structurally and functionally similar to neurons and have been shown to rich in key proteins associated with the neurons and brain (155–157). Indeed, after the brain, platelets contain the highest amounts of Amyloid precursor protein, synuclein and tau, and are major contributors to the circulating levels of these key proteins involved in

neurodegenerative diseases (158). As in the other diseases profiled, Alzheimer's presents a compromised mitochondrial ETC; in particular, the activity of cytochrome c oxidase, an oxidative metabolic component of Complex IV, has been shown to be impaired (159). Platelet mitochondria in Alzheimer's have been observed as having increased levels of oxidative stress leading to mitochondrial damage and platelet apoptosis (160). Studies of mitochondrial function in Parkinson's disease have identified mutations or defects in Complex I-IV of the ETC, α -synuclein, PARKIN, PINK1, DJ-1, and LRRK2 (139). A decrease in Complexes I and IV can develop quickly within the first year of Parkinson's (161, 162), and low levels of activity in platelet mitochondrial complexes I and II/III in early, untreated Parkinson's (163). Other key protein associations include, NADH CoQ reductase, key to Complex I dysfunction (154), coenzyme Q10, a key electron receptor in Complexes I and II of the mitochondrial ETC (164–166) and the neurotransmitter-degrading enzyme monoamine oxidase B (MAO) (167–169). Implicated in the pathogenesis of both Alzheimer's and aging in general, platelet MAO has been used as a peripheral biomarker for the onset of Alzheimer's and Parkinson's (170, 171). Also consistent with the association of oxidative stress induced mitochondrial damage and apoptosis with Parkinson's disease, levels of the oxidative protective protein methionine sulfoxide reductase type 2 (MsrB2) was recently shown to be reduced in platelets of Parkinson's disease patients leading to increased platelet apoptosis (119). Indeed, platelet mitochondria may serve as an important biomarker in PD. These neurodegenerative diseases once again demonstrate the essential nature of platelet mitochondria for survival as reduced function and damage either through genetic defects or environmental stress leads to apoptosis and premature platelet death.

With the importance of mitochondria in platelet function and the potential contributions of mitochondria dysfunction to aging (49, 130) and age-related diseases, such as diabetes mellitus (58,

93, 94), Parkinson's (51, 119), and cardiovascular disease (69), targeting platelet mitochondria may serve as adjunct therapies. Drugs targeting platelet mitochondria metabolism and apoptosis may help prevent pathological thrombosis and contributions to disease. The genomics and proteomics of the mitochondria (Figure 2) provide multiple potential targets as outlined in an excellent recent review by Fuentes et al. (172). However, selective targeting to platelets may require further coupling to platelet targeting agents.

CONCLUSION

Mitochondria in nucleated cells have been well-described. While mitochondria functions are similar, its role becomes increasingly important in the nucleus-free zone of the platelet. As described in this review, not only are mitochondria involved in energy metabolism and ATP production in the platelets, they are also the central drivers of platelet activation and apoptosis; both events critical for platelet function and lifespan. The pathophysiological role played by platelet and their mitochondria in many systemic diseases remain under intense investigation. Therapies targeting platelet mitochondria may ultimately prove beneficial for such disease processes.

AUTHOR CONTRIBUTIONS

HM and JH conceived the paper. HM wrote the first draft. KJ, TT, and JH extensively reviewed and edited the paper.

FUNDING

This manuscript was supported by grants from NIH NHLBI, grants HL115247, HL122815 and a Pilot Grant from the Claude D. Pepper Older Americans Independence Center at Yale School of Medicine (P30AG021342).

REFERENCES

- Holinstat M. Normal platelet function. *Cancer Metastasis Rev.* (2017) 36:195–8. doi: 10.1007/s10555-017-9677-x
- van der Meijden PEJ, Heemskerk JWM. Platelet biology and functions: new concepts and clinical perspectives. *Nat Rev Cardiol.* (2019) 16:166–79. doi: 10.1038/s41569-018-0110-0
- Ghoshal K, Bhattacharyya M. Overview of platelet physiology: its hemostatic and nonhemostatic role in disease pathogenesis. *ScientificWorldJournal.* (2014) 2014:781857. doi: 10.1155/2014/781857
- Herter JM, Rossaint J, Zarbock A. Platelets in inflammation and immunity. *J Thromb Haemost.* (2014) 12:1764–75. doi: 10.1111/jth.12730
- Hou Y, Carrim N, Wang Y, Gallant RC, Marshall A, Ni H. Platelets in hemostasis and thrombosis: novel mechanisms of fibrinogen-independent platelet aggregation and fibronectin-mediated protein wave of hemostasis. *J Biomed Res.* (2015) 29:437–44. doi: 10.7555/JBR.29.20150121
- Bizzozzero G. Über einen neuen Formbestandteil des Blutes und dessen Rolle bei der Thrombose und Blutgerinnung. *Virchows Arch.* (1882) 90:261–332. doi: 10.1007/BF01931360
- Wright JH. The origin and nature of the blood plates. *Boston Med Surg J.* (1906) 154:643–5. doi: 10.1056/NEJM190606071542301
- Lefrançois E, Ortiz-Muñoz G, Caudrillier A, Mallavia B, Liu F, Sayah DM, et al. The lung is a site of platelet biogenesis and a reservoir for haematopoietic progenitors. *Nature.* (2017) 544:105–9. doi: 10.1038/nature21706
- Patel SR, Hartwig JH, Italiano JE Jr. The biogenesis of platelets from megakaryocyte proplatelets. *J Clin Invest.* (2005) 115:3348–54. doi: 10.1172/JCI26891
- Thon JN, Montalvo A, Patel-Hett S, Devine MT, Richardson JL, Ehrlicher A, et al. Cytoskeletal mechanics of proplatelet maturation and platelet release. *J Cell Biol.* (2010) 191:861. doi: 10.1083/jcb.201006102
- Machlus KR, Italiano JE. The incredible journey: from megakaryocyte development to platelet formation. *J Cell Biol.* (2013) 201:785. doi: 10.1083/jcb.201304054
- Ebbeling L, Robertson C, McNicol A, Gerrard JM. Rapid ultrastructural changes in the dense tubular system following platelet activation. *Blood.* (1992) 80:718. doi: 10.1182/blood.V80.3.718.bloodjournal803718
- Escolar GG, White J. The platelet open canalicular system: a final common pathway. *Blood Cells.* (1991) 17:467–85.
- Youssefian T, Massé JM, Rendu F, Guichard J, Cramer EM. Platelet and megakaryocyte dense granules contain glycoproteins Ib and IIb-IIIa. *Blood.* (1997) 89:4047. doi: 10.1182/blood.V89.11.4047
- Lebois M, Josefsson EC. Regulation of platelet lifespan by apoptosis. *Platelets.* (2016) 27:497–504. doi: 10.3109/09537104.2016.1161739
- Garraud O, Cognasse F. Are Platelets Cells? And if Yes, are They Immune Cells? *Front Immunol.* (2015) 6:70. doi: 10.3389/fimmu.2015.00070

17. Kapur R, Zufferey A, Boilard E, Semple JW. Nouvelle cuisine: platelets served with inflammation. *J Immunol.* (2015) 194:5579–87. doi: 10.4049/jimmunol.1500259
18. Vieira-de-Abreu, Campbell RA, Weyrich AS, Zimmerman GA. Platelets: versatile effector cells in hemostasis, inflammation, and the immune continuum. *Semin Immunopathol.* (2012) 34:5–30. doi: 10.1007/s00281-011-0286-4
19. Li JL, Zarbock A, Hidalgo A. Platelets as autonomous drones for hemostatic and immune surveillance. *J Exp Med.* (2017) 214:2193. doi: 10.1084/jem.20170879
20. Walsh PN. Platelet coagulant activities and hemostasis: a hypothesis. *Blood.* (1974) 43:597. doi: 10.1182/blood.V43.4.597.597
21. Periyah MH, Halim AS, Mat Saad AZ. Mechanism action of platelets and crucial blood coagulation pathways in hemostasis. *Int J Hematol Oncol Stem Cell Res.* (2017) 11:319–27.
22. Johnson SA, Van Horn DL, Pederson HJ, Marr J. The function of platelets. *Transfusion.* (1966) 6:3–17. doi: 10.1111/j.1537-2995.1966.tb04686.x
23. Zeng Z, Xia L, Fan X, Ostrik AC, Yarovsky T, Su M, et al. Platelet-derived miR-223 promotes a phenotypic switch in arterial injury repair. *J Clin Invest.* (2019) 129:1372–86. doi: 10.1172/JCI124508
24. Ferroni P, Vazzana N, Riondino S, Cuccurullo C, Guadagni F, Davi G. Platelet function in health and disease, from molecular mechanisms, redox considerations to novel therapeutic opportunities. *Antioxidants Redox Signal.* (2012) 17:1447–85. doi: 10.1089/ars.2011.4324
25. Wang L, Wu Q, Fan Z, Xie R, Wang Z, Lu Y. Platelet mitochondrial dysfunction and the correlation with human diseases. *Biochem Soc Trans.* (2017) 45:1213. doi: 10.1042/BST20170291
26. Chae WJ, Ehrlich AK, Chan PY, Teixeira AM, Henegariu O, Hao L, et al. The Wnt Antagonist dickkopf-1 promotes pathological type 2 cell-mediated inflammation. *Immunity.* (2016) 44:246–58. doi: 10.1016/j.immuni.2016.01.008
27. Schwartz RS, Conley CL. Blood. In: *Encyclopedia Britannica*. Encyclopedia Britannica, Inc. Available online at: <https://www.britannica.com/science/blood-biochemistry>
28. Schmaier AA, Stalker TJ, Runge JJ, Lee D, Nagaswami C, Mericko P, et al. Occlusive thrombi arise in mammals but not birds in response to arterial injury: evolutionary insight into human cardiovascular disease. *Blood.* (2011) 118:3661–9. doi: 10.1182/blood-2011-02-338244
29. Belamarich FA, Shepro D, Kien M. ADP is not involved in thrombin-induced aggregation of thrombocytes of a non-mammalian vertebrate. *Nature.* (1968) 220:509–10. doi: 10.1038/220509a0
30. Alberts B. *Molecular Biology of the Cell*. New York, NY: Garland Science, Taylor and Francis Group (2015).
31. Webster M, Witkin KL, Cohen-Fix O. Sizing up the nucleus: nuclear shape, size and nuclear-envelope assembly. *J Cell Sci.* (2009) 122:1477–86. doi: 10.1242/jcs.037333
32. White JG, Clawson CC. The surface-connected canalicular system of blood platelets—a fenestrated membrane system. *Am J Pathol.* (1980) 101:353–64.
33. Weyrich AS, Lindemann S, Tolley ND, Kraiss LW, Dixon DA, Mahoney TM, et al. Change in protein phenotype without a nucleus: translational control in platelets. *Semin Thromb Hemost.* (2004) 30:491–8. doi: 10.1055/s-2004-833484
34. Rowley JW, Schwartz H, Weyrich AS. Platelet mRNA: the meaning behind the message. *Curr Opin Hematol.* (2012) 19:385–91. doi: 10.1097/MOH.0b013e328357010e
35. Weyrich AS, Schwartz H, Kraiss LW, Zimmerman GA. Protein synthesis by platelets: historical and new perspectives. *J Thromb Haemost.* (2009) 7:241–6. doi: 10.1111/j.1538-7836.2008.03211.x
36. Nilsson RJA, Balaj L, Hulleman E, van Rijn S, Pegtel DM, Walraven M, et al. Blood platelets contain tumor-derived RNA biomarkers. *Blood.* (2011) 118:3680. doi: 10.1182/blood-2011-03-344408
37. Xia L, Zeng Z, Tang WH. The role of platelet microparticle associated microRNAs in cellular crosstalk. *Front Cardiovasc Med.* (2018) 5:29. doi: 10.3389/fcvm.2018.00029
38. Clancy L, Freedman JE. Blood-derived extracellular RNA and platelet pathobiology: adding pieces to a complex circulating puzzle. *Circ Res.* (2016) 118:374–6. doi: 10.1161/CIRCRESAHA.115.308190
39. Risitano A, Beaulieu LM, Vitseva O, Freedman JE. Platelets and platelet-like particles mediate intercellular RNA transfer. *Blood.* (2012) 119:6288–95. doi: 10.1182/blood-2011-12-396440
40. Ducheze AC, Boudreau LH, Naika GS, Bollinger J, Belleannee C, Cloutier N, et al. Platelet microparticles are internalized in neutrophils via the concerted activity of 12-lipoxygenase and secreted phospholipase A2-IIA. *Proc Natl Acad Sci USA.* (2015) 112:E3564–73. doi: 10.1073/pnas.1507905112
41. Neelamegham S. The computing platelet: integrating environmental cues. *Blood.* (2012) 120:3. doi: 10.1182/blood-2012-05-426007
42. Hayashi T, Tanaka S, Hori Y, Hirayama F, Sato EF, Inoue M. Role of mitochondria in the maintenance of platelet function during in vitro storage. *Transf Med.* (2011) 21:166–74. doi: 10.1111/j.1365-3148.2010.01065.x
43. Kühlbrandt W/ Structure and function of mitochondrial membrane protein complexes. *BMC Biol.* (2015) 13:89. doi: 10.1186/s12915-015-0201-x
44. Bonora M, Patergnani S, Rimessi A, De Marchi E, Suski JM, Bononi A, et al. ATP synthesis and storage. *Purin Signal.* (2012) 8:343–57. doi: 10.1007/s11302-012-9305-8
45. Hekimi S, Wang Y, Noë A. Mitochondrial ROS and the effectors of the intrinsic apoptotic pathway in aging cells: the discerning killers! *Front Genet.* (2016) 7:161. doi: 10.3389/fgene.2016.00161
46. Sarasija S, Norman KR. A γ -Secretase independent role for presenilin in calcium homeostasis impacts mitochondrial function and morphology in *Caenorhabditis elegans*. *Genetics.* (2015) 201:1453–66. doi: 10.1534/genetics.115.182808
47. Wang C, Youle RJ. The role of mitochondria in apoptosis*. *Ann Rev Genet.* (2009) 43:95–118. doi: 10.1146/annurev-genet-102108-134850
48. H.-Kim E, Grant AR, Simic MS, Kohnz RA, Nomura DK, Durieux J, et al. Lipid biosynthesis coordinates a mitochondrial-to-cytosolic stress response. *Cell.* (2016) 166:1539–1552.e16. doi: 10.1016/j.cell.2016.08.027
49. Jain K, Tyagi T, Patell K, Xie Y, Kadado AJ, Lee SH, et al. Age associated non-linear regulation of redox homeostasis in the anucleate platelet: implications for CVD risk patients. *EBioMedicine.* (2019) 44:28–40. doi: 10.1016/j.ebiom.2019.05.022
50. Swerdlow RH. Mitochondria and mitochondrial cascades in Alzheimer's Disease. *J Alzheimer's Dis.* (2018) 62:1403–16. doi: 10.3233/JAD-170585
51. J.-Park S, Davis RL, Sue CM. Mitochondrial dysfunction in Parkinson's Disease: new mechanistic insights and therapeutic perspectives. *Curr Neurol Neurosci Rep.* (2018) 18:21. doi: 10.1007/s11910-018-0829-3
52. Kholmukhamedov A, Jobe S. Platelet respiration. *Blood Adv.* (2019) 3:599. doi: 10.1182/bloodadvances.2018025155
53. Baeten CCFMJ, Moenen FCJ, Henskens YMC, Swieringa F, Wetzels RJH, van Oerle R, et al. Impaired mitochondrial activity explains platelet dysfunction in thrombocytopenic cancer patients undergoing chemotherapy. *Haematologica.* (2018) 103:1557–67. doi: 10.3324/haematol.2017.185165
54. Menzies RA, Gold PH. The turnover of mitochondria in a variety of tissues of young adult and aged rats. *J Biol Chem.* (1971) 246:2425–9.
55. Diaz F, Moraes CT. Mitochondrial biogenesis and turnover. *Cell Calcium.* (2008) 44:24–35. doi: 10.1016/j.ccca.2007.12.004
56. Jobe SM, Wilson KM, Leo L, Raimondi A, Molkentin JD, Lentz SR, et al. Critical role for the mitochondrial permeability transition pore and cyclophilin D in platelet activation and thrombosis. *Blood.* (2008) 111:1257. doi: 10.1182/blood-2007-05-092684
57. Davizon-Castillo P, McMahon B, Aguila S, Bark D, Ashworth K, Allawzi A, et al. TNF-alpha-driven inflammation and mitochondrial dysfunction define the platelet hyperreactivity of aging. *Blood.* (2019) 134:727–40. doi: 10.1182/blood.2019000200
58. Taylor RW, Turnbull DM. Mitochondrial DNA mutations in human disease. *Nat Rev Genet.* (2005) 6:389–402. doi: 10.1038/nrg1606
59. Lodish H, Berk A, Zipursky SL, et al. *Molecular Cell Biology*. 4th Edn. Section 9.7, Organelle DNAs. New York, NY: W. H. Freeman (2000).
60. Zhang M, Chen JL, Wang PC, Fu CY, Peng H. Complete mitochondrial genome sequence of the human neuroblastoma cell line 751-NA. *Genome Announce.* (2017) 5:e01185–17. doi: 10.1128/genomeA.01185-17

61. Gilkerson R, Bravo L, Garcia I, Gaytan N, Herrera A, Maldonado A, Quintanilla B. The mitochondrial nucleoid: integrating mitochondrial DNA into cellular homeostasis. *Cold Spring Harb Persp Biol.* (2013) 5:a011080. doi: 10.1101/cshperspect.a011080
62. Moraes CT, Srivastava S, Kirkinezos I, Oca-Cossio J, vanWaveren C, Woischnick M, Diaz F. Mitochondrial DNA structure and function. *Int Rev Neurobiol.* (2002) 53:3–23. doi: 10.1016/S0074-7742(02)53002-6
63. Holt IJ, Reyes A. Human mitochondrial DNA replication. *Cold Spring Harb Perspect Biol.* (2012) 4:a012971 doi: 10.1101/cshperspect.a012971
64. Masukata H, Tomizawa J. A mechanism of formation of a persistent hybrid between elongating RNA and template DNA. *Cell.* (1990) 62:331–8. doi: 10.1016/0092-8674(90)90370-T
65. Wanrooij S, Falkenberg M. The human mitochondrial replication fork in health and disease. *Biochim Biophys Acta.* (2010) 1797:1378–88. doi: 10.1016/j.bbabi.2010.04.015
66. Holt IJ, Lorimer HE, Jacobs HT. Coupled leading- and lagging-strand synthesis of mammalian mitochondrial DNA. *Cell.* (2000) 100:515–24. doi: 10.1016/S0092-8674(00)80688-1
67. Malik AN, Czajka A. Is mitochondrial DNA content a potential biomarker of mitochondrial dysfunction? *Mitochondrion.* (2013) 13:481–92. doi: 10.1016/j.mito.2012.10.011
68. Tin A, Grams ME, Ashar FN, Lane JA, Rosenberg AZ, Grove ML, et al. Association between mitochondrial DNA copy number in peripheral blood and incident CKD in the atherosclerosis risk in communities study. *J Am Soc Nephrol.* (2016) 27:2467–73. doi: 10.1681/ASN.2015060661
69. Baccarelli AA, Byun HM. Platelet mitochondrial DNA methylation: a potential new marker of cardiovascular disease. *Clin Epigenet.* (2015) 7:44. doi: 10.1186/s13148-015-0078-0
70. Finsterer J. Genetic, pathogenetic, and phenotypic implications of the mitochondrial A3243G tRNA^{Leu}(UUR) mutation. *Acta Neurol Scand.* (2007) 116:1–14. doi: 10.1111/j.1600-0404.2007.00836.x
71. Pfanner N, Warscheid B, Wiedemann N. Mitochondrial proteins: from biogenesis to functional networks. *Nat Rev Mol Cell Biol.* (2019) 20:267–84. doi: 10.1038/s41580-018-0092-0
72. Dudek J, Rehling P, van der Laan M. Mitochondrial protein import: common principles and physiological networks. *Biochim Biophys Acta.* (2013) 1833:274–85. doi: 10.1016/j.bbamer.2012.05.028
73. Neupert W. A perspective on transport of proteins into mitochondria: a myriad of open questions. *J Mol Biol.* (2015) 427:1135–58. doi: 10.1016/j.jmb.2015.02.001
74. van der Laan M, Meinecke M, Dudek J, Hutu DP, Lind M, Perschil I, et al. Motor-free mitochondrial presequence translocase drives membrane integration of preproteins. *Nat Cell Biol.* (2007) 9:1152. doi: 10.1038/ncb1635
75. Alberts B, Johnson A, Lewis J, et al. *Molecular Biology of the Cell.* 4th Edn. The Transport of Proteins into Mitochondria and Chloroplasts. New York, NY: Garland Science (2002).
76. Kramer PA, Ravi S, Chacko B, Johnson MS, Darley-Usmar VM. A review of the mitochondrial and glycolytic metabolism in human platelets and leukocytes: implications for their use as bioenergetic biomarkers. *Redox Biol.* (2014) 2:206–10. doi: 10.1016/j.redox.2013.12.026
77. Aibibula M, Naseem KM, Sturmeier RG. Glucose metabolism and metabolic flexibility in blood platelets. *J Thromb Haemost.* (2018) 16:2300–14. doi: 10.1111/jth.14274
78. Doery JC G, Hirsh J, Cooper I. Energy metabolism in human platelets: interrelationship between glycolysis and oxidative metabolism. *Blood.* (1970) 36:159. doi: 10.1182/blood.V36.2.159.159
79. Holmsen H, Setkowsky CA, Day HJ. Effects of antimycin and 2-deoxyglucose on adenine nucleotides in human platelets. Role of metabolic adenosine triphosphate in primary aggregation, secondary aggregation and shape change of platelets. *Biochem J.* (1974) 144:385–96. doi: 10.1042/bj1440385
80. Fidler TP, Campbell RA, Funari T, Dunne N, Balderas Angeles E, Middleton EA, Chaudhuri D, Weyrich AS, Abel ED. Deletion of GLUT1 and GLUT3 reveals multiple roles for glucose metabolism in platelet and megakaryocyte function. *Cell Rep.* (2017) 20:881–94. doi: 10.1016/j.celrep.2017.06.083
81. Clemetson KJ. Platelets and primary haemostasis. *Thromb Res.* (2012) 129:220–4. doi: 10.1016/j.thromres.2011.11.036
82. Remenyi G, Szasz R, Friese P, Dale George L. Role of mitochondrial permeability transition pore in coated-platelet formation. *Arterioscler Thromb Vasc Biol.* (2005) 25:467–71. doi: 10.1161/01.ATV.0000152726.49229.bf
83. Leytin V, Allen DJ, Mutlu A, Gyulkhandanyan AV, Mykhaylov S, Freedman J. Mitochondrial control of platelet apoptosis: effect of cyclosporin A, an inhibitor of the mitochondrial permeability transition pore. *Lab Invest.* (2009) 89:374. doi: 10.1038/labinvest.2009.13
84. Lopez JJ, Salido GM E, GÓmez-Arteta, Rosado JA, Pariente JA. Thrombin induces apoptotic events through the generation of reactive oxygen species in human platelets. *J Thromb Haemost.* (2007) 5:1283–91. doi: 10.1111/j.1538-7836.2007.02505.x
85. Yamagishi SI, Edelstein D, Du XL, Brownlee M. Hyperglycemia potentiates collagen-induced platelet activation through mitochondrial superoxide overproduction. *Diabetes.* (2001) 50:1491. doi: 10.2337/diabetes.50.6.1491
86. Choo HJ, Saafir Talib B, Mkumba L, Wagner Mary B, Jobe Shawn M. Mitochondrial calcium and reactive oxygen species regulate agonist-initiated platelet phosphatidylserine exposure. *Arterioscler Thromb Vasc Biol.* (2012) 32:2946–55. doi: 10.1161/ATVBAHA.112.300433
87. Davi G, Patrono C. Platelet activation and atherothrombosis. *N Engl J Med.* (2007) 357:2482–94. doi: 10.1056/NEJMra071014
88. Garcia-Souza LF, Oliveira MF. Mitochondria, biological roles in platelet physiology and pathology. *Int J Biochem Cell Biol.* (2014) 50:156–60. doi: 10.1016/j.biocel.2014.02.015
89. Brass LF. Ca²⁺ homeostasis in unstimulated platelets. *J Biol Chem.* (1984) 259:12563–70.
90. Figueira TR, Barros MH, Camargo AA, Castilho RF, Ferreira JC B, Kowaltowski AJ, et al. Mitochondria as a source of reactive oxygen and nitrogen species: from molecular mechanisms to human health. *Antioxid Redox Sign.* (2012) 18:2029–74. doi: 10.1089/ars.2012.4729
91. Lopez JJ, Salido GM, Pariente JA, Rosado JA. Thrombin induces activation and translocation of Bid, Bax and Bak to the mitochondria in human platelets. *J Thromb Haemost.* (2008) 6:1780–8. doi: 10.1111/j.1538-7836.2008.03111.x
92. Kodama T, Takehara T, Hikita H, Shimizu S, Shigekawa M, Li W, et al. BH3-only activator proteins bid and bim are dispensable for bak/bax-dependent thrombocyte apoptosis induced by Bcl-xL deficiency: molecular requisites for the mitochondrial pathway to apoptosis in platelets. *J Biol Chem.* (2011) 286:13905–13. doi: 10.1074/jbc.M110.195370
93. Tang WH, Stitham J, Gleim S, Di Febbo C, Porreca E, Fava C, et al. Glucose and collagen regulate human platelet activity through aldose reductase induction of thromboxane. *J Clin Invest.* (2011) 121:4462–76. doi: 10.1172/JCI59291
94. Tang WH, Stitham J, Jin Y, Liu R, Lee SH, Du J, et al. Aldose reductase-mediated phosphorylation of p53 leads to mitochondrial dysfunction, and damage in diabetic platelets. *Circulation.* (2014) 129:1598–609. doi: 10.1161/CIRCULATIONAHA.113.005224
95. Lee SH, Du J, Stitham J, Attaya G, Lee S, Xiang Y, et al. Inducing mitophagy in diabetic platelets protects against severe oxidative stress. *EMBO Mol Med.* (2016) 8:779–95. doi: 10.15252/emmm.201506046
96. Gyulkhandanyan AV, Mutlu A, Freedman J, Leytin V. Markers of platelet apoptosis: methodology and applications. *J Thromb Thrombolysis.* (2012) 33:397–411. doi: 10.1007/s11239-012-0688-8
97. Perrotta PL, Perrotta CL, Snyder EL. Apoptotic activity in stored human platelets. *Transfusion.* (2003) 43:526–35. doi: 10.1046/j.1537-2995.2003.00349.x
98. Kile BT. The role of the intrinsic apoptosis pathway in platelet life and death. *J Thromb Haemos.* (2009) 7:214–7. doi: 10.1111/j.1538-7836.2009.03366.x
99. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol.* (2008) 9:47. doi: 10.1038/nrm2308
100. Mason KD, Carpinelli MR, Fletcher JI, Collinge JE, Hilton AA, Ellis S, et al. Programmed anuclear cell death delimits platelet life span. *Cell.* (2007) 128:1173–86. doi: 10.1016/j.cell.2007.01.037
101. Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA, et al. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature.* (2005) 435:677–81. doi: 10.1038/nature03579
102. Josefsson EC, James C, Henley KJ, Debrincat MA, Rogers KL, Dowling MR, et al. Megakaryocytes possess a functional intrinsic apoptosis pathway that

- must be restrained to survive and produce platelets. *J Exp Med.* (2011) 208:2017–31. doi: 10.1084/jem.20110750
103. Vandenberg CJ, Josefsson EC, Campbell KJ, James C, Lawlor KE, Kile BT, et al. Loss of Bak enhances lymphocytosis but does not ameliorate thrombocytopenia in BCL-2 transgenic mice. *Cell Death Differ.* (2014) 21:676–84. doi: 10.1038/cdd.2013.201
 104. Debrincat MA, Pleines I, Lebois M, Lane RM, Holmes ML, Corbin J, et al. BCL-2 is dispensable for thrombopoiesis and platelet survival. *Cell Death Dis.* (2015) 6:e1721. doi: 10.1038/cddis.2015.97
 105. McArthur K, Chappaz S, Kile BT. Apoptosis in megakaryocytes and platelets: the life and death of a lineage. *Blood.* (2018) 131:605. doi: 10.1182/blood-2017-11-742684
 106. Julien O, Wells JA. Caspases and their substrates. *Cell Death Differ.* (2017) 24:1380. doi: 10.1038/cdd.2017.44
 107. Bhatlekar S, Basak I, Edelstein LC, Campbell RA, Lindsey CR, Italiano JE Jr, et al. Anti-apoptotic BCL2L2 increases megakaryocyte proplatelet formation in cultures of human cord blood. *Haematologica.* (2019) 104:2075–83. doi: 10.3324/haematol.2018.204685
 108. Feng W, Chang C, Luo D, Su H, Yu S, Hua W, et al. Dissection of autophagy in human platelets. *Autophagy.* (2014) 10:642–51. doi: 10.4161/auto.27832
 109. Cao Y, Cai J, Zhang S, Yuan N, Li X, Fang Y, et al. Loss of autophagy leads to failure in megakaryopoiesis, megakaryocyte differentiation, and thrombopoiesis in mice. *Exp Hematol.* (2015) 43:488–94. doi: 10.1016/j.exphem.2015.01.001
 110. Ouseph MM, Huang Y, Banerjee M, Joshi S, MacDonald L, Zhong Y, et al. Autophagy is induced upon platelet activation and is essential for hemostasis and thrombosis. *Blood.* (2015) 126:1224–33. doi: 10.1182/blood-2014-09-598722
 111. Allen GF, Toth R, James J, Ganley IG. Loss of iron triggers PINK1/Parkin-independent mitophagy. *EMBO Rep.* (2013) 14:1127–35. doi: 10.1038/embor.2013.168
 112. Gegg ME, Cooper JM, Chau KY, Rojo M, Schapira AH, Taanman JW. Mitofusin 1 and mitofusin 2 are ubiquitinated in a PINK1/parkin-dependent manner upon induction of mitophagy. *Hum Mol Genet.* (2010) 19:4861–70. doi: 10.1093/hmg/ddq419
 113. Geisler S, Holmstrom KM, Skujat D, Fiesel FC, Rothfuss OC, Kahle PJ, et al. PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nat Cell Biol.* (2010) 12:119–31. doi: 10.1038/ncb2012
 114. Shiba-Fukushima K, Imai Y, Yoshida S, Ishihama Y, Kanao T, Sato S, et al. PINK1-mediated phosphorylation of the Parkin ubiquitin-like domain primes mitochondrial translocation of Parkin and regulates mitophagy. *Sci Rep.* (2012) 2:1002. doi: 10.1038/srep01002
 115. Wauer T, Simicek M, Schubert A, Komander D. Mechanism of phospho-ubiquitin-induced PARKIN activation. *Nature.* (2015) 524:370–4. doi: 10.1038/nature14879
 116. Choi AM, Ryter SW, Levine B. Autophagy in human health and disease. *N Engl J Med.* (2013) 368:1845–6. doi: 10.1056/NEJMc1303158
 117. Shintani T, Klionsky DJ. Autophagy in health and disease: a double-edged sword. *Science.* (2004) 306:990–5. doi: 10.1126/science.1099993
 118. Kubli DA, Gustafsson AB. Mitochondria and mitophagy: the yin and yang of cell death control. *Circ Res.* (2012) 111:1208–21. doi: 10.1161/CIRCRESAHA.112.265819
 119. Lee SH, Lee S, Du J, Jain K, Ding M, Kadado AJ, et al. Mitochondrial MsrB2 serves as a switch and transducer for mitophagy. *EMBO Mol Med.* (2019) 11:e10409. doi: 10.15252/emmm.201910409
 120. Koçer A, Yaman A, Niftaliyev E, Dürüyen H, Eryilmaz M, Koçer E. Assessment of platelet indices in patients with neurodegenerative diseases: mean platelet volume was increased in patients with Parkinson's disease. *Curr Gerontol Geriatr Res.* (2013) 2013:986254. doi: 10.1155/2013/986254
 121. Willoughby S, Holmes A, Loscalzo J. Platelets and cardiovascular disease. *Euro J Cardiovasc Nurs.* (2002) 1:273–88. doi: 10.1016/S1474-51510200038-5
 122. Santilli F, Simeone P, Liani R, Davi G. Platelets and diabetes mellitus. *Prostaglandins & Other Lipid Mediat.* (2015) 120:28–39. doi: 10.1016/j.prostaglandins.2015.05.002
 123. Dewitte A, Lepreux S, Villeneuve J, Rigotherier C, Combe C, Ouattara A, et al. Blood platelets and sepsis pathophysiology: A new therapeutic prospect in critically [corrected] ill patients? *Ann Intens Care.* (2017) 7:115. doi: 10.1186/s13613-017-0337-7
 124. Lannan KL, Phipps RP, White RJ. Thrombosis, platelets, microparticles and PAH: more than a clot. *Drug Discov Today.* (2014) 19:1230–5. doi: 10.1016/j.drudis.2014.04.001
 125. Sinauridze EI, Kireev DA, Popenko NY, Pichugin AV, Panteleev MA, Krymskaya OV, et al. Platelet microparticle membranes have 50- to 100-fold higher specific procoagulant activity than activated platelets. *Thromb Haemost.* (2007) 97:425–34. doi: 10.1160/TH06-06-0313
 126. Gawaz M, Vogel S. Platelets in tissue repair: control of apoptosis and interactions with regenerative cells. *Blood.* (2013) 122:2550. doi: 10.1182/blood-2013-05-468694
 127. Song S, Pursell ZF, Copeland WC, Longley MJ, Kunkel TA, Mathews CK. DNA precursor asymmetries in mammalian tissue mitochondria and possible contribution to mutagenesis through reduced replication fidelity. *Proc Natl Acad Sci USA.* (2005) 102:4990. doi: 10.1073/pnas.0500253102
 128. Chial H, Craig J. mtDNA and mitochondrial diseases. *Nat Educ.* (2008) 1:217.
 129. Ryzhkova AI, Sazonova MA, Sinyov VV, Galitsyna EV, Chicheva MM, Melnichenko AA, et al. Mitochondrial diseases caused by mtDNA mutations: a mini-review. *Therap Clin Risk Manag.* (2018) 14:1933–42. doi: 10.2147/TCRM.S154863
 130. Shokolenko IN, Wilson GL, Alexeyev MF. Aging: a mitochondrial DNA perspective, critical analysis and an update. *World J Exp Med.* (2014) 4:46–57. doi: 10.5493/wjem.v4.i4.46
 131. Ito S, Ohta S, Nishimaki K, Kagawa Y, Soma R, Kuno SY, et al. Functional integrity of mitochondrial genomes in human platelets and autopsied brain tissues from elderly patients with Alzheimer's disease. *Proc Natl Acad Sci USA.* (1999) 96:2099–103. doi: 10.1073/pnas.96.5.2099
 132. Hurtado-Roca Y, Ledesma M, Gonzalez-Lazaro M, Moreno-Loshuertos R, Fernandez-Silva P, Enriquez JA, et al. Adjusting MtDNA quantification in whole blood for peripheral blood platelet and leukocyte counts. *PLoS ONE.* (2016) 11:e0163770. doi: 10.1371/journal.pone.0163770
 133. Lindqvist D, Wolkowitz OM, Picard M, Ohlsson L, Bersani FS, Fernstrom J, et al. Circulating cell-free mitochondrial DNA, but not leukocyte mitochondrial DNA copy number, is elevated in major depressive disorder. *Neuropsychopharmacology.* (2018) 43:1557–64. doi: 10.1038/s41386-017-0001-9
 134. Chait A, Bornfeldt KE. Diabetes and atherosclerosis: is there a role for hyperglycemia? *J Lipid Res.* (2009) 50(Suppl):S335–9. doi: 10.1194/jlr.R800059-JLR200
 135. Madamanchi Nageswara R, Runge Marschall S. Mitochondrial dysfunction in atherosclerosis. *Circ Res.* (2007) 100:460–73. doi: 10.1161/01.RES.0000258450.44413.96
 136. Lindemann S, KrÄMer B, Seizer P, Gawaz M. Platelets, inflammation and atherosclerosis. *J Thromb Haemost.* (2007) 5:203–11. doi: 10.1111/j.1538-7836.2007.02517.x
 137. Cybulsky MI, Iiyama K, Li H, Zhu S, Chen M, Iiyama M, et al. A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. *J Clin Invest.* (2001) 107:1255–62. doi: 10.1172/JCI11871
 138. Ran J, Guo X, Li Q, Mei G, Lao G. Platelets of type 2 diabetic patients are characterized by high ATP content and low mitochondrial membrane potential. *Platelets.* (2009) 20:588–93. doi: 10.3109/09537100903288422
 139. Zharikov S, Shiva S. Platelet mitochondrial function: from regulation of thrombosis to biomarker of disease. *Biochem Soc Trans.* (2013) 41:118. doi: 10.1042/BST20120327
 140. Schneider DJ. Factors contributing to increased platelet reactivity in people with diabetes. *Diab Care.* (2009) 32:525–7. doi: 10.2337/dc08-1865
 141. Tang WH, Martin KA, Hwa J. Aldose reductase, oxidative stress, and diabetic mellitus. *Front Pharmacol.* (2012) 3:87. doi: 10.3389/fphar.2012.00087
 142. Xin G, Wei Z, Ji C, Zheng H, Gu J, Ma L, et al. Metformin uniquely prevents thrombosis by inhibiting platelet activation and mtDNA release. *Sci Rep.* (2016) 6:36222. doi: 10.1038/srep36222
 143. Semple JW, Italiano JE, Freedman J. Platelets and the immune continuum. *Nat Rev Immunol.* (2011) 11:264. doi: 10.1038/nri2956
 144. Hottz ED, Bozza FA, Bozza PT. Platelets in immune response to virus and immunopathology of viral infections. *Front Med.* (2018) 5:121. doi: 10.3389/fmed.2018.00121
 145. Ali RA, Wuescher LM, Worth RG. Platelets: essential components of the immune system. *Curr Trends Immunol.* (2015) 16:65–78.

146. Morrell CN, Aggrey AA, Chapman LM, Modjeski KL. Emerging roles for platelets as immune and inflammatory cells. *Blood*. (2014) 123:2759. doi: 10.1182/blood-2013-11-462432
147. Protti A, Fortunato E, Caspani ML, Pluderer M, Lucchini V, Grimoldi N, et al. Mitochondrial changes in platelets are not related to those in skeletal muscle during human septic shock. *PLoS ONE*. (2014) 9:e96205 doi: 10.1371/journal.pone.0096205
148. Singer M. The role of mitochondrial dysfunction in sepsis-induced multi-organ failure. *Virulence*. (2014) 5:66–72. doi: 10.4161/viru.26907
149. Sharma B, Sharma M, Majumder M, Steier W, Sangal A, Kalawar M. Thrombocytopenia in septic shock patients—a prospective observational study of incidence, risk factors and correlation with clinical outcome. *Anaes Intens Care*. (2007) 35:833–1018. doi: 10.1177/0310057X0703500604
150. Claushuis TAM, van Vught LA, Scicluna BP, Wiewel MA, Klein Klouwenberg PMC, Hoogendijk AJ, et al. Thrombocytopenia is associated with a dysregulated host response in critically ill sepsis patients. *Blood*. (2016) 127:3062. doi: 10.1182/blood-2015-11-680744
151. Boudreau LH, Duchez AC, Cloutier N, Soulet D, Martin N, Bollinger J, et al. Platelets release mitochondria serving as substrate for bactericidal group IIA-secreted phospholipase A2 to promote inflammation. *Blood*. (2014) 124:2173–83. doi: 10.1182/blood-2014-05-573543
152. Andersson SG, Zomorodipour A, Andersson JO, Sicheritz-Ponten T, Alsmark UC, Podowski RM, et al. The genome sequence of Rickettsia prowazekii and the origin of mitochondria. *Nature*. (1998) 396:133–40. doi: 10.1038/24094
153. Veitinger M, Varga B, Guterres SB, Zellner M. Platelets, a reliable source for peripheral Alzheimer's disease biomarkers? *Acta Neuropathol Commun*. (2014) 2:65. doi: 10.1186/2051-5960-2-65
154. Krige D, Carroll MT, Cooper JM, Marsden CD, Schapira AHV. Platelet mitochondria function in Parkinson's disease. *Ann Neurol*. (1992) 32:782–8. doi: 10.1002/ana.410320612
155. Catricala S, Torti M, Ricevuti G. Alzheimer disease and platelets: how's that relevant. *Immun Ageing*. (2012) 9:20. doi: 10.1186/1742-4933-9-20
156. Talib LL, Joaquim HP, Forlenza OV. Platelet biomarkers in Alzheimer's disease. *World J Psychiatry*. (2012) 2:95–101. doi: 10.5498/wjp.v2.i6.95
157. Behari M, Shrivastava M. Role of platelets in neurodegenerative diseases: a universal pathophysiology. *Int J Neurosci*. (2013) 123:287–99. doi: 10.3109/00207454.2012.751534
158. Bush AI, Martins RN, Rumble B, Moir R, Fuller S, Milward E, et al. The amyloid precursor protein of Alzheimer's disease is released by human platelets. *J Biol Chem*. (1990) 265:15977–83.
159. Valla J, Berndt JD, Gonzalez-Lima F. Energy hypometabolism in posterior cingulate cortex of Alzheimer's patients: superficial laminar cytochrome oxidase associated with disease duration. *J Neurosci*. (2001) 21:4923–30. doi: 10.1523/JNEUROSCI.21-13-04923.2001
160. Swerdlow RH, Parks JK, Cassarino DS, Maguire DJ, Maguire RS, Bennett JP, et al. Cybrids in Alzheimer's disease: a cellular model of the disease? *Neurology*. (1997) 49:918. doi: 10.1212/WNL.49.4.918
161. Winklhofer KE, Haass C. Mitochondrial dysfunction in Parkinson's disease. *Biochim Biophys Acta*. (2010) 1802:29–44. doi: 10.1016/j.bbdis.2009.08.013
162. Benecke R, Strümper P, Weiss H. Electron transfer complexes I and IV of platelets are abnormal in Parkinson's disease but normal in Parkinson-plus syndromes. *Brain*. (1993) 116:1451–63. doi: 10.1093/brain/116.6.1451
163. Haas RH, Nasirian F, Nakano K, Ward D, Pay M, Hill R, et al. Low platelet mitochondrial complex I and complex II/III activity in early untreated parkinson's disease. *Ann Neurol*. (1995) 37:714–22. doi: 10.1002/ana.410370604
164. Shults CW. Therapeutic role of coenzyme Q10 in Parkinson's disease. *Pharmacol Therap*. (2005) 107:120–30. doi: 10.1016/j.pharmthera.2005.02.002
165. Kingsbury AE, David Mardsen C, Foster OJF. DNA fragmentation in human substantia nigra: Apoptosis or perimortem effect? *Mov Disord*. (1998) 13:877–84. doi: 10.1002/mds.870130604
166. Andersen JK. Does neuronal loss in Parkinson's disease involve programmed cell death? *BioEssays*. (2001) 23:640–6. doi: 10.1002/bies.1089
167. Bortolato M, Chen K, Shih JC. Monoamine oxidase inactivation: from pathophysiology to therapeutics. *Adv Drug Deliv Rev*. (2008) 60:1527–33. doi: 10.1016/j.addr.2008.06.002
168. Nagatsu T, Sawada M. Molecular mechanism of the relation of monoamine oxidase B and its inhibitors to Parkinson's disease: possible implications of glial cells. *J Neural Transm*. (2006) 113:53–65. doi: 10.1007/978-3-211-33328-0_7
169. Dias V, Junn E, Mouradian MM. The role of oxidative stress in Parkinson's disease. *J Parkinsons Dis*. (2013) 3:461–91. doi: 10.3233/JPD-130230
170. Adolfsson R, Gottfries CG, Orelund L, Wiberg Å, Winblad B. Increased activity of brain and platelet monoamine oxidase in dementia of Alzheimer type. *Life Sci*. (1980) 27:1029–34. doi: 10.1016/0024-3205(80)90025-9
171. Cesura AM, Pletscher A. The new generation of monoamine oxidase inhibitors. In: Mitsuhashi S, Kojima T, Nakanishi N, Fujimoto T, Goto O, Miyusaki S, et al. editors. *Progress in Drug Research/Fortschritte der Arzneimittelforschung/Progrès des recherches pharmaceutiques*. Basel: Birkhäuser Basel (1992). p. 171–297.
172. Fuentes E, Araya-Maturana R, Urra FA. Regulation of mitochondrial function as a promising target in platelet activation-related diseases. *Free Radic Biol Med*. (2019) 136:172–182. doi: 10.1016/j.freeradbiomed.2019.01.007

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Melchinger, Jain, Tyagi and Hwa. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Stratified Approaches to Antiplatelet Therapies Based on Platelet Reactivity Testing

Małgorzata Ostrowska¹, Jacek Kubica¹, Piotr Adamski¹, Aldona Kubica², Ceren Eyileten³, Marek Postula³, Aurel Toma⁴, Christian Hengstenberg⁴ and Jolanta M. Siller-Matula^{3,4*}

¹ Department of Cardiology and Internal Medicine, Collegium Medicum, Nicolaus Copernicus University, Bydgoszcz, Poland, ² Department of Health Promotion, Collegium Medicum, Nicolaus Copernicus University, Bydgoszcz, Poland, ³ Department of Experimental and Clinical Pharmacology, Centre for Preclinical Research and Technology (CEPT), Medical University of Warsaw, Warsaw, Poland, ⁴ Department of Cardiology, Medical University of Vienna, Vienna, Austria

OPEN ACCESS

Edited by:

Marie Lordkipanidzé,
Université de Montréal, Canada

Reviewed by:

Mark Thomas,
University of Birmingham,
United Kingdom
Marie-Christine Alessi,
Aix Marseille Université, France

*Correspondence:

Jolanta M. Siller-Matula
jolanta.siller-matula@
meduniwien.ac.at

Specialty section:

This article was submitted to
Atherosclerosis and Vascular
Medicine,
a section of the journal
Frontiers in Cardiovascular Medicine

Received: 07 September 2019

Accepted: 14 November 2019

Published: 03 December 2019

Citation:

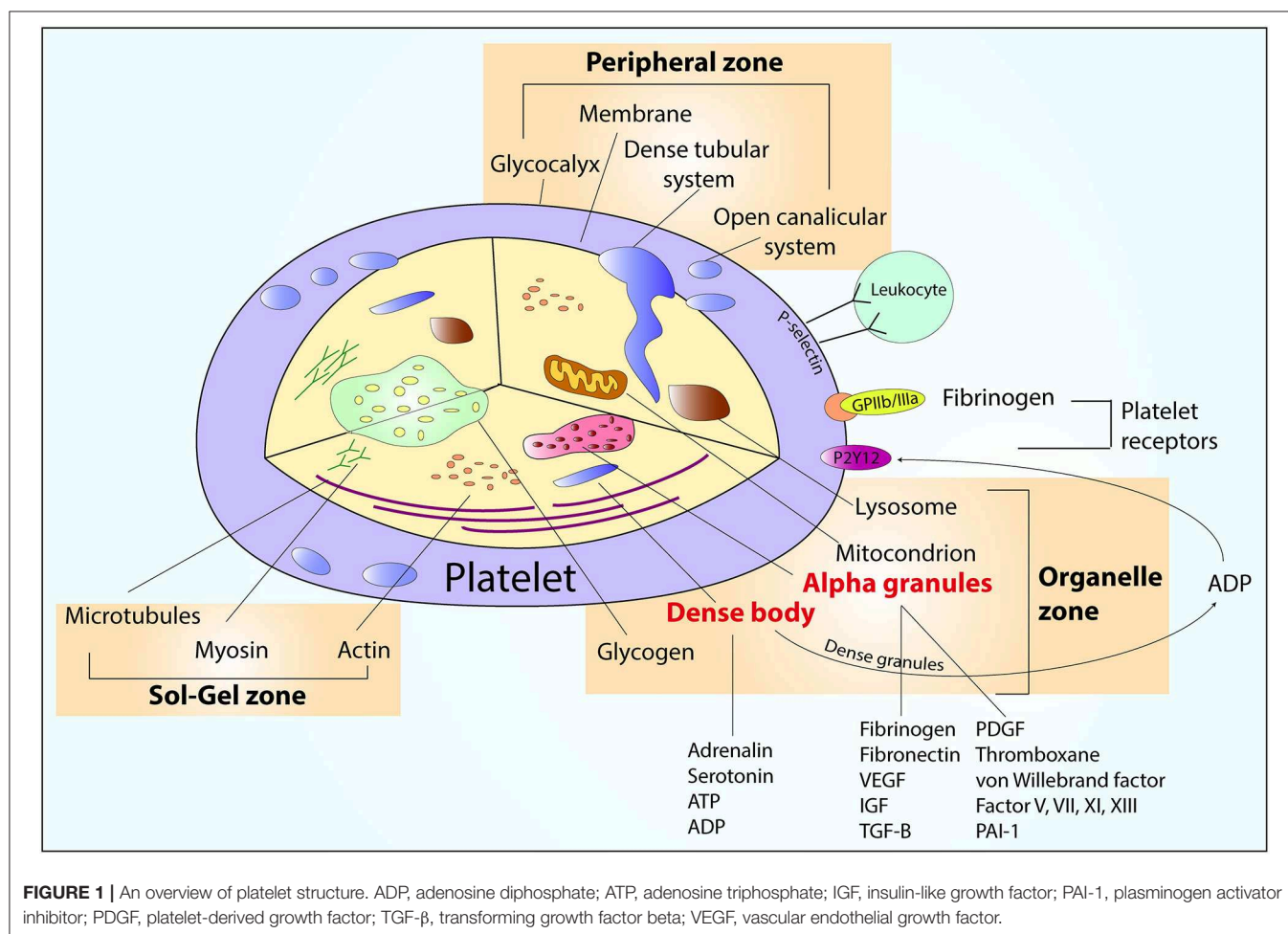
Ostrowska M, Kubica J, Adamski P,
Kubica A, Eyileten C, Postula M,
Toma A, Hengstenberg C and
Siller-Matula JM (2019) Stratified
Approaches to Antiplatelet Therapies
Based on Platelet Reactivity Testing.
Front. Cardiovasc. Med. 6:176.
doi: 10.3389/fcvm.2019.00176

Antiplatelet therapy with P2Y₁₂ receptor inhibitors (clopidogrel, prasugrel, ticagrelor, cangrelor) is a cornerstone of medical therapy after percutaneous coronary interventions. Significant prevalence of high on-treatment platelet reactivity (HTPR) on clopidogrel treatment led to introduction of more potent P2Y₁₂ inhibitors: prasugrel (a third generation thienopyridine), ticagrelor, and cangrelor (cyclopentyl-triazolo-pyrimidines). Nevertheless, more potent platelet inhibition and resulting low on-treatment platelet reactivity (LTPR) has led to increased risk of major bleeding events. These limitations resulted in a need for an individualized antiplatelet therapy approach. This review discusses the current role and future perspectives of diagnostic tools such as platelet function testing to optimize antiplatelet therapy with a focus on deescalating therapies to reduce bleeding risks.

Keywords: P2Y₁₂ inhibitors, antiplatelet therapy, ACS, HPR, LPR, precision medicine

ROLE OF PLATELETS IN ARTERIAL THROMBOSIS

Myocardial infarction (MI) is generally a consequence of unstable atherosclerotic plaque rupture or erosion, caused by endothelial damage (1). In patients with ST-segment elevation myocardial infarction (STEMI), the rupture of atherosclerotic plaque is associated with exposure of the lipid core and subendothelial collagen fibers, both of which initiate activation of platelets, and thrombus formation that usually lead to acute obstruction of the coronary artery (1). On the other hand, in patients with non-ST segment elevation myocardial infarction (NSTEMI) the MI is usually caused by a clot formed on unstable coronary plaque, which does not produce complete obstruction of the artery lumen (1). Excessive activation and aggregation of platelets play a pivotal role in the pathogenesis of both types of MI (2). Platelets are the smallest, anuclear morphotic elements of the blood, which derive from megakaryocytes and live 7–10 days. Their surface is covered with multiple receptors and their organelle include factors promoting the clot formation (**Figure 1**). Platelets are responsible for the primary hemostasis, that consists of platelet adhesion, secretion, and aggregation (2). Vascular injury and exposure of the von Willebrand factor initiates platelets adhesion and activation, as a result the surface integrins $\alpha_2\beta_1$ and $\alpha_{2b}\beta_3$ (also called GP IIb/IIIa) gain high affinity to collagen and fibrinogen (3, 4). One of the most powerful modulators of platelet function is ADP, the main agonist of platelet P2Y₁ and P2Y₁₂ receptors (5). Stimulation of the P2Y₁ receptor results in phospholipase C activation (6, 7), while stimulation of the P2Y₁₂



receptor deactivates adenylyl cyclase resulting in termination of cyclic adenosine monophosphate production, translating into lack of inhibition of the phospholipase C (8). Stimulation of both P2Y receptors leads to hydrolysis of phosphatidylinositol by the activated phospholipase C to triphosphate inositol and diacylglycerol (3). Triphosphate inositol is responsible for opening of the membrane calcium channels and influx of calcium, which facilitates cytoskeleton modification and change of shape to spherical, transport of α granules and dense bodies to the central part and release of their components (6). The process of aggregation is based on bridging of two neighboring platelets with fibrinogen, via activated GP IIb/IIIa platelet membrane receptors, allowing formation of the primary clot (9). Further platelet activation initiates next phase of platelet aggregation associated with cyclooxygenase-1 mediated production of thromboxane A_2 from arachidonic acid. Thromboxane A_2 further promotes platelet aggregation and vasoconstriction (10). The next stage is secondary hemostasis, it is initiated by platelet products and mediated by coagulation factors (2). Undesired platelet activation, leading to clot formation inside the coronary arteries explains the pathomechanism of MI and stent thrombosis—a possibly lethal complication of percutaneous coronary intervention (PCI) with stent implantation (1, 11).

ANTIPLATELET AGENTS

Current armamentarium of antiplatelet agents includes four groups of drugs and is summarized in Table 1.

Aspirin

Aspirin represents the cornerstone of antithrombotic therapy. Aspirin is an irreversible antagonist of the cyclooxygenase-1, that blocks the production of the thromboxane A_2 —one of the most powerful promoters of platelet aggregation (13). In patients with acute coronary syndrome (ACS), an initial oral loading dose of 150–300 mg of non-enteric coated formulation is recommended, followed by 75–100 mg once daily regimen (12, 14, 15). Life-long maintenance therapy with acetylsalicylic acid is indicated in all patients in secondary prevention of coronary artery disease (16). Although arachidonic acid induced platelet aggregation varies according to several variables (as age or sex), no routine monitoring of its treatment is required (15, 17). In case of aspirin intolerance, chronic clopidogrel therapy is recommended as an alternative (16).

TABLE 1 | Groups of antiplatelet agents.

Group of antiplatelet agents	Receptor agonist	Drug/s	Route of administration	Mode of platelet inhibition	Current place in ACS treatment in ESC guidelines (12)	
					Class of recommendation	Level of evidence
Cyclooxygenase-1 antagonist	-	Acetylsalicylic acid	Oral/intravenous	Irreversible	I	A
P2Y ₁₂ antagonists	ADP	Clopidogrel	Oral	Irreversible	I	A/B*
		Prasugrel				
		Ticagrelor	Intravenous	Reversible	IIb	A
		Cangrelor				
GP IIb/IIIa antagonists	Fibrinogen	Abciximab	Intravenous	Noncompetitive	IIa	C
		Tirofiban				
		Eptifibatide	Intravenous	Competitive	IIa	C
		Eptifibatide				
PAR-1 antagonist	Thrombin	Vorapaxar	Oral	Competitive	-	-

*Level of evidence A in ST-elevation acute myocardial infarction, level of evidence B in non-ST-elevation acute coronary syndromes. ACS, acute coronary syndrome; ADP, adenosine diphosphate; ESC, European Society of Cardiology; PAR-1, protease-activated receptor 1.

P2Y₁₂ Receptor Antagonists

Dual antiplatelet therapy, composed of an aspirin and an antagonist of the platelet P2Y₁₂ receptor, is a foundation of modern ACS therapy. There are three types of purinergic receptors: P2X₁, P2Y₁, and P2Y₁₂ on the platelet surface, but only the P2Y₁₂ has become a target for antithrombotic therapies that is used in everyday clinical practice (17). ADP is an agonist of the P2Y₁₂ receptors. It activates the P2Y₁₂ receptor via stimulation of the G_{αi2} protein, which deactivates adenylyl cyclase translating into decreased cyclic adenosine monophosphate synthesis, which is responsible for phospholipase C inhibition, thus leading to platelet aggregation (8). Stimulation of the G_{αi2} protein by ADP activates also the GP IIb/IIIa receptor leading to induction of fibrinogen bridging, and initiation of the secretion of platelet derived products (18).

Contemporary armamentarium of the P2Y₁₂ receptor inhibitors includes two thienopyridines: clopidogrel and prasugrel, and two non-thienopyridine drugs: ticagrelor and cangrelor (19). Thienopyridines are oral pro-drugs demanding hepatic activation via cytochrome P450, their metabolites irreversibly bind to the P2Y₁₂ receptors for 7–10 days, which may impact the time to surgery after cessation (20). Whereas, both non-thienopyridines are potent, reversible and direct acting drugs, characterized by different route of administration—ticagrelor is administered orally, while cangrelor intravenously. Beside antiplatelet action, P2Y₁₂ receptor inhibitors seem to exert a whole palette of pleiotropic effects including: increased adenosine plasma concentration in ticagrelor treated patients leading to increase in adenosine-related coronary blood flow, cardioprotection, promotion of the release of anticoagulative factors (21). These off-platelet effects are also associated with dyspnea and bradycardia. Additional off-target effects include improvement in peripheral arterial function and endothelial function, plaque stabilization and post-conditioning mimetic effect with cangrelor observed in animal models (22). Basic

characteristic of all four P2Y₁₂ receptor antagonists is presented in **Table 2**.

European Society of Cardiology (ESC) guidelines clearly recommend in patients with ACS the use of more potent antiplatelet agents like ticagrelor and prasugrel, with loading doses (ticagrelor 180 mg, prasugrel 60 mg), followed by maintenance doses (ticagrelor 90 mg twice daily, prasugrel 10 mg once daily), limiting the use of clopidogrel to situations when newer agents are not available or contraindicated (600 mg loading dose, followed by 75 mg maintenance dose) (12, 14, 15). Ticagrelor can also be used in STEMI patients after fibrinolysis (23). Moreover, guidelines advise to consider the use of cangrelor in P2Y₁₂ receptor inhibitor naïve patients and to continue the infusion for 2 h or until the end of PCI (12). Dual antithrombotic therapy post ACS shall be continued for up to 12 months, unless there are contraindications such as excessive risk of bleeding. Noteworthy, in high ischemic-risk patients, who have not suffered from bleeding, dual antiplatelet therapy with ticagrelor in reduced dose of 60 mg twice daily may be maintained beyond 1 year for up to 3 years (12). In patients with chronic coronary syndromes undergoing PCI, dual antithrombotic regimen composed of aspirin and clopidogrel remains the well-known standard of care, while ticagrelor or prasugrel use is limited to high-risk situations like previous stent thrombosis (16). In general, after elective stent implantation P2Y₁₂ inhibitor should be continued for up to 6 months (12). Of note, in high bleeding risk patients with chronic coronary syndrome, dual antiplatelet therapy can be shortened to 1 month, while in those post ACS with high ischemic and low bleeding risk it may prolonged for up to 30 months (12, 16). A special population of interest represents patients who require combination of antiplatelet therapy and anticoagulation. Recently, it has been shown that among patients with atrial fibrillation and chronic coronary syndrome (>1-year after the index event), the addition of antiplatelet drugs, as a monotherapy or dual antiplatelet therapy, does not provide added protection against coronary events, but increases the risk of major bleeding (24).

TABLE 2 | Characteristics of P2Y₁₂ receptor antagonists.

	Clopidogrel	Prasugrel	Ticagrelor	Cangrelor
Chemical group	Thienopyridine	Thienopyridine	Cyclopentyl -triazolo-pyrimidine	Cyclopentyl -triazolo-pyrimidine
Prodrug (requiring hepatic activation)	Yes	Yes	No	No
Loading dose	300/600 mg	60 mg	180 mg	30 µg/kg bolus
Maintenance dose	75 mg	10 mg	2 × 90 mg	4 µg/kg/min infusion
Onset of action	2–4 h	30 min	30 min	2 min
Duration of antiplatelet effect	3–10 days	5–10 days	3–4 days	1–2 h
Excretion route	Renal and biliary	Renal and feces	Biliary	Renal and feces
Recommended stop of treatment before surgery	5 days	7 days	3 days	1 h

Glycoprotein IIb/IIIa Receptor Antagonists

Another group of antithrombotic agents are inhibitors of the GP IIb/IIIa receptors. These glycoproteins belong to adhesion molecules and are the most abundant platelet surface receptors. Their role in platelet aggregation is pivotal, after activation, and change of conformation they gain high affinity for fibrinogen, von Willebrand factor and prothrombin (25). Fibrinogen molecule has binding sites on both sides allowing bridging between two neighboring platelets, thus initiating aggregation. In everyday clinical practice we use three intravenous GP IIb/IIIa inhibitors: abciximab, eptifibatide, and tirofiban. Beside potent antiplatelet effect they can exert some off-target actions, mainly anti-inflammatory (26). The use of GP IIb/IIIa antagonists should be reserved for bail-out situations, if there is evidence of no-reflow or a thrombotic complication (class of recommendation IIa, level of evidence C) (12). Their use in patients in whom coronary anatomy is not known is not recommended (class of recommendation III, level of evidence A) (12).

ASSESSMENT OF PLATELET INHIBITION UNDER ANTIPLATELET THERAPIES

Platelet Function Testing

Many different methods to assess platelet function exist, beginning with the historic golden standard—light transmission aggregometry, that measures the difference between light transmission through platelet rich plasma and platelet poor plasma, assessment of platelet aggregation on fibrinogen-coated microparticles (VerifyNow assay) or metal electrodes (Multiplate analyzer), measurement of the VASP protein phosphorylation (VASP assay), assessment of platelet aggregation *in vitro* in conditions similar to physiological blood flow (PFA-100, PFA-200, Innovance P2Y₁₂, IMPACT-R), assessment of the clot strength (thromboelastography), measurement of the thrombocytes number before and after the addition of an agonist (Plateletworks) (27–29). It has to be acknowledged that due to great differences in assessment of platelet reactivity between available tests, a diagnosis of either HTPR or LTPR based on one method can be unconfirmed with the use

of a different method. According to both American and European groups of experts there are three recommended platelet function tests: the VerifyNow assay, the Multiplate analyzer, and the VASP assay for clinical guidance (30, 31). In the HARMONIC study platelet reactivity values assessed with all three recommended platelet function tests in MI patients treated with ticagrelor correlated well with each other, however a significantly higher correlation was demonstrated between the VerifyNow and Multiplate tests than in other assay combinations (32). Interestingly, emerging concepts as platelet redox assessment (intracellular concentration of reactive oxygen species, activity of antioxidant enzymes, reduced/oxidized glutathione ratio, level of lipid peroxidation, Cu/Zn ratio, and molecular oxygen consumption) might be potentially useful to establish the platelet-related etiological factors in different disorders and to evaluate the antiplatelet therapies (33).

High On-Treatment Platelet Reactivity (HTPR)

Numerous studies have shown that up to 40% of patients exhibit HTPR under clopidogrel treatment (34–42). There are many potential causes of this phenomenon including clinical variables such as ACS at admission, diabetes mellitus, renal failure, drug-drug interactions, non-adherence to therapy, genetic polymorphism of genes coding cytochrome P450 enzymes (crucial in clopidogrel bioactivation) or glycoprotein P (responsible for clopidogrel absorption in intestines) (37, 43–48). Recently, an association between the circulating proprotein convertase subtilisin/kexin type 9 (PCSK9) levels, HTPR and ischemic events in ACS patients undergoing PCI were described (49). There is a clear evidence showing that HTPR on clopidogrel is a significant risk factor for atherothrombotic events, including MI, stent thrombosis, cardiovascular death and cerebrovascular events (40, 50–52). There are some therapeutic options to overcome HTPR on clopidogrel. HTPR may also affect patients treated with newer, more potent antithrombotic agents such as prasugrel or ticagrelor, mainly within the first hours post loading dose in ACS patients undergoing PCI, when sufficient antiplatelet blockade is particularly desired (52–56). However,

in a recently published systematic review and meta-analysis, early (>2 h pre-PCI) vs. late (<2 h pre-PCI or post-PCI) administration of loading doses of potent antiplatelet agents did not improve ischemic outcomes in more than 60,000 patients, questioning the importance of early loading (57). In contrast, early clopidogrel loading in ACS or STEMI patients reduced the risk of adverse events (57). The prevalence of HTPR in patients treated with ticagrelor was significantly lower as compared with those receiving prasugrel in a meta-analysis by Lemesle et al. (58). It was previously documented that age, gender, food, preloading with clopidogrel or genetic polymorphisms do not affect ticagrelor metabolism or its antiplatelet effect (59–61). Diversely, morphine which used to be a golden standard of care for all patients presenting with acute MI, was found to attenuate ticagrelor bioavailability and its antiplatelet action, mainly due to vomiting and decelerating the intestinal passage and absorption of ticagrelor (53, 62). There are few disputed strategies to overcome the morphine-ticagrelor interaction, either by crushing ticagrelor tablets, giving other analgesic, co-administering naloxone or metoclopramide (62–64). In a prospective, observational PINPOINT trial it has been found that ticagrelor concentration was reduced and antiplatelet response was delayed in the initial hours of treatment in STEMI patients as compared with NSTEMI patients (65). In a subsequent analysis, it has been reported that the main determinants of HTPR at 1 and 2 h after ticagrelor loading dose are presence of STEMI and morphine co-administration (66). Furthermore, the presence of STEMI and diabetes mellitus were found to be associated with impaired metabolism of ticagrelor within first 6 h post ticagrelor loading dose in ACS patients (67). It has been recently published, that bioavailability of ticagrelor in MI patients managed with mild therapeutic hypothermia after out-of-hospital cardiac arrest is significantly decreased, thus increasing the risk of stent thrombosis, a possibly lethal complication, which is not uncommon in this specific subset of patients (68, 69). The main reasons of insufficient antiplatelet effect of the P2Y₁₂ inhibitors in out-of-hospital cardiac arrest survivors treated with mild therapeutic hypothermia are probably impaired gastrointestinal absorption and altered cytochrome activity causing a delay in drug metabolism (69–71). The temporary use of ticagrelor may be a solution to overcome HTPR while oral antiplatelet agents start to work in resuscitated patients undergoing mild therapeutic hypothermia (72). A single dose of intravenous morphine in STEMI patients was associated with a delay in the onset of prasugrel action (73), 65% of critically ill patients display HTPR on prasugrel, mainly due to poor absorption from gastrointestinal tract, as well as increased platelet reactivity induced by generalized inflammation (74).

Conclusion of the Chapter

- i) HTPR is a significant and modifiable risk factor for cardiac ischemic events and it is present frequently in clopidogrel treated patients.
- ii) Patients treated with prasugrel and ticagrelor can display HTPR mainly in the acute phase of treatment, which can be in part related to opioid use.
- iii) The routine use of platelet function testing to detect HTPR and undertake action is not recommended by the ESC guidelines. Nevertheless, HTPR should be taken into account, if de-escalation is undertaken from potent P2Y₁₂ inhibitors to clopidogrel (12).
- iv) HTPR can be detected by a variety of platelet reactivity testing.

Low On-Treatment Platelet Reactivity (LTPR)

With the introduction of more potent antiplatelet agents the problem of LTPR associated with elevated bleeding risk became a major concern. In the TRITON-TIMI 38 trial the use of prasugrel as compared with clopidogrel was associated with significant increase of non-coronary artery bypass grafting related major bleeding, as well as life-threatening bleeding and bleeding leading to death according to the Thrombolysis in Myocardial Infarction (TIMI) criteria (75). While in the PLATO trial the administration of ticagrelor as compared with clopidogrel carried similar risk of major bleeding according to the PLATO trial criteria. However non-coronary artery bypass grafting related major bleeding and both major and minor bleeding occurred more frequently in the ticagrelor group (76).

Data on LTPR and bleeding on clopidogrel therapy comes from few small studies adapting different bleeding scales. Another limitation is low amount of serious bleeding events in those trials and predominant inclusion of low risk stable patients. First study reporting a link between LTPR and bleeding was conducted in 597 ACS patients treated with clopidogrel (77). In a 1-month observation period there were 16 bleeding episodes (5 serious and 11 small). Patients suffering from bleeding events were characterized by stronger platelet inhibition measured with the light transmission aggregometry (a previous golden standard) or the VASP assay. In a study by Sibbing et al. LTPR on clopidogrel (the cut-off value was based on the ROC curve analysis accounting 18.8 U according to the Multiplate analyzer) affected 39% of 2,533 patients with stable coronary artery disease undergoing PCI. Furthermore, those with LTPR had significantly higher risk of major in-hospital bleeding according to the TIMI criteria (78). Another study including 246 stable coronary artery disease patients receiving clopidogrel showed a relationship between >50% platelet inhibition measured with the light transmission aggregometry and occurrence of any bleeding event assessed with the use of very liberal bleeding scale called the BleedScore: 88% of all included bleeding events were superficial bleeding (79). Importantly, older age and female sex are important predictors of LTPR and of bleeding odds (80, 81).

There are few prospective studies providing a head-to-head comparison of platelet reactivity and bleeding risk in patients on prasugrel vs. ticagrelor (55, 82, 83). The first randomized trial included only 96 ACS patients treated with PCI (82). The half of them received ticagrelor, the other half received prasugrel, and platelet reactivity measurements were performed after 1 month. LTPR was defined as PRI ≤ 20% in the VASP assay and occurred in 58% of ticagrelor recipients and 33% of prasugrel recipients with a lack of relationship between LTPR

and bleeding events. Another prospective registry including 512 patients with ACS treated with PCI (278 on ticagrelor, 234 on prasugrel) has shown that patients treated with ticagrelor were characterized by lower platelet reactivity assessed with the use of the VerifyNow device at 1 month post PCI, as compared with prasugrel (33.3 Platelet Reactivity Units (PRU) vs. 84.6 PRU; $p < 0.001$) (83). Grade 1 Bleeding Academic Research Consortium (BARC) bleeding events were more frequent in the ticagrelor arm, while grade ≥ 2 BARC bleeding events rate was similar irrespective of antiplatelet agent used. Another observational study assessing the relationship between occurrence of clinical events and platelet reactivity in 226 ACS patients (105 on ticagrelor and 121 on prasugrel) (55) indicated that bleeding episodes occurred in patients with platelet reactivity values ≤ 23 U as assessed with the Multiplate Analyzer.

Conclusion of the Chapter

- i) Due to the widespread use of potent P2Y₁₂ inhibitors, the LTPR phenotype is frequent.
- ii) LTPR is a well-documented risk factor for bleeding complications. Platelet function guided dose-adjustment of potent P2Y₁₂ inhibitors may be a potential solution in patients who are presenting with a bleeding event (12).

Therapeutic Window Strategy

Based on the growing body of evidence showing an association between HTPR and ischemic events, and LTPR with bleeding events, the therapeutic window hypothesis was developed. It suggests that patients with platelet reactivity values within the middle range achieve the best net clinical benefit (28, 84). According to the European group of experts, the cut-off values for HTPR are as following: the VerifyNow assay >208 PRU, the Multiplate analyzer >46 Units (U) and the VASP assay $>50\%$ Platelet Reactivity Index (PRI) (31). The cut-off values for LTPR are as following: the VerifyNow assay <95 PRU, the Multiplate analyzer <19 U and the VASP assay $<16\%$ PRI.

Conclusion of the Chapter

- i) The therapeutic window strategy to guide antiplatelet therapy might be an attractive strategy to improve patients net clinical benefit in terms of precision medicine.
- ii) Clinical randomized trials aiming to answer that question are missing yet.

STUDIES INVESTIGATING INDIVIDUALIZED ANTIPLATELET TREATMENT TO OVERCOME HTPR (TABLE 3)

First small randomized trials comparing individualized antiplatelet therapy vs. standard of care antithrombotic treatment showed favorable results of antithrombotic adjusted therapy with either higher clopidogrel dose or addition of GP IIb/IIIa antagonist (104, 106, 107, 110, 113, 114). First large randomized trial that has brought huge disappointment to advocates of

the individualized approach was the Gauging Responsiveness with a VerifyNow assay, Impact on Thrombosis and Safety (GRAVITAS) trial showing no benefit of administration of higher clopidogrel dose vs. standard clopidogrel dosing in 2200 low-to-moderate cardiovascular risk patients undergoing PCI with HTPR on-clopidogrel when it comes to death from cardiovascular causes, MI or stent thrombosis (hazard ratio [HR] 1.01; 95% confidence interval [CI] 0.58–1.76; $P = 0.97$) in a 6 month follow-up (96). The next negative, similar size study—The Assessment by a Double Randomization of a Conventional Antiplatelet Strategy for Drug-Eluting Stent Implantation and of Treatment Interruption vs. Continuation 1 Year after Stenting (ARCTIC) trial demonstrated that the addition of acetylsalicylic acid, clopidogrel or switch to prasugrel as compared with conventional approach did not show any significant differences in the occurrence of the primary end point composed of death from cardiovascular causes, MI, stent thrombosis, urgent revascularization or stroke (HR 1.13; 95% CI 0.98–1.29; $P = 0.10$) 1 year after stent implantation in a group of 2,440 low-to-moderate cardiovascular risk patients (95). The third large trial that was prematurely ended and almost entombed the individualized approach was The Testing Platelet Reactivity in Patients Undergoing Elective Stent Placement on Clopidogrel to Guide Alternative Therapy with Prasugrel (TRIGGER-PCI). Trial included only 423 low cardiovascular risk patients with stable coronary artery disease undergoing elective PCI, and the strategy of switch from clopidogrel to prasugrel in those with HTPR on-clopidogrel did not bring reduction in the primary endpoint composed of death from cardiovascular causes and MI with concomitant increase in TIMI major bleeding at 6 months (94).

More promising results on the conception of individualized approach were shown by some prospective registries (89, 90, 92). The MADONNA registry, which included 798 patients (more than one third of them had MI), has shown that the non-guided group had significantly higher risk of stent thrombosis (odds ratio [OR] 7.9; 95% CI 1.08–69.2; $p = 0.048$) at 30 days as compared with individualized therapy group (92). In the IDEAL-PCI registry the main strategy to overcome HTPR was a switch to a more potent antithrombotic agent (mainly prasugrel). At 30 days, there was only one definite stent thrombosis in the non-guided group (90). In the PECS registry, including only ACS patients, those with HTPR received either higher clopidogrel dose or prasugrel, while those below HTPR threshold received conventional clopidogrel therapy (89). The primary endpoint composed of all-cause death, MI, stent thrombosis or stroke at 1 year occurred more frequently in patients treated with higher clopidogrel doses than in conventional treatment group (HR 2.27; 95% CI 1.45–3.55; $p < 0.0001$), while the risk of ischemic events in prasugrel recipients was similar to conventional treatment arm (HR 0.90; 95% CI 0.44–1.81; $p = 0.76$). Worth adding is the fact that bleeding events (3/5 according to the BARC scale) were also more frequent in the higher clopidogrel dose group vs. conventional group (HR 2.09; 95% CI 1.05–4.17; $p = 0.04$), while in prasugrel recipients it was similar to conventional treatment arm (HR: 1.90; 95% CI 1.17–3.08; $p = 0.01$).

TABLE 3 | Studies investigating individualized antiplatelet treatment.

Study author/acronym	Population	n	Follow-up	Outcome	Method	Cut-off value	Study type
ELECTRA (85)	MI	52	14 days	Level of platelet inhibition	VASP assay and MEA	16% for LTPR, 50% for HTPR; 19 U for LTPR 46 U for HTPR	CRT: ticagrelor standard maintenance dose 2 × 90 mg vs. ticagrelor reduced maintenance dose 2 × 60 mg in patients 30 days post MI
TOPIC (86)	PCI for ACS	646	1 year	MACE, BARC ≥ 2 bleeding	n/a	n/a	CRT: continuation of ticagrelor/prasugrel vs. switch to clopidogrel in patients 1 month post ACS
TROPICAL-ACS (87)	PCI for ACS	2,610	1 year	Net clinical benefit: MACE or BARC ≥ 2 bleeding	MEA	46 U	CRT: guided de-escalation: 7 days prasugrel 5 or 10 mg + 7 days clopidogrel 75 mg after 14 days if HTPR prasugrel 5 or 10 mg, if no HTPR clopidogrel 75 mg vs. non-guided prasugrel 5 or 10 mg
ANTARCTIC (88)	PCI for ACS	877	1 year	ST, MACE, BARC major bleeding	VerifyNow	208PRU for HTPR 85 for LTPR	CRT: guided: in case of HTPR on prasugrel 5 mg switch to prasugrel 10 mg, in case of LTPR on prasugrel 5 mg switch to clopidogrel 75 mg vs. non-guided prasugrel 5 mg
PECS REGISTRY (89)	ACS+PCI	741	1 year	ST, MACE, BARC major bleeding	MEA	46 U	Observational: 600/150 mg clopidogrel vs. prasugrel in patients with HTPR
IDEAL-PCI (90)	PCI	1,008	1 month	ST	MEA	50 U	Observational: non-HTPR on clopidogrel arm vs. HTPR on clopidogrel individualized approach (reloading with clopidogrel, ticagrelor, or prasugrel; re-testing)
ISAR-HPR (91)	PCI for CAD or ACS	999	1 month	ST, MACE, TIMI bleeding	MEA	468Auxmin	Retrospective HTPR on clopidogrel arm vs. prospective HTPR on clopidogrel individualized approach arm (reloading with clopidogrel, switch to prasugrel, re-testing)
MADONNA (92)	PCI	798	1 month	ST, MACE, TIMI major bleeding	MEA	50 U	Non-randomized, controlled: non-guided vs. guided group (up to 4 loadings with 600 mg clopidogrel or 1 loading with prasugrel in patients with HTPR)
Kozinski et al. (93)	ACS+PCI	71	1 month	Level of platelet inhibition	VASP assay	50%	Parallel-group, open-label study: patients with HTPR were assigned to prasugrel (30 mg loading dose, 10 mg maintenance dose) or clopidogrel (150 mg maintenance dose for 6 days and thereafter 75 mg maintenance dose)
TRIGGER-PCI (94)	Elective PCI	423	6 months	MACE, bleeding	VerifyNow	208PRU	CRT: prasugrel (loading of 60 mg and maintenance 10 mg) vs. clopidogrel (maintenance 75 mg) in patients with HTPR
ARCTIC (95)	PCI with DES	2,440	1 year	MACE	VerifyNow	235PRU	CRT: guided: clopidogrel (600 mg reloading and 75 mg or 150 mg maintenance) or prasugrel (60 mg loading and 10 mg maintenance) or GP IIb/IIIa inhibitors vs. non-guided: clopidogrel (maintenance 75 mg) in patients with HTPR
GRAVITAS (96)	PCI for CAD or NSTEMI-ACS	2,214	6 months	MACE	VerifyNow	230PRU	CRT: 300/75 mg clopidogrel vs. 600/75 mg clopidogrel in patients with HTPR
Alexopoulos et al. (97)	CAD with clopidogrel treatment	31	1 month	Level of platelet inhibition	VerifyNow	235PRU	Randomized, crossover: 10 m prasugrel vs. 150 mg clopidogrel in patients with htp
Alexopolus et al. (98)	HD with clopidogrel treatment	21	1 month	Level of platelet inhibition	VerifyNow	235PRU	Randomized, crossover: 10 m prasugrel vs. 150 mg clopidogrel c

(Continued)

TABLE 3 | Continued

Study author/acronym	Population	n	Follow-up	Outcome	Method	Cut-off value	Study type
Capranzano et al. (99)	Clopidogrel treatment + age > 75	100		Level of platelet inhibition	VerifyNow	230PRU	Observational: prasugrel in patients with htp
Ferreiro et al. (100)	DMII	30		Level of platelet inhibition	VASP assay	50%	Observational: cilostazol vs. 150 mg clopidogrel in patients with HTPR
BOCLA Plan (101)	PCI	504		Level of platelet inhibition	IA	5 Ω	Observational: 600/150 mg clopidogrel vs. ticlopidine vs. prasugrel in patients with HTPR
Gurbel et al. (102)	Stable CAD + previous PCI	20	7 days	level of platelet inhibition	LTA	43%	Observational: single dose elinogrel 60 mg in patients with HTPR
RESPOND (103)	Stable CAD + clopidogrel	41	1 month	Level of platelet inhibition	LTA	43%	CRT crossover: ticagrelor 180/90 mg vs. clopidogrel 600/75 mg
Valgimigli et al. (104)	Elective PCI	263	In hospital	MACE	VerifyNow	235PRU	CRT: tirofiban vs. placebo in patients with HTPR
ACCEL-RESISTANCE (105)	PCI	60	1 month	Level of platelet inhibition	LTA	50%	CRT: adjunctive cilostazol vs. 150 mg clopidogrel in patients with HTPR
Bonello et al. (106)	PCI	429	1 month	MACE, ST, bleeding	VASP assay	50%	CRT: guided (repeated loading with clopidogrel 600 mg) vs. non-guided group
Bonello et al. (107)	PCI	162	1 month	MACE	VASP assay	50%	CRT: guided (repeated loading with clopidogrel 600 mg) vs. non-guided group
VASP-02 (108)	Elective PCI	153	1 month	MACE, level of platelet inhibition	VASP assay	69%	Observational: 150 mg clopidogrel in patients with HTPR
Trenk et al. (109)	Elective PCI	117	14 days	Level of platelet inhibition	LTA	14%	Observational: 150 mg clopidogrel vs. control in patients with HTPR
Cuisset et al. (110)	Elective PCI	149	1 month	MACE	LTA	70%	CRT: GP IIb/IIIa antagonists vs. control in patients with HTPR
Matezky et al. (111)	MI	200	10 weeks	Level of platelet inhibition	LTA	80%	Observational: 600/150 mg clopidogrel in patients with HTPR
Neubauer et al. (112)	Elective PCI	161		Level of platelet inhibition	IA	5 Ω	Observational: 600/150 mg clopidogrel vs. ticlopidine in patients with HTPR

ACS, acute coronary syndrome; BARC, Bleeding Academic Research Consortium; CAD, coronary artery disease; CRT, controlled randomized trial; CYP, cytochrome P450; DM, diabetes mellitus; HD, haemodialysis; HTPR, high on-treatment platelet reactivity; IA, impedance aggregometry; LTA, light transmission aggregometry; LTPR, low on-treatment platelet reactivity; MACE, major adverse cardiovascular event; MEA, multiple electrode aggregometry; MI, myocardial infarction; n/a, not applicable; NSTEMI-ACS, non ST-elevation acute coronary syndrome; PCI, percutaneous coronary intervention; PRU, platelet reactivity unit; ST, stent thrombosis; TIMI, thrombolysis in myocardial infarction; U, unit; VASP, vasodilator stimulated phosphoprotein.

After failure of the first randomized trials investigating individualized antithrombotic therapy and some favorable data from registries, long-awaited results of the Platelet function monitoring to adjust antiplatelet therapy in elderly patients stented for an acute coronary syndrome (ANTARCTIC) randomized study were recently published (88). The study was designed for elderly population including patients over 75 years old undergoing PCI for ACS. Participants were divided into two groups. In the monitoring group, patients received prasugrel 5 mg daily with dose or drug adjustment in case of HTPR, while in conventional group patients were treated with prasugrel 5 mg daily. Platelet function was tested with the VerifyNow assay. The cutoff values for HTPR and LTPR were based on the American consensus of experts, accounting ≥ 208 for ischemic events and ≤ 85 for bleeding events (30). The primary endpoint composed of cardiovascular death, myocardial infarction, stroke, stent thrombosis, urgent revascularization, and BARC-defined bleeding complications (types 2, 3, or 5) occurred in 120 (28%) patients in the monitoring group vs. 123 (28%) in the conventional group (HR 1.003, 95% CI 0.78–1.29; $p = 0.98$). Rates of bleeding events did not differ significantly between groups. Drug or dose adjustment based on platelet reactivity measurements did not improve the clinical outcome in a group of elderly patients undergoing PCI for ACS.

Conclusion of the Chapter

- i) Trials on individualized antiplatelet approach had many limitations. These concerns are mainly due to the chosen low cardiovascular risk populations (mostly stable coronary disease patients), use of different cut-off points for HTPR, predominant use of higher clopidogrel doses instead of more potent antiplatelet agents to overcome HTPR, only single switch to other dose or antiplatelet agent, delayed time of randomization (after PCI or even day after PCI) and chosen compounds of the primary endpoint (34).
- ii) Real life data from the registries showed more promising results.

STUDIES INVESTIGATING DE-ESCALATION OF ANTIPLATELET TREATMENT

TROPICAL

The randomized trial Testing Responsiveness to Platelet Inhibition on Chronic Antiplatelet Treatment for Acute Coronary Syndromes (TROPICAL-ACS) assessed guided de-escalation of antiplatelet treatment in patients with MI treated with PCI in 2,610 patients (87). Investigators of the TROPICAL-ACS trial aimed to test safety and efficacy of antithrombotic treatment de-escalation from prasugrel in the acute phase of ACS to clopidogrel in the chronic phase based on platelet reactivity measured with the Multiplate analyzer. 1,304 patients were included to the de-escalation study arm. Participants were treated with prasugrel for a week, and then switched to clopidogrel for a week and after 14

days platelet reactivity assessment was performed resulting in either continuation of clopidogrel therapy or in case of HTPR switch back to prasugrel. In the conventional study arm, 1,306 patients were treated with prasugrel for 12 months. The primary endpoint was the net clinical benefit (cardiovascular death, MI, stroke or bleeding grade 2 or higher according to BARC criteria) and it occurred in 95 patients (7%) in the guided de-escalation group and in 118 patients (9%) in the control group ($p_{\text{non-inferiority}} = 0.0004$; HR 0.81; 95% CI 0.62–1.06; $p_{\text{superiority}} = 0.12$). Despite early de-escalation, there was no increase in the primary endpoint of ischemic events in the de-escalation group (32 patients [3%]) vs. the control group (42 patients [3%]; $p_{\text{non-inferiority}} = 0.0115$), with similar frequency of BARC 2 or higher bleeding events in the de-escalation group vs. control group (64 [5%] vs. 79 [6%]; HR 0.82; 95% CI 0.59–1.13; $p = 0.23$). It is worth underlining that the trial was designed to test the non-inferiority hypothesis and the analysis was intention to treat. As a consequence, the platelet reactivity-guided antithrombotic drug de-escalation was non-inferior to recommended conventional 12 months prasugrel therapy at 1 year after PCI in MI patients in terms of the net clinical benefit.

TOPIC

In the TOPIC (timing of platelet inhibition after acute coronary syndrome) randomized study, 645 patients 1 month after ACS were randomly assigned to either continuation of dual antiplatelet therapy composed of aspirin and potent antiplatelet agent or de-escalation to aspirin and clopidogrel (86). Drug de-escalation occurred without platelet function testing, however all patients underwent platelet reactivity assessment with the use the VASP assay at the time of randomization. The primary endpoint combining cardiovascular death, urgent revascularization, stroke and bleeding as defined as BARC ≥ 2 occurred in 85 (26.3%) patients in the unchanged drug group vs. 43 (13.4%) patients in the de-escalation group (HR 95%CI 0.48 (0.34–0.68; $P < 0.01$), with significant reduction in the occurrence of BARC ≥ 2 bleeding (48 [14.9%] vs. 13 [4%]; HR 95%CI 0.30 (0.18–0.50), $P < 0.01$). Additionally, the subanalysis revealed that at the time of randomization based on the results of platelet function testing, 47% of patients were classified with LTPR. Among this subpopulation, drug de-escalation brought the most prominent reduction in the primary endpoint incidence as compared with continued potent antiplatelet regimen (HR 0.29; 95% CI 0.17–0.51; $p < 0.01$). Nevertheless, the reduction of bleedings in the de-escalation group was mainly due to TIMI minimal and minor bleedings, with no difference in the major bleeding events.

ELECTRA

In the recently published Effectiveness of LowEr maintenance dose of TicagRelor early After myocardial infarction (ELECTRA) study, the antiplatelet efficacy of two ticagrelor maintenance dose regimens (reduced dose of 60 mg twice daily vs. standard dose of 90 mg twice daily) in stable patients at 30 days after acute MI were compared (85). The trial included 52 patients randomized in 1:1 ratio to the reduced or standard ticagrelor

maintenance dose. Platelet function testing with the use of the VASP assay and the Multiplate analyzer were performed 2 weeks after the treatment initiation. There were no significant differences in platelet reactivity between patients treated with reduced vs. standard ticagrelor dose (VASP: 10.4 [5.6–22.2] vs. 14.1 [9.4–22.1] %PRI; $p = 0.30$; Multiplate: 30.0 [24.0–39.0] vs. 26.5 [22.0–35.0] U; $p = 0.26$). Importantly, the percentage of patients with HTPR was similar in reduced vs. standard ticagrelor dose (VASP: 4% vs. 8%; $p = 0.67$; Multiplate: 15% vs. 8%; $p = 0.54$). In conclusion, the lower ticagrelor dose provided similar antiplatelet effect to the standard regimen.

Three observational registries aimed to assess the incidence of switching between P2Y₁₂ receptor blockers:

TRANSLATE-ACS

The Longitudinal Assessment of Treatment Patterns and Events after Acute Coronary Syndrome (TRANSLATE-ACS) observational study in 8672 MI patients has reported that P2Y₁₂ inhibitor switch occurred in 7.6% of participants (115). The switches were usually de-escalations from more potent agents to clopidogrel mainly due to economic reasons, while escalations from clopidogrel were mainly promoted by ischemic events.

ATLANTIS-SWITCH

The recently published, prospective, observational, multicenter ATLANTIS-SWITCH study included 571 ACS patients undergoing PCI treated with ticagrelor (45%) or prasugrel (55%) and investigated the frequency and predictors of either switch or drug discontinuation (116). The prevalence of P2Y₁₂ antagonist stop was 5.9%, and of switch was 6.7% and it was more frequent in ticagrelor recipients as compared with prasugrel (15.9% vs. 9.2%; $p = 0.016$). The majority of stop/switch choices were prompted by physicians (75%), they did not increase the risk of adverse cardiovascular events and were motivated by one of four identified independent predictors: major surgery, need for oral anticoagulation, TIMI major bleeding, or drug intolerance (116).

SCOPE

The SCOPE registry investigated the incidence of P2Y₁₂ inhibitor switching in 1363 patients undergoing PCI (117). The P2Y₁₂ inhibitor switch occurred in 10.5% and was not platelet function based. The authors concluded that de-escalation of antiplatelet treatment from more potent drugs to clopidogrel was an independent predictor of net cerebrovascular event (NACE) defined as a combination of adverse cardiovascular event and any bleeding event (OR 5.3; CI: 2.1–18.2; $p = 0.04$).

Conclusion of the Chapter

De-escalation strategies with use of platelet function testing seem to be safe.

CURRENT PLACE OF PLATELET FUNCTION TESTING IN EVERYDAY CLINICAL PRACTICE

The ischemic risk in ACS patients undergoing PCI is relatively high in clopidogrel treated patients due to its heterogeneous and unpredictable antiplatelet effect (40, 50). With the common use of more potent antiplatelet agents, increased ischemic risk occurs mainly within first months after ACS, whereas bleeding events are proportional to the duration and intensity of antiplatelet treatment (75, 76). Recently, the idea of de-escalation of antiplatelet therapy was investigated and focused on the net clinical benefit and to minimize the bleeding risk (86, 87). The choice of P2Y₁₂ inhibitors offers a chance for individualization of the therapy based on patient characteristics (81, 118). However, the de-escalation trials were powered for minor bleeding events and not for ischemic events. The prolongation studies with P2Y₁₂ receptor inhibitors as the DAPT trial or the PEGASUS-TIMI 54 trial indicated benefit for longer treatment with potent drugs as prasugrel or ticagrelor (119, 120). Therefore, in the era of personalized medicine, according to the latest guidelines on myocardial revascularization, platelet function testing guided P2Y₁₂ inhibitor de-escalation (e.g., switch from newer more potent drug to clopidogrel after an acute phase) may be considered in ACS patients, particularly those unsuitable for 12-month potent antithrombotic therapy due to the increased bleeding risk (class of recommendation IIb, level of evidence B) (12). Such drug de-escalation could be deliberated highly risky without platelet function testing guidance, especially when we take under consideration very high variability in response to P2Y₁₂ receptor inhibitors. In ACS patients undergoing cardiac surgery, platelet function testing is recommended to guide antiplatelet treatment interruption (class of recommendation IIb, level of evidence B), because the preoperative use of P2Y₁₂ inhibitors plus aspirin is associated with increased risk of bleeding and mortality (12, 87).

CONCLUSIONS

According to the recent guidelines, platelet function testing use is narrowed to certain clinical scenarios, as P2Y₁₂ inhibitor de-escalation and guidance of antiplatelet treatment interruption in ACS patients undergoing cardiac surgery (12). Due to unfavorable results of previous randomized trials its use is not recommended in everyday clinical practice (12, 87). In the course of modern ACS treatment, as directed in the guidelines, a potent P2Y₁₂ inhibitor, like prasugrel or ticagrelor, are initiated to prevent ischemic complications, but at the same time taking a risk of increased bleeding. When it comes to a major bleed, a switch to a less potent agent is performed, this time risking possible ischemic complications, leading to a vicious circle. The main goal of the precision-based therapy concept is to provide the right drug in the right dose to fit the needs of an individual patient from the very beginning

of the treatment process (29). The physician's choice would then be based on clinical, genetic, cellular and environmental variables. All these data would have to be integrated in an algorithm, as previously proposed (28). The gathered clinical information (e.g., based on the PREDICT score), results of platelet function testing and genetic status (CYP2C19 carrier) could be used to personalize antiplatelet therapy in patients with high-thrombotic or bleeding risk. Moreover, the precision-based antiplatelet therapies are also cost-effective, as this would reduce unnecessary hospitalizations due to either ischemic or bleeding complications. Such a test should be simple, fast, not expensive,

well-validated, user-friendly, and platelet function testing fits pretty well to this description.

AUTHOR CONTRIBUTIONS

JS-M, MO, and JK contributed conception and design of the manuscript. MO, PA, CE, and AK searched the literature. MO and PA wrote the first draft of the manuscript. JS-M, JK, AK, CE, MP, AT, and CH wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

REFERENCES

- Falk E, Nakano M, Bentzon JF, Finn AV, Virmani R. Update on acute coronary syndromes: the pathologists' view. *Eur Heart J*. (2013) 34:719–28. doi: 10.1093/eurheartj/ehs411
- Davi G, Patrono C. Platelet activation and atherothrombosis. *N Engl J Med*. (2007) 357:2482–94. doi: 10.1056/NEJMra071014
- Siller-Matula JM, Lang IM, Schoergenhofer C, Roest M, Jilma B. Interdependence between osteoprotegerin and active von Willebrand factor in long-term cardiovascular mortality prediction in patients undergoing percutaneous coronary intervention. *Thromb Haemost*. (2017) 117:1730–8. doi: 10.1160/TH17-02-0087
- Varga-Szabo D, Pleines I, Nieswandt B. Cell adhesion mechanisms in platelets. *Arterioscler Thromb Vasc Biol*. (2008) 28:403–12. doi: 10.1161/ATVBAHA.107.150474
- Raju NC, Eikelboom JW, Hirsh J. Platelet ADP-receptor antagonists for cardiovascular disease: past, present and future. *Nat Clin Pract Cardiovasc Med*. (2008) 5:766–80. doi: 10.1038/ncpcardio1372
- Hechler B, Leon C, Vial C, Vigne P, Frelon C, Cazenave JP, et al. The P2Y₁ receptor is necessary for adenosine 5'-diphosphate-induced platelet aggregation. *Blood*. (1998) 92:152–9. doi: 10.1182/blood.V92.1.152.413k27_152_159
- Jin J, Daniel JL, Kunapuli SP. Molecular basis for ADP-induced platelet activation: II. The P2Y₁ receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. *J Biol Chem*. (1998) 273:2030–4. doi: 10.1074/jbc.273.4.2030
- Hollopeter G, Jantzen H, Vincent D, Li G, England L, Ramakrishnan V, et al. Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature*. (2001) 409:202–7. doi: 10.1038/35051599
- Lindemann S, Krämer B, Seizer P, Gawaz M. Platelets, inflammation and atherosclerosis. *J Thromb Haemost*. (2007) 5:203–11. doi: 10.1111/j.1538-7836.2007.02517.x
- Awtry EH, Loscalzo J. Aspirin. *Circulation*. (2000) 101:1206–18. doi: 10.1161/01.CIR.101.10.1206
- Iakovou I, Schmidt T, Bonizzi E, Ge L, Sangiorgi GM, Stankovic G, et al. Incidence, predictors, and outcome of thrombosis after successful implantation of drug-eluting stents. *J Am Med Assoc*. (2005) 293:2126–30. doi: 10.1001/jama.293.17.2126
- Neumann FJ, Sousa-Uva M, Ahlsson A, Alfonso F, Banning AP, Benedetto U, et al. 2018 ESC/EACTS Guidelines on myocardial revascularization. *Eur Heart J*. (2019) 40:87–165. doi: 10.1093/eurheartj/ehy394
- Smith J, Willis A. Aspirin selectively inhibits prostaglandin production in human platelets. *Nat New Biol*. (1971) 231:235–7. doi: 10.1038/newbio231235a0
- Ibanez B, James S, Agewall S, Antunes MJ, Bucciarelli-Ducci C, Bueno H, et al. 2017 ESC Guidelines for the management of acute myocardial infarction in patients presenting with ST-segment elevation: the task Force for the management of acute myocardial infarction in patients presenting with ST-segment elevation of the European Society of Cardiology (ESC). *Eur Heart J*. (2018) 39:119–77. doi: 10.1093/eurheartj/ehx393
- Roffi M, Patrono C, Collet JP, Mueller C, Valgimigli M, Andreotti F, et al. 2015 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: Task Force for the Management of Acute Coronary Syndromes in Patients Presenting without Persistent ST-Segment Elevation of the European Society of Cardiology (ESC). *Eur Heart J*. (2016) 37:267–315. doi: 10.1093/eurheartj/ehv320
- Knuuti J, Wijns W, Saraste A, Capodanno D, Barbato E, Funck-Brentano C, et al. 2019 ESC Guidelines for the diagnosis and management of chronic coronary syndromes. *Eur Heart J*. (2019). [Epub ahead of print].
- Winter MP, Schneeweiss T, Cremer R, Biesinger B, Hengstenberg C, Prüller F, et al. Platelet reactivity patterns in patients treated with dual antiplatelet therapy. *Eur J Clin Invest*. (2019) 49:e13102. doi: 10.1111/eci.13102
- Ibanez B, Vilahur G, Badimon JJ. Pharmacology of thienopyridines: rationale for dual pathway inhibition. *Eur Heart J Suppl*. (2006) 8:G3–9. doi: 10.1093/eurheartj/sul047
- Adamski P, Adamska U, Ostrowska M, Navarese EP, Kubica J. Evaluating current and emerging antithrombotic therapy currently available for the treatment of acute coronary syndrome in geriatric populations. *Expert Opin Pharmacother*. (2018) 19:1415–25. doi: 10.1080/14656566.2018.1510487
- Siller-Matula JM, Petre A, Delle-Karth G, Huber K, Ay C, Lordkipanidzé M, et al. Impact of preoperative use of P2Y₁₂ receptor inhibitors on clinical outcomes in cardiac and non-cardiac surgery: a systematic review and meta-analysis. *Eur Heart J Acute Cardiovasc Care*. (2017) 6:753–70. doi: 10.1177/2048872615585516
- Kubisa MJ, Jezewski MP, Gasecka A, Siller-Matula JM, Postuła M. Ticagrelor - toward more efficient platelet inhibition and beyond. *Ther Clin Risk Manag*. (2018) 14:129–40. doi: 10.2147/TCRM.S152369
- Adamski P, Kozinski M, Ostrowska M, Fabiszak T, Navarese EP, Paciorek P, et al. Overview of pleiotropic effects of platelet P2Y₁₂ receptor inhibitors. *Thromb Haemost*. (2014) 112:224–42. doi: 10.1160/TH13-11-0915
- Hengstenberg C, Siller-Matula JM. Shedding light on long-term effects of early antiplatelet strategies after fibrinolytic treatment in STEMI. *J Am Coll Cardiol*. (2019) 73:2829–31. doi: 10.1016/j.jacc.2019.04.008
- Patti G, Pecan L, Lucerna M, Huber K, Rohla M, Renda G, et al. Outcomes of anticoagulated patients with atrial fibrillation treated with or without antiplatelet therapy - a pooled analysis from the PREFER in AF and PREFER in AF PROLONGATON registries. *Int J Cardiol*. (2018) 270:160–6. doi: 10.1016/j.ijcard.2018.06.098
- Schrör K, Weber AA. Comparative pharmacology of GP IIb/IIIa antagonists. *J Thromb Thrombolysis*. (2003) 15:71–80. doi: 10.1023/B:THRO.0000003308.63022.8d
- Ostrowska M, Adamski P, Kozinski M, Navarese EP, Fabiszak T, Grześk G, et al. Off-target effects of glycoprotein IIb/IIIa receptor inhibitors. *Cardiol J*. (2014) 21:458–64. doi: 10.5603/CJ.a2014.0020
- Siller-Matula JM, Trenk D, Schrör K, Gawaz M, Kristensen SD, Storey RF, et al. EPA (European Platelet Academy). Response variability to P2Y₁₂ receptor inhibitors: expectations and reality. *JACC Cardiovasc Interv*. (2013) 6:1111–28. doi: 10.1016/j.jcin.2013.06.011
- Siller-Matula JM, Trenk D, Schrör K, Gawaz M, Kristensen SD, Storey RF, et al. How to improve the concept of individualised antiplatelet therapy with P2Y₁₂ receptor inhibitors-is an algorithm the answer? *Thromb Haemost*. (2015) 113:37–52. doi: 10.1160/TH14-03-0238
- Winter MP, Grove EL, De Caterina R, Gorog DA, Ahrens I, Geisler T, et al. Advocating cardiovascular precision medicine with P2Y₁₂

- receptor inhibitors. *Heart J Cardiovasc Pharmacother.* (2017) 3:221–34. doi: 10.1093/ehjcvp/pvw044
30. Tantry US, Bonello L, Aradi D, Price MJ, Jeong YH, Angiolillo DJ, et al. Consensus and update on the definition of on-treatment platelet reactivity to adenosine diphosphate associated with ischemia and bleeding. *J Am Coll Cardiol.* (2013) 62:2261–73. doi: 10.1016/j.jacc.2013.07.101
 31. Aradi D, Kirtane A, Bonello L, Gurbel PA, Tantry US, Huber K, et al. Bleeding and stent thrombosis on P2Y12-inhibitors: collaborative analysis on the role of platelet reactivity for risk stratification after percutaneous coronary intervention. *Eur Heart J.* (2015) 36:1762–71. doi: 10.1093/eurheartj/ehv104
 32. Kozinski M, Ostrowska M, Adamski P, Sikora J, Sikora A, Karczmarzka-Wodzka A, et al. Which platelet function test best reflects the *in vivo* plasma concentrations of ticagrelor and its active metabolite? The HARMONIC study. *Thromb Haemost.* (2016) 116:1140–9. doi: 10.1160/TH16-07-0535
 33. Komosa A, Rzymiski P, Perek B, Ropacka-Lesiak M, Lesiak M, Siller-Matula JM, et al. Platelets redox balance assessment: Current evidence and methodological considerations. *Vascul Pharmacol.* (2017) 93–95:6–13. doi: 10.1016/j.vph.2017.06.002
 34. Gurbel PA, Bliden KP, Hiatt BL, O'Connor CM. Clopidogrel for coronary stenting: response variability, drug resistance, and the effect of pretreatment platelet reactivity. *Circulation.* (2003) 107:2908–13. doi: 10.1161/01.CIR.0000072771.11429.83
 35. Gurbel PA, Bliden KP, Hayes KM, Yoho JA, Herzog WR, Tantry US. The relation of dosing to clopidogrel responsiveness and the incidence of high post-treatment platelet aggregation in patients undergoing coronary stenting. *J Am Coll Cardiol.* (2005) 45:1392–6. doi: 10.1016/j.jacc.2005.01.030
 36. Angiolillo DJ, Fernandez-Ortiz A, Bernardo E, Ramirez C, Sabaté M, Bañuelos C, et al. High clopidogrel loading dose during coronary stenting: effects on drug response and interindividual variability. *Eur Heart J.* (2004) 25:1903–10. doi: 10.1016/j.ehj.2004.07.036
 37. Mega JL, Simon T. Pharmacology of antithrombotic drugs: an assessment of oral antiplatelet and anticoagulant treatments. *Lancet.* (2015) 386:281–91. doi: 10.1016/S0140-6736(15)60243-4
 38. Gurbel PA, Tantry US. Drug insight: clopidogrel nonresponsiveness. *Nat Clin Pract Cardiovasc Med.* (2006) 3:387–95. doi: 10.1038/ncpcardio0602
 39. Gurbel PA, Becker RC, Mann KG, Steinhubl SR, Michelson AD. Platelet function monitoring in patients with coronary artery disease. *J Am Coll Cardiol.* (2007) 50:1822–34. doi: 10.1016/j.jacc.2007.07.051
 40. Aradi D, Komócsi A, Vorobcsuk A, Rideg O, Tokés-Füzesi M, Magyarlaci T, et al. Prognostic significance of high on-clopidogrel platelet reactivity after percutaneous coronary intervention: systematic review and meta-analysis. *Am Heart J.* (2010) 160:543–51. doi: 10.1016/j.ahj.2010.06.004
 41. Ait-Mokhtara O, Bonello L, Benamarab S, Paganelli F. High on treatment platelet reactivity. *Heart Lung Circ.* (2012) 21:12–21. doi: 10.1016/j.hlc.2011.08.069
 42. Mallouk N, Labruyère C, Reny JL, Chapelle C, Piot M, Fontana P, et al. Prevalence of poor biological response to clopidogrel: a systematic review. *Thromb Haemost.* (2012) 107:494–506. doi: 10.1160/TH11-03-0202
 43. Siller-Matula JM, Delle-Karth G, Lang IM, Neunteufl T, Kozinski M, Kubica J, et al. Phenotyping vs. genotyping for prediction of clopidogrel efficacy and safety: the PEGASUS-PCI study. *J Thromb Haemost.* (2012) 10:529–42. doi: 10.1111/j.1538-7836.2012.04639.x
 44. Navarese EP, Verdoia M, Schaffer A, Suriano P, Kozinski M, Castriota F, et al. Ischaemic and bleeding complications with new, compared to standard, ADP-antagonist regimens in acute coronary syndromes: a meta-analysis of randomized trials. *QJM.* (2011) 104:561–9. doi: 10.1093/qjmed/hcr069
 45. Kubica A, Kozinski M, Grześ G, Goch A. Znaczenie kliniczne interakcji między kłpidogrelem a inhibitorami pompy protonowej. *Kardiologia Pol.* (2011) 69:610–6.
 46. Kubica A, Kosobucka A, Fabiszak T, Gorog DA, Siller-Matula JM. Assessment of adherence to medication in patients after myocardial infarction treated with percutaneous coronary intervention. Is there a place for new self-reported questionnaires? *Curr Med Res Opin.* (2019) 35:341–9. doi: 10.1080/03007995.2018.1510385
 47. Bergmeijer TO, Reny JL, Pakyz RE, Gong L, Lewis JP, Kim EY, et al. Genome-wide and candidate gene approaches of clopidogrel efficacy using pharmacodynamic and clinical end points-Rationale and design of the International Clopidogrel Pharmacogenomics Consortium (ICPC). *Am Heart J.* (2018) 198:152–9. doi: 10.1016/j.ahj.2017.12.010
 48. Milanowski L, Rasul F, Gajda SN, Eyleten C, Siller-Matula JM, Postula M. Genetic variability of SRC family kinases and its association with platelet hyperreactivity and clinical outcomes: a systematic review. *Curr Pharm Des.* (2018) 24:628–40. doi: 10.2174/1381612824666171213105002
 49. Navarese EP, Kolodziejczak M, Winter MP, Alimohammadi A, Lang IM, Buffon A, et al. Association of PCSK9 with platelet reactivity in patients with acute coronary syndrome treated with prasugrel or ticagrelor: The PCSK9-REACT study. *Int J Cardiol.* (2017) 227:644–9. doi: 10.1016/j.ijcard.2016.10.084
 50. Stone GW, Witzensichler B, Weisz G, Rinaldi MJ, Neumann FJ, Metzger DC, et al. Platelet reactivity and clinical outcomes after coronary artery implantation of drug-eluting stents (ADAPT-DES): a prospective multicentre registry study. *Lancet.* (2013) 382:614–23. doi: 10.1016/S0140-6736(13)61170-8
 51. Komosa A, Siller-Matula JM, Lesiak M, Michalak M, Kowal J, Maczynski M, et al. Association between high on-treatment platelet reactivity and occurrence of cerebral ischemic events in patients undergoing percutaneous coronary intervention. *Thromb Res.* (2016) 138:49–54. doi: 10.1016/j.thromres.2015.12.021
 52. Parodi G, Valenti R, Bellandi B, Migliorini A, Marcucci R, Comito V, et al. Comparison of prasugrel and ticagrelor loading doses in ST-segment elevation myocardial infarction patients: RAPID (Rapid Activity of Platelet Inhibitor Drugs) primary PCI study. *J Am Coll Cardiol.* (2013) 61:1601–6. doi: 10.1016/j.jacc.2013.01.024
 53. Kubica J, Adamski P, Ostrowska M, Sikora J, Kubica JM, Sroka WD, et al. Morphine delays and attenuates ticagrelor exposure and action in patients with myocardial infarction: the randomized, double-blind, placebo-controlled IMPRESSION trial. *Eur Heart J.* (2016) 37:245–52. doi: 10.1093/eurheartj/ehv547
 54. Siller-Matula JM, Akca B, Neunteufl T, Maurer G, Lang IM, Kreiner G, et al. Inter-patient variability of platelet reactivity in patients treated with prasugrel and ticagrelor. *Platelets.* (2016) 27:373–7. doi: 10.3109/09537104.2015.1095874
 55. Siller-Matula JM, Hintermeier A, Kastner J, Kreiner G, Maurer G, Kratochwil C, et al. Distribution of clinical events across platelet aggregation values in all-comers treated with prasugrel and ticagrelor. *Vascul Pharmacol.* (2016) 79:6–10. doi: 10.1016/j.vph.2016.01.003
 56. Winter MP, Kozinski M, Kubica J, Aradi D, Siller-Matula JM. Personalized antiplatelet therapy with P2Y12 receptor inhibitors: benefits and pitfalls. *Postępy Kardiologii Interwencyjnej.* (2015) 11:259–80. doi: 10.5114/pwki.2015.55596
 57. Komosa A, Lesiak M, Krasinski Z, Grygier M, Siniawski A, Skorupski W, et al. Optimal timing of P2Y12 inhibitor loading in patients undergoing PCI: a meta-analysis. *Thromb Haemost.* (2019) 119:1000–20. doi: 10.1055/s-0039-1683421
 58. Lemesle G, Schurtz G, Bauters C, Hamon M. High on-treatment platelet reactivity with ticagrelor versus prasugrel: a systematic review and meta-analysis. *J Thromb Haemost.* (2015) 13:931–42. doi: 10.1111/jth.12907
 59. Teng R, Mitchell P, Butler K. Effect of age and gender on pharmacokinetics and pharmacodynamics of a single ticagrelor dose in healthy individuals. *Eur J Clin Pharmacol.* (2012) 68:1175–82. doi: 10.1007/s00228-012-1227-4
 60. Teng R, Mitchell PD, Butler K. Lack of significant food effect on the pharmacokinetics of ticagrelor in healthy volunteers. *J Clin Pharm Ther.* (2012) 37:464–8. doi: 10.1111/j.1365-2710.2011.01307.x
 61. Varenhorst C, Eriksson N, Johansson B, Barratt BJ, Hagstroem E, Åkerblom A, et al. Effect of genetic variations on ticagrelor plasma levels and clinical outcomes. *Eur Heart J.* (2015) 36:1901–12. doi: 10.1093/eurheartj/ehv116
 62. Kubica J, Kubica A, Jilma B, Adamski P, Hobl EL, Navarese EP, et al. Impact of morphine on antiplatelet effects of oral P2Y12 receptor inhibitors. *Int J Cardiol.* (2016) 215:201–8. doi: 10.1016/j.ijcard.2016.04.077
 63. Sikora J, Niezgoda P, Baranska M, Buszko K, Skibinska N, Sroka W, et al. METoclopramide Administration as a Strategy to Overcome MORPHine-ticagrelor Interaction in Patients with Unstable Angina Pectoris-The METAMORPHOSIS Trial. *Thromb Haemost.* (2018) 118:2126–33. doi: 10.1055/s-0038-1675605

64. Niezgoda P, Sikora J, Baranska M, Sikora A, Buszko K, Sieminska E, et al. Crushed sublingual versus oral ticagrelor administration strategies in patients with unstable angina. A pharmacokinetic/pharmacodynamic study. *Thromb Haemost.* (2017) 117:718–26. doi: 10.1160/TH16-08-0670
65. Adamski P, Sikora J, Laskowska E, Buszko K, Ostrowska M, Uminska JM, et al. Comparison of bioavailability and antiplatelet action of ticagrelor in patients with ST-elevation myocardial infarction and non-ST-elevation myocardial infarction: A prospective, observational, single-centre study. *PLoS ONE.* (2017) 12:e0186013. doi: 10.1371/journal.pone.0186013
66. Adamski P, Buszko K, Sikora J, Niezgoda P, Fabiszak T, Ostrowska M, et al. Determinants of high platelet reactivity in patients with acute coronary syndromes treated with ticagrelor. *Sci Rep.* (2019) 9:3924. doi: 10.1038/s41598-019-40628-0
67. Adamski P, Buszko K, Sikora J, Niezgoda P, Baranska M, Ostrowska M, et al. Metabolism of ticagrelor in patients with acute coronary syndromes. *Sci Rep.* (2018) 8:11746. doi: 10.1038/s41598-018-29619-9
68. Schoergenhofer C, Hobl EL, Schellongowski P, Heinz G, Speidl WS, Siller-Matula JM, et al. Clopidogrel in critically ill patients. *Clin Pharmacol Ther.* (2018) 103:217–23. doi: 10.1002/cpt.878
69. Uminska JM, Ratajczak J, Buszko K, Sobczak P, Sroka W, Marszałł MP, et al. Impact of mild therapeutic hypothermia on bioavailability of ticagrelor in patients with acute myocardial infarction after out-of-hospital cardiac arrest. *Cardiol J.* (2019). [Epub ahead of print].
70. Eyleten C, Sopłinska AM, Pordzik J, Siller-Matula JM, Postuła M. Effectiveness of antiplatelet drugs under therapeutic hypothermia: a comprehensive review. *Clin. Pharmacol. Ther.* (2019) 106:993–1005. doi: 10.1002/cpt.1492
71. Prüller F, Milke OL, Bis L, Fruhwald F, Scherr D, Eller P, et al. Impaired aspirin-mediated platelet function inhibition in resuscitated patients with acute myocardial infarction treated with therapeutic hypothermia: a prospective, observational, non-randomized single-centre study. *Ann Intensive Care.* (2018) 8:28. doi: 10.1186/s13613-018-0366-x
72. Prüller F, Bis L, Milke OL, Fruhwald F, Pätzold S, Altmanninger-Sock S, et al. Cangrelor induces more potent platelet inhibition without increasing bleeding in resuscitated patients. *J Clin Med.* (2018) 7:E442. doi: 10.3390/jcm7110442
73. Siller-Matula JM, Specht S, Kubica J, Alexopoulos D, De Caterina R, Hobl EL, et al. Abciximab as a bridging strategy to overcome morphine-prasugrel interaction in STEMI patients. *Br J Clin Pharmacol.* (2016) 82:1343–50. doi: 10.1111/bcp.13053
74. Schoergenhofer C, Hobl EL, Staudinger T, Speidl WS, Heinz G, Siller-Matula JM, et al. Prasugrel in critically ill patients. *Thromb Haemost.* (2017) 117:1582–7. doi: 10.1160/TH17-03-0154
75. Wiviott SD, Braunwald E, McCabe CH, Montalescot G, Ruzyllo W, Gottlieb S, et al. Prasugrel versus clopidogrel in patients with acute coronary syndromes. *N Engl J Med.* (2007) 357:2001–15. doi: 10.1056/NEJMoa0706482
76. Wallentin L, Becker RC, Budaj A, Cannon CP, Emanuelsson H, Held C, et al. Ticagrelor versus clopidogrel in patients with acute coronary syndromes. *N Engl J Med.* (2009) 361:1045–57. doi: 10.1056/NEJMoa0904327
77. Cuisset T, Cayla G, Frere C, Quilici J, Poyet R, Gaborit B, et al. Predictive value of post-treatment platelet reactivity for occurrence of post-discharge bleeding after non-ST elevation acute coronary syndrome. Shifting from antiplatelet resistance to bleeding risk assessment? *EuroIntervention.* (2009) 5:325–9. doi: 10.4244/51
78. Sibbing D, Schulz S, Braun S, Morath T, Stegherr J, Mehilli J, et al. Antiplatelet effects of clopidogrel and bleeding in patients undergoing coronary stent placement. *J Thromb Haemost.* (2010) 8:250–6. doi: 10.1111/j.1538-7836.2009.03709.x
79. Serebruany V, Rao SV, Silva MA, Donovan JL, Kannan AO, Makarov L, et al. Correlation of inhibition of platelet aggregation after clopidogrel with post discharge bleeding events: assessment by different bleeding classifications. *Eur Heart J.* (2010) 31:227–35. doi: 10.1093/eurheartj/ehp434
80. Patti G, Lucerna M, Pecan L, Siller-Matula JM, Cavallari I, Kirchhof P, et al. Thromboembolic risk, bleeding outcomes and effect of different antithrombotic strategies in very elderly patients with atrial fibrillation: a sub-analysis from the PREFER in AF (PREvention of Thromboembolic Events-European Registry in Atrial Fibrillation). *J. Am Heart Assoc.* (2017) 6:e005657. doi: 10.1161/JAHA.117.005657
81. Renda G, Patti G, Lang IM, Siller-Matula JM, Hylek EM, Ambrosio G, et al. Thrombotic and hemorrhagic burden in women: gender-related issues in the response to antithrombotic therapies. *Int J Cardiol.* (2019) 286:198–207. doi: 10.1016/j.ijcard.2019.02.004
82. Deharo P, Bassez C, Bonnet G, Pankert M, Quilici J, Lambert M, et al. Prasugrel versus ticagrelor in acute coronary syndrome: a randomized comparison. *Int J Cardiol.* (2013) 170:e21–2. doi: 10.1016/j.ijcard.2013.10.043
83. Alexopoulos D, Stavrou K, Koniari I, Gkizas V, Perperis A, Kontoprias K, et al. Ticagrelor vs prasugrel one-month maintenance therapy: impact on platelet reactivity and bleeding events. *Thromb Haemost.* (2014) 112:551–7. doi: 10.1160/TH14-02-0119
84. Sibbing D, Steinhubl SR, Schulz S, Schömig A, Kastrati A. Platelet aggregation and its association with stent thrombosis and bleeding in clopidogrel-treated patients: initial evidence of a therapeutic window. *J Am Coll Cardiol.* (2010) 56:317–8. doi: 10.1016/j.jacc.2010.03.048
85. Kubica J, Adamski P, Buszko K, Baranska M, Sikora J, Marszałł MP, et al. Platelet inhibition with standard versus lower maintenance dose of ticagrelor early after myocardial infarction (ELECTRA): a randomized, open-label, active-controlled pharmacodynamic and pharmacokinetic study. *Eur Heart J Cardiovasc Pharmacother.* (2019) 5:139–48. doi: 10.1093/ehjcvp/pvz004
86. Cuisset T, Deharo P, Quilici J, Johnson TW, Deffarges S, Bassez C, et al. Benefit of switching dual antiplatelet therapy after acute coronary syndrome: the TOPIC (timing of platelet inhibition after acute coronary syndrome) randomized study. *Eur Heart J.* (2017) 38:3070–8. doi: 10.1093/eurheartj/ehx175
87. Sibbing D, Aradi D, Jacobshagen C, Gross L, Trenk D, Geisler T, et al. Guided de-escalation of antiplatelet treatment in patients with acute coronary syndrome undergoing percutaneous coronary intervention (TROPICAL-ACS): a randomised, open-label, multicentre trial. *Lancet.* (2017) 390:1747–57. doi: 10.1016/S0140-6736(17)32155-4
88. Cayla G, Cuisset T, Silvain J, Leclercq F, Manzo-Silberman S, Saint-Etienne C, et al. Platelet function monitoring to adjust antiplatelet therapy in elderly patients stented for an acute coronary syndrome (ANTARCTIC): an open-label, blinded-endpoint, randomised controlled superiority trial. *Lancet.* (2016) 388:2015–22. doi: 10.1016/S0140-6736(16)31323-X
89. Aradi D, Tornóyos A, Pintér T, Voróbcsek A, Kónyi A, Faluközy J, et al. Optimizing P2Y₁₂ receptor inhibition in patients with acute coronary syndrome on the basis of platelet function testing: impact of prasugrel and high-dose clopidogrel. *J Am Coll Cardiol.* (2014) 63:1061–70. doi: 10.1016/j.jacc.2013.12.023
90. Christ G, Siller-Matula JM, Francesconi M, Dechant C, Grohs K, Podczec-Schweighofer A. Individualising dual antiplatelet therapy after percutaneous coronary intervention: the IDEAL-PCI registry. *BMJ Open.* (2014) 4:e005781. doi: 10.1136/bmjopen-2014-005781
91. Mayer K, Schulz S, Bernlochner I, Morath T, Braun S, Hausleiter J, et al. A comparative cohort study on personalised antiplatelet therapy in PCI-treated patients with high on-clopidogrel platelet reactivity. Results of the ISAR-HPR registry. *Thromb Haemost.* (2014) 112:342–51. doi: 10.1160/TH13-10-0874
92. Siller-Matula JM, Francesconi M, Dechant C, Jilma B, Maurer G, Delle-Karth G, et al. Personalized antiplatelet treatment after percutaneous coronary intervention: the MADONNA study. *Int J Cardiol.* (2013) 167:2018–23. doi: 10.1016/j.ijcard.2012.05.040
93. Kozinski M, Obonska K, Stankowska K, Navarese EP, Fabiszak T, Stolarek W, et al. Prasugrel overcomes high on-clopidogrel platelet reactivity in the acute phase of acute coronary syndrome and maintains its antiplatelet potency at 30-day follow-up. *Cardiol J.* (2014) 21:547–56. doi: 10.5603/CJ.a2014.0026
94. Trenk D, Stone GW, Gawaz M, Kastrati A, Angiolillo DJ, Müller U, et al. A randomized trial of prasugrel versus clopidogrel in patients with high platelet reactivity on clopidogrel after elective percutaneous coronary intervention with implantation of drug-eluting stents: results of the TRIGGER-PCI (Testing Platelet Reactivity In Patients Undergoing Elective Stent Placement on Clopidogrel to Guide Alternative Therapy With Prasugrel) study. *J Am Coll Cardiol.* (2012) 59:2159–64. doi: 10.1016/j.jacc.2012.02.026

95. Collet JP, Cuisset T, Rangé G, Cayla G, Elhadad S, Pouillot C, et al. Bedside monitoring to adjust antiplatelet therapy for coronary stenting. *N Engl J Med*. (2012) 367:2100–9. doi: 10.1056/NEJMoa1209979
96. Price MJ, Berger PB, Teirstein PS, Tanguay JF, Angiolillo DJ, Spriggs D, et al. GRAVITAS Investigators. Standard- vs high-dose clopidogrel based on platelet function testing after percutaneous coronary intervention: the GRAVITAS randomized trial. *JAMA*. (2011) 305:1097–105. doi: 10.1001/jama.2011.290
97. Alexopoulos D, Xanthopoulou I, Davlouros P, Plakomiti TE, Panagiotou A, Mavronasiou E, et al. Prasugrel overcomes high on-clopidogrel platelet reactivity in chronic coronary artery disease patients more effectively than high dose (150 mg) clopidogrel. *Am Heart J*. (2011) 162:733–9. doi: 10.1016/j.ahj.2011.07.026
98. Alexopoulos D, Panagiotou A, Xanthopoulou I, Komninakis D, Kassimis G, Davlouros P, et al. Antiplatelet effects of prasugrel vs double clopidogrel in patients on hemodialysis and high on-treatment platelet reactivity. *J Thromb Haemost*. (2011) 9:2379–85. doi: 10.1111/j.1538-7836.2011.04531.x
99. Capranzano P, Tamburino C, Capodanno D, Micciché E, D'Urso L, Calvi V, et al. Platelet function profiles in the elderly: Results of a pharmacodynamic study in patients on clopidogrel therapy and effects of switching to prasugrel 5 mg in patients with high platelet reactivity. *Thromb Haemost*. (2011) 106:1149–57. doi: 10.1160/TH11-05-0346
100. Ferreira JL, Ueno M, Desai B, Capranzano P, Capodanno D, Angiolillo DJ. Impact of adjunctive cilostazol therapy versus high maintenance dose of clopidogrel in suboptimal responders with diabetes mellitus. *Rev Esp Cardiol*. (2011) 65:105–6. doi: 10.1016/j.recesp.2011.04.008
101. Neubauer H, Kaiser AF, Endres HG, Krüger JC, Engelhardt A, Lask S, et al. Tailored antiplatelet therapy can overcome clopidogrel and aspirin resistance—the BOchum CLopidogrel and Aspirin Plan (BOCLA-Plan) to improve antiplatelet therapy. *BMC Med*. (2011) 9:3. doi: 10.1186/1741-7015-9-3
102. Gurbel PA, Bliden KP, Antonino MJ, Stephens G, Gretler DD, Jurek MM, et al. The effect of elinogrel on high platelet reactivity during dual antiplatelet therapy and the relation to CYP2C19*2 genotype: first experience in patients. *J Thromb Haemost*. (2010) 8:43–53. doi: 10.1111/j.1538-7836.2009.03648.x
103. Gurbel PA, Bliden KP, Butler K, Antonino MJ, Wei C, Teng R, et al. Response to ticagrelor in clopidogrel nonresponders and responders and effect of switching therapies: the VA study. *Circulation*. (2010) 121:1188–99. doi: 10.1161/CIRCULATIONAHA.109.919456
104. Valgimigli M, Campo G, de Cesare N, Meliga E, Vranckx P, Furgieri A, et al. Intensifying platelet inhibition with tirofiban in poor responders to aspirin, clopidogrel, or both agents undergoing elective coronary intervention: results from the double-blind, prospective, randomized Tailoring Treatment with Tirofiban in Patients Showing Resistance to Aspirin and/or Resistance to Clopidogrel study. *Circulation*. (2009) 119:3215–22. doi: 10.1161/CIRCULATIONAHA.108.83236
105. Jeong YH, Lee SW, Choi BR, Kim IS, Seo MK, Kwak CH, et al. Randomized comparison of adjunctive cilostazol versus high maintenance dose clopidogrel in patients with high post-treatment platelet reactivity: results of the ACCEL-RESISTANCE (Adjunctive Cilostazol Versus High Maintenance Dose Clopidogrel in Patients With Clopidogrel Resistance) randomized study. *J Am Coll Cardiol*. (2009) 53:1101–9. doi: 10.1016/j.jacc.2008.12.025
106. Bonello L, Camoin-Jau L, Armero S, Com O, Arques S, Burignat-Bonello C, et al. Tailored clopidogrel loading dose according to platelet reactivity monitoring to prevent acute and subacute stent thrombosis. *Am J Cardiol*. (2009) 103:5–10. doi: 10.1016/j.amjcard.2008.08.048
107. Bonello L, Camoin-Jau L, Arques S, Boyer C, Panagides D, Wittenberg O, et al. Adjusted clopidogrel loading doses according to vasodilator-stimulated phosphoprotein phosphorylation index decrease rate of major adverse cardiovascular events in patients with clopidogrel resistance: a multicenter randomized prospective study. *J Am Coll Cardiol*. (2008) 51:1404–11. doi: 10.1016/j.jacc.2007.12.044
108. Aleil B, Jacquemin L, De Poli F, Zaehring M, Collet JP, Montalescot G, et al. Clopidogrel 150 mg/day to overcome low responsiveness in patients undergoing elective percutaneous coronary intervention: results from the VASP-02 (Vasodilator-Stimulated Phosphoprotein-02) randomized study. *JACC Cardiovasc Interv*. (2008) 1:631–8. doi: 10.1016/j.jcin.2008.09.004
109. Trenk D, Hochholzer W, Muller B, Stratz C, Valina CM, Schmiebusch P, et al. Antiplatelet response to the 150-mg maintenance dose of clopidogrel in patients with insufficient platelet inhibition after clopidogrel loading for elective coronary stent placement. *EuroIntervention*. (2008) 4:214–21. doi: 10.4244/EIJV4I2A39
110. Cuisset T, Frere C, Quilici J, Morange PE, Mouret JP, Bali L, et al. Glycoprotein IIb/IIIa inhibitors improve outcome after coronary stenting in clopidogrel nonresponders: a prospective, randomized study. *J Am Coll Cardiol*. (2008) 1:649–53. doi: 10.1016/j.jcin.2008.08.018
111. Matetzky S, Fefer P, Shenkman B, Varon D, Savion N, Hod H. Effectiveness of reloading to overcome clopidogrel nonresponsiveness in patients with acute myocardial infarction. *Am J Cardiol*. (2008) 102:524–9. doi: 10.1016/j.amjcard.2008.04.028
112. Neubauer H, Lask S, Engelhardt A, Mügge A. How to optimise clopidogrel therapy? Reducing the low-response incidence by aggregometry-guided therapy modification. *Thromb Haemost*. (2008) 99:357–62. doi: 10.1160/TH07-10-0624
113. Wang XD, Zhang DF, Zhuang SW, Lai Y. Modifying clopidogrel maintenance doses according to vasodilator-stimulated phosphoprotein phosphorylation index improves clinical outcome in patients with clopidogrel resistance. *Clin Cardiol*. (2011) 34:332–8. doi: 10.1002/clc.20884
114. Ari H, Ozkan H, Karacinar A, Ari S, Koca V, Bozat T. The Effect of high-dose Clopidogrel treatment in patients with clopidogrel resistance (the EFFICIENT trial). *Int J Cardiol*. (2012) 157:374–80. doi: 10.1016/j.ijcard.2010.12.083
115. Zettler ME, Peterson ED, McCoy LA, Effron MB, Anstrom KJ, Henry TD, et al. Switching of adenosine diphosphate receptor inhibitor after hospital discharge among myocardial infarction patients: Insights from the Treatment with Adenosine Diphosphate Receptor Inhibitors: Longitudinal Assessment of Treatment Patterns and Events after Acute Coronary Syndrome (TRANSLATE-ACS) observational study. *Am Heart J*. (2017) 183:62–8. doi: 10.1016/j.ahj.2016.10.006
116. Winter MP, von Lewinski D, Wallner M, Prüller F, Kolesnik E, Hengstenberg C, et al. Incidence, predictors, and prognosis of premature discontinuation or switch of prasugrel or ticagrelor: the ATLANTIS - SWITCH study. *Sci Rep*. (2019) 9:8194. doi: 10.1038/s41598-019-44673-7
117. De Luca L, D'Ascenzo F, Musumeci G, Saia F, Parodi G, Varbella F, et al. Incidence and outcome of switching of oral platelet P2Y12 receptor inhibitors in patients with acute coronary syndromes undergoing percutaneous coronary intervention: the SCOPE registry. *EuroIntervention*. (2017) 13:459–66. doi: 10.4244/EIJ-D-17-00092
118. Gasecka A, Konwerski M, Pordzik J, Soplińska A, Filipiak KJ, Siller-Matula JM, et al. Switching between P2Y12 antagonists - From bench to bedside. *Vascul Pharmacol*. (2019) 115:1–12. doi: 10.1016/j.vph.2019.01.003
119. Mauri L, Kereiakes DJ, Yeh RW, Driscoll-Shempp P, Cutlip DE, Steg PG, et al. Twelve or 30 months of dual antiplatelet therapy after drug-eluting stents. *N Engl J Med*. (2014) 371:2155–66. doi: 10.1056/NEJMoa1409312
120. Bonaca MP, Bhatt DL, Cohen M, Steg PG, Storey RF, Jensen EC, et al. Long-term use of ticagrelor in patients with prior MI. *N Engl J Med*. (2015) 372:1791–800. doi: 10.1056/NEJMoa1500857

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Ostrowska, Kubica, Adamski, Kubica, Eyileten, Postula, Toma, Hengstenberg and Siller-Matula. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Procoagulant Phosphatidylserine-Exposing Platelets *in vitro* and *in vivo*

Emily C. Reddy^{1†} and Margaret L. Rand^{2,3*†}

¹ Developmental and Stem Cell Biology, Research Institute, The Hospital for Sick Children, Toronto, ON, Canada, ² Division of Haematology/Oncology, Translational Medicine, Research Institute, The Hospital for Sick Children, Toronto, ON, Canada,

³ Departments of Laboratory Medicine & Pathobiology, Biochemistry, and Paediatrics, University of Toronto, Toronto, ON, Canada

OPEN ACCESS

Edited by:

Marie Lordkipanidzé,
Université de Montréal, Canada

Reviewed by:

Matthew Harper,
University of Cambridge,
United Kingdom
Mikhail Panteleev,
Lomonosov Moscow State
University, Russia
Marina Camera,
University of Milan, Italy

*Correspondence:

Margaret L. Rand
margaret.rand@sickkids.ca

[†]These authors have contributed
equally to this work

Specialty section:

This article was submitted to
Atherosclerosis and Vascular
Medicine,
a section of the journal
Frontiers in Cardiovascular Medicine

Received: 15 October 2019

Accepted: 30 January 2020

Published: 03 March 2020

Citation:

Reddy EC and Rand ML (2020)
Procoagulant
Phosphatidylserine-Exposing Platelets
in vitro and *in vivo*.
Front. Cardiovasc. Med. 7:15.
doi: 10.3389/fcvm.2020.00015

The physiological heterogeneity of platelets leads to diverse responses and the formation of discrete subpopulations upon platelet stimulation. Procoagulant platelets are an example of such subpopulations, a key characteristic of which is exposure either of the anionic aminophospholipid phosphatidylserine (PS) or of tissue factor on the activated platelet surface. This review focuses on the former, in which PS exposure on a subpopulation of platelets facilitates assembly of the intrinsic tenase and prothrombinase complexes, thereby accelerating thrombin generation on the activated platelet surface, contributing importantly to the hemostatic process. Mechanisms involved in platelet PS exposure, and accompanying events, induced by physiologically relevant agonists are considered then contrasted with PS exposure resulting from intrinsic pathway-mediated apoptosis in platelets. Pathologies of PS exposure, both inherited and acquired, are described. A consideration of platelet PS exposure as an antithrombotic target concludes the review.

Keywords: platelets, procoagulant, phosphatidylserine (PS) exposure, platelet activation, platelet apoptosis, hemostasis, thrombosis

INTRODUCTION

Blood vessel wall injury sets into play processes that lead to the formation of a hemostatic plug that stops the bleeding from the injury site. In primary hemostasis, platelets adhere to exposed subendothelium, resulting in their activation and aggregation, forming a platelet plug. Secondary hemostasis is initiated by tissue factor exposure at the site of vessel wall damage, resulting in formation, *via* the coagulation pathway, of covalently cross-linked fibrin that binds to, and stabilizes, the platelet plug (1).

It has long been recognized that activated platelets contribute in a major way to fibrin formation; this is well-exemplified in the cell-based model of coagulation (2). This procoagulant property of platelets, earlier termed platelet factor 3 availability (PF3a) and assayed by measuring the ability of platelets to promote thrombin and fibrin formation (3), results from exposure of the anionic aminophospholipid phosphatidylserine (PS) on the surface of activated platelets (4). PS, translocated from the internal to the external platelet membrane leaflet, facilitates the assembly of the intrinsic tenase complex [factor (F)VIIIa; FIXa; FX] and prothrombinase complex (FVa; FXa; prothrombin), contributing to the burst of thrombin generation in the propagation phase of coagulation. Specifically, the negatively charged γ -carboxyglutamate (Gla) residues at the NH₂ termini of the vitamin K-dependent factors, FIX(a), FX(a), and prothrombin, interact with negatively charged PS *via* Ca²⁺. FVIII binds to PS *via* its C2 domain and FVa *via* its C1 and C2

domains (5, 6). Tenase and prothrombinase activities are enhanced by PS-containing membranes by up to three orders of magnitude (7–9). Phosphatidylethanolamine (PE), that also becomes exposed on the surface of activated platelets, can contribute to the enhanced thrombin formation (7, 10); the fatty acid chain length of PE, but not PS, regulates the ability to support coagulation, with platelet-specific PEs demonstrating optimum activity (11). Oxidized PE, specifically 12-hydroxyeicosatetraenoic acid (HETE)-PE, formed by activated platelets, is even more potent than native PE in enhancing thrombin generation (12).

It is recognized that tissue factor-expressing platelets also comprise a subpopulation of procoagulant platelets. However, a discussion of this type of procoagulant platelet is beyond the scope of this mini-review, and the reader is referred to several recent, relevant publications on the topic (13–18).

Procoagulant Phosphatidylserine-Exposing Platelet Subpopulations and Nomenclature

A unique feature of procoagulant platelet formation is that only a subpopulation of activated platelets exposes PS. This was recognized over 25 years ago by flow cytometry (19) using fluorescently labeled annexin A5 that binds PS with high affinity in a Ca^{2+} -dependent manner. Fluorescently labeled lactadherin that does not require Ca^{2+} is also used to detect PS-exposing platelets [e.g., Dasgupta et al. (20)]. Flow cytometric and microscopy studies have shown colocalization of FVIII(a), FIX(a), FX(a), FV(a), and prothrombin with PS-exposing platelets, confirming that these platelets serve as assembly sites for the intrinsic tenase and prothrombinase complexes (21–25).

Procoagulant platelet subpopulations have been referred to by a myriad of names in the literature; however, it is recognized that these platelets share the key characteristic of PS exposure. An early description of a subpopulation of PS-exposing platelets was as COAT (Collagen And Thrombin)-FV platelets formed in response to dual agonist activation. These platelets were characterized by high levels of FV on their surface, in addition to PS (22, 26). COAT-FV was later abbreviated to COAT when it was demonstrated that these platelets are also coated with fibrinogen, fibronectin, von Willebrand factor (VWF), and thrombospondin, among many other α -granule proteins, on their surface (27). Subsequently, this subpopulation has been termed coated platelets, denoting the coating of the platelets with procoagulant proteins, including fibrin (28–30). The distinct morphology of procoagulant platelets has led to the terminology of blebbing, balloon(ing), or balloon-like platelets (31–35). Procoagulant platelets have also been referred to as SCIP (sustained calcium-induced platelet morphology) platelets (36), necrotic/4-[N-(S-glutathionylacetyl)amino]phenylarsonous acid (GSAO)-binding platelets (37–39), superactivated platelets (40), capped platelets (21, 41), and zombie platelets (42). Agbani and Poole (43) recently proposed “procoagulant platelets” as the unifying term for this activated platelet subpopulation.

In this brief review, we focus on mechanisms involved in PS exposure induced by platelet activation to form a subpopulation

of procoagulant platelets, then contrasting it with PS exposure resulting from platelet apoptosis. Pathologies of PS exposure, inherited and acquired, are described. We conclude with a consideration of platelet PS exposure as an antithrombotic target.

PLATELET MEMBRANE PHOSPHOLIPID ASYMMETRY: MAINTENANCE AND COLLAPSE

Flippase [(Aminophospholipid) Translocase]

Similar to other biological membranes, resting platelets possess an asymmetrical phospholipid plasma membrane bilayer (4), with the minor phospholipid PS sequestered to the inner cytoplasmic leaflet. This PS asymmetry is created by a flippase/(amino)phospholipid translocase enzyme, a member of the Type IV subfamily of P-type ATPases (P4-ATPases) (9, 44), that rapidly and specifically shuttles PS from the outer to the inner membrane leaflet against the concentration gradient, in an ATP-dependent fashion (45). Its activity is abrogated when cytoplasmic Ca^{2+} ($\text{Ca}^{2+}_{\text{cyt}}$) increases to low micromolar levels (46).

Scramblase and TMEM16F

Scramblase is a Ca^{2+} -dependent, ATP-independent enzyme that regulates the rapid, non-specific bidirectional movement, i.e., “scrambling,” of phospholipids between membrane leaflets, resulting in a loss of normal membrane phospholipid bilayer asymmetry. Although scramblase activity has long been described in platelets (4), the protein involved in Ca^{2+} -dependent PS exposure was only identified a decade ago as TMEM16F (anoctamin 6) (47–49). It is a member of the multiple transmembrane (TMEM)16 (anoctamin) domain family of proteins, of which the first-described member, TMEM16A, is a Ca^{2+} -activated Cl^- channel. TMEM16F has been described to be a Ca^{2+} -dependent Cl^- channel, a Ca^{2+} -regulated non-selective cation channel permeable for Ca^{2+} , or a Ca^{2+} -dependent phospholipid scramblase (49). Evidence is accumulating that TMEM16F is indeed itself a scramblase [e.g., Watanabe et al. (50); Le et al. (51)].

PLATELET PHOSPHATIDYLSERINE EXPOSURE

Several different pathways result in procoagulant platelet formation. In one, PS exposure occurs rapidly *via* platelet activation by strong agonists. A second is the intrinsic apoptosis pathway *via* which PS exposure occurs more slowly (9, 37, 52, 53). These pathways are considered in turn below, and key characteristics are summarized in **Table 1**. In a recently described third pathway that is distinct from the aforementioned canonical pathways, binding of oxidized low-density lipoprotein to platelet membrane glycoprotein (GP)IV (CD36) and signaling through extracellular signal-regulated protein kinase (ERK)5 mitogen-activated protein (MAP) kinase leads to PS exposure. This pathway may be relevant in thrombotic events that occur in dyslipidemia (54).

TABLE 1 | Summary of key characteristics of platelet agonist- and apoptosis-induced PS exposure (Agonist-Induced Phosphatidylserine Exposure and Apoptosis-Induced Phosphatidylserine Exposure).

	Agonist-induced platelet PS exposure (fast response)	Apoptosis-induced platelet PS exposure (slow response)
Trigger	<i>Physiologically relevant:</i> engagement of both GPVI (collagen/convulxin/CRP) and PAR1/PAR4 (thrombin) receptors <i>Non-physiological:</i> Ca ²⁺ ionophores A23187, ionomycin	Inhibition of pro-survival Bcl-xL, resulting in activation of proapoptotic Bak and Bax <i>Non-physiological:</i> ABT-737
Cytoplasmic Ca ²⁺ (Ca ²⁺ _{cyt}) concentrations	Sustained, elevated Ca ²⁺ _{cyt} levels required	Not dependent on sustained, elevated Ca ²⁺ _{cyt} levels
Mitochondrial integrity	Loss of IMM integrity: • Dependence on MPTP formation • ΔΨ _m depolarization occurs	MOMP occurs early, with loss of IMM integrity occurring concomitant with PS exposure
Intracellular protease activation	• Calpain activated • Caspase activation occurs, but PS exposure is not dependent on it	• Dependent on caspase activation
TMEM16F	Required	Not essential
Morphology	• Rounded, blebbing platelets essentially empty of cytoplasmic contents • EVs are shed	• Rounded, blebbing platelets with cytoplasmic contents remaining • EVs are shed
<i>In vivo</i> effects (in animal models)	• In arterial thrombi, PS-exposing platelets form microdomains that do not participate in platelet aggregation • PS-exposing platelets continue to circulate in the bloodstream	• Formation of venous thrombi inhibited • Thrombocytopenia, with PS exposure persisting on remaining circulating platelets

Ca²⁺_{cyt}, cytoplasmic Ca²⁺; CRP, collagen-related peptide; EV, extracellular vesicle; GP, glycoprotein; IMM, inner mitochondrial membrane; MOMP, mitochondrial outer membrane permeabilization; MPTP, mitochondrial permeability transition pore; PAR, protease-activated receptor; PS, phosphatidylserine; ΔΨ_m, inner mitochondrial membrane potential.

Agonist-Induced Phosphatidylserine Exposure

Agonist-stimulated platelet surface PS exposure is a rapid process, occurring in seconds to minutes, and is accompanied by other apoptotic-like events, including mitochondrial membrane permeabilization and depolarization, and plasma membrane blebbing, with extracellular vesicle (EV) formation. The proportion of PS-exposing platelets formed depends on the agonist(s) used for platelet stimulation, with the most potent *in vitro*, physiologically relevant stimulus being the combination of collagen/convulxin/collagen-related peptide (CRP) plus thrombin (C+T). The former binds to GPVI, and the latter cleaves protease-activated receptor (PAR)1 and PAR4 (23), synergizing to set into motion the signaling pathways that result in the sustained, supramaximal levels of Ca²⁺_{cyt} (55) (see below) that are required for PS exposure on a substantial proportion of platelets. Anywhere from 20 to 40% of C+T-stimulated platelets become PS-exposing, with a wide variation between donors; singly, these agonists are not as potent, with a smaller proportion of PS-exposing platelets being formed (56). ADP or thromboxane A₂ (TxA₂) (using the stable mimetic U46619) does not play a major role (23, 33), while shear forces are effective (57, 58). The non-physiological, non-receptor-mediated ionophores A23187, and ionomycin, that directly increase Ca²⁺_{cyt}, are the most potent stimulators of PS exposure, with typically >90% of platelets taking on the procoagulant phenotype (19, 56).

Platelet stimulation by collagen (or convulxin/CRP) or thrombin [or the PAR-specific thrombin receptor activating peptides (TRAPs)] alone activates phospholipase (PL)Cγ

(via GPVI) and PLCβ (via PAR1/4), resulting in a rise in Ca²⁺_{cyt} to the micromolar range (23). Cleavage of membrane phosphatidylinositol-4,5-bisphosphate by PLC forms inositol trisphosphate (IP₃) and diacylglycerol (DAG); the former induces the release of Ca²⁺ from internal stores, the dense tubular system (DTS), via IP₃ receptors. Depletion of internal Ca²⁺ stores allows store-operated Ca²⁺ entry (SOCE) from the platelet exterior: briefly, stromal interaction molecule 1 (STIM1) in the DTS membrane undergoes a conformational change, allowing activation of Orai1, the major Ca²⁺ release-activated Ca²⁺ channel in the platelet plasma membrane (23). DAG, together with Ca²⁺, activates protein kinase C (PKC)α that enhances Na⁺/Ca²⁺ exchange during SOCE (59, 60). Sustained increases in Ca²⁺_{cyt} via release from internal stores, SOCE, and release of mitochondrial Ca²⁺ upon mitochondrial permeability transition pore (MPTP) formation (see below) in a small proportion of platelets activate scramblase. It is the dual stimulation of GPVI and PAR1/4 by C+T that leads to the sustained, elevated levels of Ca²⁺_{cyt} necessary for PS exposure in a substantial proportion of platelets; the combination of C+T activates store-independent, receptor-operated Ca²⁺ entry (ROCE). This involves non-selective cation transient receptor potential C (TRPC) channels, TRPC3 and TRPC6, that allow Na⁺ entry. Coupling to reverse-mode Na⁺/Ca²⁺ exchange then leads to the sustained elevated Ca²⁺_{cyt} that activates scramblase (61).

Ca²⁺_{cyt} increases activation of the Ca²⁺-dependent cysteine protease calpain that has a number of substrates in platelets, including cytoskeletal components, signaling molecules, and

the $\beta 3$ integrin subunit, thereby regulating many platelet responses, including spreading, secretion, aggregation, and EV formation (36, 62–64). In platelets stimulated to expose PS, calpain-2-mediated proteolysis of $\alpha \text{IIb}\beta 3$ -associated proteins and $\beta 3$ results in inactivation of $\alpha \text{IIb}\beta 3$, the integrin necessary for platelet aggregation (65); thus, procoagulant platelets are unable to participate in aggregation. To that end, both in flow chambers coated with collagen and in mouse models of arterial thrombosis, two distinct microdomains of platelets are visualized in thrombi: 1) aggregated, non-PS-exposing platelets with extended pseudopods and activated $\alpha \text{IIb}\beta 3$; surrounded by 2) PS-exposing platelets that have elevated $\text{Ca}^{2+}_{\text{cyt}}$, inactivated $\alpha \text{IIb}\beta 3$, a rounded morphology, and are shedding EVs (see below) (32, 36). Further, in these *ex vivo* and *in vivo* systems, PS-exposing platelets are observed to translocate to the surface of thrombi where they accelerate fibrin formation (66).

Mitochondrial integrity loss is an apoptosis hallmark that precedes agonist-induced PS exposure, with involvement of the inner mitochondrial membrane (IMM) Formation of the cyclophilin D-regulated MPTP, a non-selective multiprotein pore that spans the IMM, is a key step (67, 68), as PS exposure is reduced in convulxin+thrombin-stimulated cyclophilin D-deficient platelets or platelets treated with cyclosporin A, an MPTP inhibitor (69–71). Reactive oxygen species, e.g., hydrogen peroxide (H_2O_2), that can trigger MPTP formation, synergize with thrombin to expose platelet PS, indicating a role for oxidative stress in procoagulant platelet formation (69). Sustained MPTP formation leads to disruption of the IMM potential ($\Delta\Psi_{\text{m}}$) (72, 73), and $\Delta\Psi_{\text{m}}$ depolarization is associated with PS exposure both in agonist-stimulated platelets *in vitro* and platelets aging *in vivo* (69, 71, 74–77).

In convulxin+thrombin-stimulated platelets, PS exposure is entirely dependent on $\Delta\Psi_{\text{m}}$ loss and TMEM16F. However, there is evidence of a second minor pathway of PS exposure that occurs with collagen+thrombin stimulation that is independent of these (76, 78). This pathway may also be involved in the mitochondrial depolarization-independent PS exposure observed in A23187-stimulated platelets in the presence of cyclosporin A (79). Heterogeneity within the PS-exposing platelet subpopulation has also been reported by Topalov et al. (80): one subset with high $\text{Ca}^{2+}_{\text{cyt}}$, $\Delta\Psi_{\text{m}}$ loss, and inactive $\alpha \text{IIb}\beta 3$; and another with low $\text{Ca}^{2+}_{\text{cyt}}$, intact $\Delta\Psi_{\text{m}}$, and active $\alpha \text{IIb}\beta 3$. Subsequently, this latter subset was described to be the result of the interaction between a procoagulant platelet and an aggregatory (non-PS-exposing) platelet (81).

Although caspase activation occurs upon mitochondrial depolarization and has been used as a marker in studies of agonist-induced platelet PS exposure, e.g., caspase-3 (75, 79), the agonist-induced pathway of procoagulant platelet formation appears to be independent of caspase activation (52, 82).

Near-complete shedding of GPIIb α and GPVI mediated by ADAM17 and 10, respectively, is accompanied by PS exposure and modulates platelet function from less adhesive to more procoagulant (83).

In becoming procoagulant, platelets undergo remarkable morphological changes. Platelets adherent to collagen/CRP, but

not fibrinogen, spread and transform into blebbing, rounded, balloon-like structures (31, 34). These collagen-adherent balloon platelets are PS exposing, as determined by annexin A5 binding (32, 33). Similarly, platelets stimulated in suspension by C+T or A23187 form a distinct PS-exposing platelet subpopulation with a spherical, balloon-like morphology, almost devoid of granules and normal internal architecture (**Figure 1**) (35, 84). This ballooning has been attributed to activation of Ca^{2+} -activated Cl^- channels, resulting in initial salt entry into platelets, which is then followed by the influx of water (33). There is increased permeability of plasma membrane of the PS-exposing platelets to low-molecular-weight molecules (33, 71).

The unique surface protein coating of procoagulant platelets (Introduction) has been observed primarily localized to a small, convex structure, or cap, rather than distributed uniformly on the PS-exposing platelet surface; 85% of PS-exposing platelets possess caps, with one per platelet (21, 41). It was subsequently demonstrated that procoagulant activity is located in the cap, or remnant platelet body, early in balloon-platelet development, then becomes predominant in the balloon-like structure at later time points (85). Recently, it was observed that more than 90% of PS-exposing platelets possess one or multiple associated EVs that exhibit heterogeneity in platelet membrane glycoproteins and activation markers different from platelet-derived EVs free in suspension (see below) (**Figure 1**) (35).

PS exposure is accompanied by the release of membrane-bound EVs (previously referred to as microparticles or microvesicles) from the platelet plasma membrane (86); indeed, Scott syndrome platelets, that are deficient in PS exposure upon activation (Pathologies of Phosphatidylserine Exposure), are also deficient in EV formation (87, 88). EV surface membranes are heterogeneous in their expression of platelet membrane glycoproteins, e.g., $\alpha \text{IIb}\beta 3$ and GPIIb-IX-V, and activation markers, e.g., CD62P (P-selectin), CD63, and activated $\alpha \text{IIb}\beta 3$ (35, 89, 90), and only about half expose PS (86). Platelet-derived EVs support hemostasis and also play a role in platelet–cell communications, delivering bioactive molecules, e.g., cytokines, eicosanoids, RNA species, to target cells [e.g., Boilard et al. (91)]. Elevated circulating EV levels have been reported in thrombotic conditions, immune-mediated conditions, and malignancy and inflammatory conditions, but whether they are “active contributors” or “passive indicators” of these conditions is not known (86). EVs are cleared rapidly (92), implying that they must be produced continuously for circulating levels to be detected.

Once procoagulant platelet formation has been stimulated *in vitro*, PS exposure is not readily reversed; this is likely due, at least in part, to inhibition of translocase activity (56, 76, 93), preventing the flipping of PS to the internal membrane leaflet. Even *in vivo*, PS exposure persists on activated platelets, as demonstrated by a study in which rabbit platelets stimulated to expose PS *in vitro* continued to circulate when injected into recipient rabbits (94). Although PS is a clearance signal of apoptotic cells by macrophages (see below), there are examples of cells that express PS constitutively and are viable (95, 96).

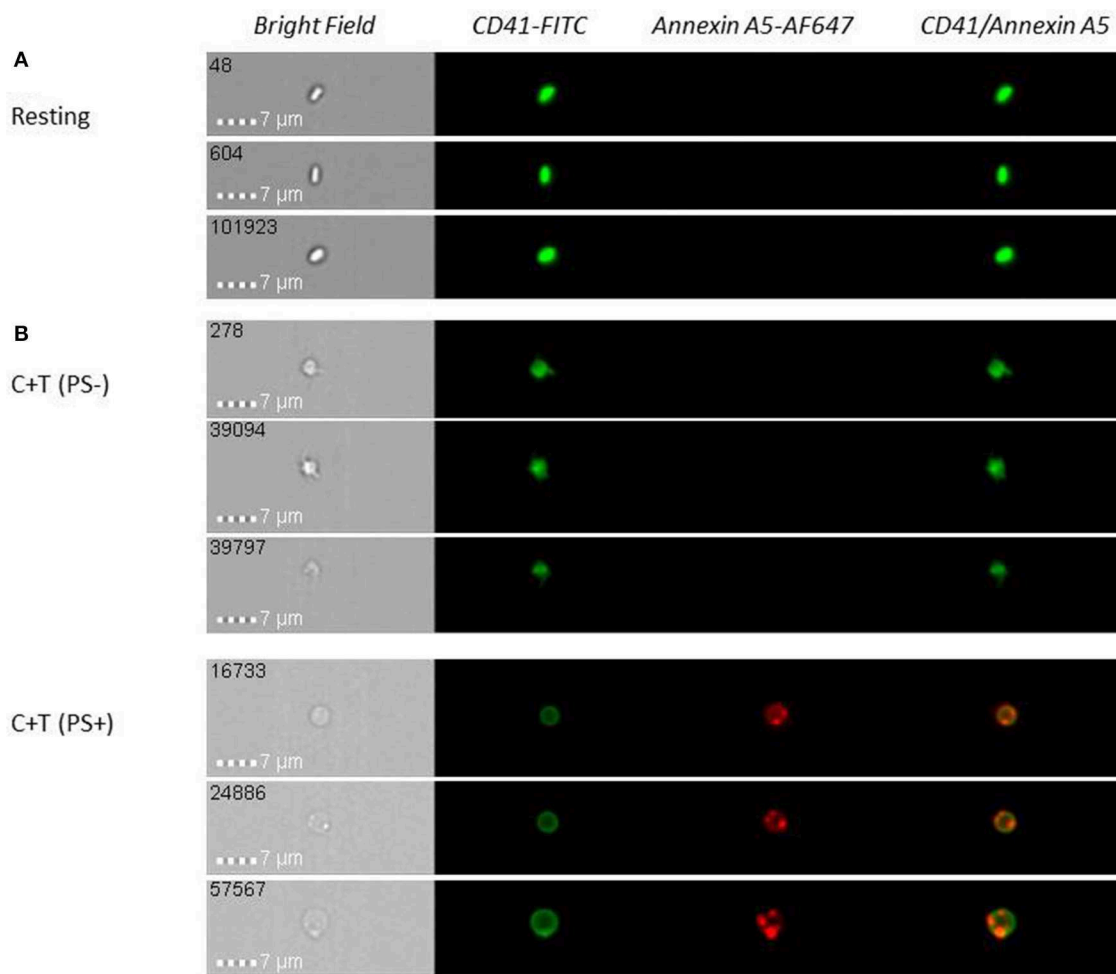


FIGURE 1 | Upon platelet activation with collagen (10 μg/ml) + thrombin (1 U/ml) (C + T), a subpopulation of phosphatidylserine (PS)-exposing platelets with a round, balloon-like morphology and one or more associated extracellular vesicles (EVs) is formed. Resting (unstimulated) (A) or stimulated (B) washed platelets were labeled with anti-CD41-FITC (platelet marker) and annexin A5-Alexa Fluor 647 (PS exposure) and analyzed on an Amnis ImageStream^X Mark II (IS^X) multispectral imaging flow cytometer. Images representative of *n* = 4 independent experiments.

It is still not clear why there is platelet response heterogeneity to becoming PS exposing. Certainly, it is not due to differences in overall platelet reactivity, as PS-exposing platelets express CD62P to the same extent as non-PS-exposing platelets (9), indicating that they are capable of the secretion event. Although it had previously been attributed to platelet age, with young platelets, identified by increased thiazole orange staining, having an enhanced capacity to take on a procoagulant phenotype (22, 97), uptake of this dye is increased in large platelets that are not necessarily young platelets in the steady state (98). Recently, it has been shown that young, newly produced, steady-state (rabbit) platelets are indeed less responsive in exposing PS than older platelets (98). It is speculated that differences in receptor expression levels and in Ca^{2+} -flux machinery activity are involved (99), and levels of adhesive receptors, including GPVI, GPIbα, αIIb, and β3, have been reported to be increased on procoagulant platelets (97, 100).

Apoptosis-Induced Phosphatidylserine Exposure

It is now well-recognized that platelets, although they are anucleate, can undergo apoptosis. This occurs *via* the intrinsic, mitochondrial-dependent pathway, with platelets possessing the necessary cytosolic machinery, while likely lacking the death receptors required for the extrinsic pathway (37, 101, 102). Platelet apoptosis can be initiated *in vitro* by ABT-737, a BH3-only protein mimetic, that inhibits the pro-survival Bcl-2 family protein Bcl-xL, resulting in activation of the proapoptotic Bcl-2 proteins Bak and Bax; these then go on to initiate mitochondrial damage, cytochrome c release, caspase activation, PS exposure, and membrane blebbing and EV formation (37, 52, 101), all hallmarks of apoptosis in nucleated cells.

In contrast to platelet agonist-induced PS exposure, which occurs rapidly, apoptosis-induced PS exposure occurs more slowly, on the order of hours (9, 37, 52, 53). This apoptosis

pathway is indeed Bak and Bax dependent, as platelets lacking these proteins do not externalize PS when incubated with ABT-737. It is also caspase dependent, as responses are abolished in the presence of a caspase inhibitor (52, 78). However, it does not require increases in $\text{Ca}^{2+}_{\text{cyt}}$ or calpain activity, which are necessary for agonist-induced PS exposure (52) (Agonist-Induced Phosphatidylserine Exposure). There is early mitochondrial outer membrane permeabilization (MOMP), followed by later IMM disruption concomitant with PS exposure (81): MPTP formation may not be involved, and $\Delta\Psi_m$ depolarization may occur (52, 103–105). PS-exposing platelets take on a rounded morphology but maintain cytoplasmic components (105, 106); EV formation is not observed early on, but increases with time (103, 104, 107).

ABT-737 treatment of platelets has also been reported to result in PS exposure on a second platelet population at a higher level, observed with agonist-induced PS exposure, than described above. PS exposure on this population is dependent on increases in $\text{Ca}^{2+}_{\text{cyt}}$ and TMEM16F but unlike agonist-induced PS exposure is dependent on caspase activation (78, 107).

Apoptosis, being the process of programmed cell death, is a major physiological mechanism that regulates the life span of cells, with externalized PS being the “eat me” recognition signal for phagocytic cells to mediate clearance of damaged cells (108). Platelet apoptosis regulates circulating platelet life span, as mutations in Bcl-xL result in shortened platelet survival, while deletion of Bak and Bax prolongs it by almost 2-fold (109, 110). Administration of ABT-737 to dogs and mice causes dramatic thrombocytopenia within 2 h (109, 111), but PS exposure persists on the circulating platelets (106); formation of venous thrombi is inhibited (106). Platelet PS exposure may be involved in physiological platelet clearance; the proportion of PS-exposing platelets increases as rabbit platelets age in the circulation under steady-state conditions (77).

PATHOLOGIES OF PHOSPHATIDYLSERINE EXPOSURE

The importance of activated platelet membrane phospholipid bilayer scrambling with resulting PS exposure in hemostasis is highlighted in the very rare inherited autosomal recessive disorder Scott syndrome. The first-described patient, Mrs. M.A. Scott, had a relatively severe bleeding phenotype: she was found to have an isolated defect in PF3a (Introduction) (112); impaired PS exposure upon platelet activation, thereby resulting in deficient procoagulant activity and abrogated fibrin formation at sites of vascular damage (3, 113); and diminished EV formation (Agonist-Induced Phosphatidylserine Exposure). The genetic defect in four of the six known Scott syndrome patients for whom mutational analysis is available, as well as in canine Scott syndrome, in German Shepherd dogs, involves homozygous and heterozygous variants in the *TMEM16F* gene (47, 49, 114–118), resulting in an absence of expression of the TMEM16F protein (Scramblase and TMEM16F). Knockout of *TMEM16F* in genetically modified mice recapitulates the Scott syndrome phenotype (119–122).

Studies of Scott syndrome platelets have shown that, in humans, TMEM16F is required for the major agonist-induced PS exposure pathway but is not essential for apoptosis-induced PS exposure (78). In contrast, in dogs, both agonist- and apoptosis-induced platelet PS exposure requires TMEM16F (116). Detailed proteomic profiling of human Scott syndrome platelets has provided insight into protein modifications that occur when platelets are activated to expose PS (123).

In contrast with the Scott syndrome, in the rare autosomal dominant Stormorken syndrome, resting platelets have elevated surface PS exposure; resting $\text{Ca}^{2+}_{\text{cyt}}$ is increased due to a novel *STIM1* gain-of-function variant. Stormorken syndrome patients have thrombocytopenia and a mild bleeding diathesis along with their thrombocytopathy (124).

Another inherited disorder in which elevated PS exposure and $\text{Ca}^{2+}_{\text{cyt}}$ is observed with resting platelets is the microthrombocytopenia, Wiskott–Aldrich syndrome (WAS). Upon stimulation, WAS platelets have increased susceptibility to PS exposure that occurs as a result of MPTP opening (125, 126).

Resting platelets from Bernard–Soulier syndrome patients also have elevated PS exposure, independent of their large size (127), indicating a role for GPIb-IX-V. PS exposure is generally increased in activated BSS platelets as well and is accompanied by $\Delta\Psi_m$ depolarization in a proportion of platelets (127).

Aberrant PS exposure has been described in certain acquired platelet disorders. Platelets from patients with immune thrombocytopenia have increased (apoptosis-induced) PS exposure likely contributing to the decreased platelet counts (128, 129). Recently, there has been the report of increased circulating PS-exposing ballooned platelets in trauma hemorrhage in response to the damage-associated molecular pattern histone H4, demonstrating a mechanism by which platelets respond to tissue damage (130).

CONCLUSION: POTENTIAL OF PROCOAGULANT PHOSPHATIDYLSERINE-EXPOSING PLATELETS AS AN ANTITHROMBOTIC TARGET

Understanding of the mechanisms involved in the formation of the procoagulant PS-exposing platelet phenotype and its role in hemostasis has increased dramatically. Since traditional antiplatelet agents do not completely reduce the risk of thromboembolic events, the question arises: Is PS exposure a useful antithrombotic target? Not surprisingly, drugs that target the ADP and TxA_2 pathways of platelet activation and aggregation have no major effect on inhibiting platelet procoagulant activity (38, 131, 132) since these agonists are not particularly potent in stimulating the formation of PS-exposing platelets (Agonist-Induced Phosphatidylserine Exposure). There are certainly indications that the procoagulant platelet might be a useful target in reducing thrombosis. Firstly, knockout of *TMEM16F* in platelets of genetically

modified mice (Pathologies of Phosphatidylserine Exposure) decreases platelet thrombus formation *in vitro* on collagen-coated coverslips under flow conditions and in models of arterial and venous thrombosis (119–122). Secondly, there is evidence that in clinical conditions of thrombosis, specifically coronary artery disease, and essential thrombocythemia, the procoagulant platelet response is increased, and that increased levels of procoagulant platelets are associated with increased risk for recurrent infarction in lacunar and non-lacunar stroke and predict incident stroke after transient ischemic attack (131, 133–136). Thirdly, procoagulant platelets have recently been shown to play a critical role in forming neutrophil macroaggregates that promote pulmonary thrombosis after gut ischemia that is a potent inducer of platelet PS exposure on the endothelium in the intestines, liver, and lungs; large membrane fragments ripped from PS-exposing platelets in a shear-dependent fashion wrap around the neutrophils to form adhesive bridges (137).

Thus, since PS exposure persists on activated platelets not only *in vitro* but *in vivo* as well (76, 94), blocking of the procoagulant surface could be an effective, novel strategy to reduce thrombosis. By binding to PS, annexin A5, diannexin

[a recombinant annexin A5 homodimer with a longer circulating half-life than annexin A5 and a 10-fold higher binding affinity to PS (138, 139)] and lactadherin inhibit thrombus formation *in vitro* and in animal models of arterial and venous thrombosis (139–145). However, diannexin and lactadherin impair hemostasis as well, increasing murine tail bleeding time blood loss (139, 145); thus, if a strategy of blocking exposed PS is to be pursued, dosages of blocking compounds must be finely tuned.

Alternatively, mitochondrial depolarization (70), scramblase activity (146, 147), or water entry into platelets (33, 148) are potential targets to inhibit formation of the thrombin-generating subpopulation of platelets while still allowing platelet aggregation to occur. It may be that inhibition of procoagulant platelet formation could be an alternative approach to reduce thrombosis without impairing hemostasis.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

- Revel-Vilk S, Rand ML, Israels SJ. Primary and secondary hemostasis, regulators of coagulation, and fibrinolysis: understanding the basics. In: Blanchette VS, Brandao LR, Breakey VR, Revel-Vilk S, editors. *SickKids Handbook of Pediatric Thrombosis and Hemostasis*. 2nd ed, Basel: Karger (2017). p. 8–16. doi: 10.1159/000446684
- Monroe DM, Hoffman M, Roberts HR. Platelets and thrombin generation. *Arterioscler Thromb Vasc Biol.* (2002) 22:1381–9. doi: 10.1161/01.ATV.0000031340.68494.34
- Weiss HJ. Impaired platelet procoagulant mechanisms in patients with bleeding disorders. *Semin Thromb Hemost.* (2009) 35:233–41. doi: 10.1055/s-0029-1220331
- Zwaal RF, Schroit AJ. Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. *Blood.* (1997) 89:1121–32. doi: 10.1182/blood.V89.4.1121
- Schreuder M, Reitsma PH, Bos MHA. Blood coagulation factor Va's key interactive residues and regions for prothrombinase assembly and prothrombin binding. *J Thromb Haemost.* (2019) 17:1229–39. doi: 10.1111/jth.14487
- Gilbert GE, Novakovic VA, Kaufman RJ, Miao H, Pipe SW. Conservative mutations in the C2 domains of factor VIII and factor V alter phospholipid binding and cofactor activity. *Blood.* (2012) 120:1923–32. doi: 10.1182/blood-2012-01-408245
- Zwaal RF, Comfurius P, and Bevers EM. Lipid-protein interactions in blood coagulation. *Biochim Biophys Acta.* (1998) 1376:433–53. doi: 10.1016/S0304-4157(98)00018-5
- Heemskerk JW, Bevers EM, Lindhout T. Platelet activation and blood coagulation. *Thromb Haemost.* (2002) 88:186–93. doi: 10.1055/s-0037-1613209
- Bevers EM, Williamson PL. Getting to the outer leaflet: physiology of phosphatidylserine exposure at the plasma membrane. *Physiol Rev.* (2016) 96:605–45. doi: 10.1152/physrev.00020.2015
- Tavoosi N, Davis-Harrison RL, Pogorelov TV, Ohkubo YZ, Arcario MJ, Clay MC, et al. Molecular determinants of phospholipid synergy in blood clotting. *J Biol Chem.* (2011) 286:23247–53. doi: 10.1074/jbc.M111.251769
- Clark SR, Thomas CP, Hammond VJ, Aldrovandi M, Wilkinson GW, Hart KW, et al. Characterization of platelet aminophospholipid externalization reveals fatty acids as molecular determinants that regulate coagulation. *Proc Natl Acad Sci USA.* (2013) 110:5875–80. doi: 10.1073/pnas.1222419110
- Lauder SN, Allen-Redpath K, Slatter DA, Aldrovandi M, O'Connor A, Farewell D, et al. Networks of enzymatically oxidized membrane lipids support calcium-dependent coagulation factor binding to maintain hemostasis. *Sci Signal.* (2017) 10:eaan2787. doi: 10.1126/scisignal.aan2787
- Camera M, Toschi V, Brambilla M, Lettino M, Rossetti L, Canzano P, et al. The role of tissue factor in atherothrombosis and coronary artery disease: insights into platelet tissue factor. *Semin Thromb Hemost.* (2015) 41:737–46. doi: 10.1055/s-0035-1564041
- Brambilla M, Facchinetti L, Canzano P, Rossetti L, Ferri N, Balduini A, et al. Camera: human megakaryocytes confer tissue factor to a subset of shed platelets to stimulate thrombin generation. *Thromb Haemost.* (2015) 114:579–92. doi: 10.1160/TH14-10-0830
- Panes O, González C, Hidalgo P, Valderas JP, Acevedo M, Contreras S, et al. Platelet tissue factor activity and membrane cholesterol are increased in hypercholesterolemia and normalized by rosuvastatin, but not by atorvastatin. *Atherosclerosis.* (2017) 257:164–171. doi: 10.1016/j.atherosclerosis.2016.12.019
- Matus V, Valenzuela JG, Hidalgo P, Pozo LM, Panes O, Wozniak A. Human platelet interaction with *E. coli* O111 promotes tissue-factor-dependent procoagulant activity, involving Toll like receptor 4. *PLoS ONE.* (2017) 12:e0185431. doi: 10.1371/journal.pone.0185431
- Brambilla M, Gelosa P, Rossetti L, Castiglioni L, Zara C, Canzano P, et al. Impact of angiotensin-converting enzyme inhibition on platelet tissue factor expression in stroke-prone rats. *J Hypertens.* (2018) 36:1360–1371. doi: 10.1097/HJH.0000000000001702
- Capozzi A, Manganelli V, Riitano G, Recalchi S, Truglia S, Alessandri C, et al. Tissue factor over-expression in platelets of patients with anti-phospholipid syndrome: induction role of anti-β2-GPI antibodies. *Clin Exp Immunol.* (2019) 196:59–66. doi: 10.1111/cei.13248
- Dachary-Prigent J, Freyssinet JM, Pasquet JM, Carron JC, Nurden AT. Annexin V as a probe of aminophospholipid exposure and platelet membrane vesiculation: a flow cytometry study showing a role for free sulfhydryl groups. *Blood.* (1993) 81:2554–65. doi: 10.1182/blood.V81.10.2554.2554
- Dasgupta SK, Guchhait P, Thiagarajan P. Lactadherin binding and phosphatidylserine expression on cell surface-comparison with annexin A5. *Transl Res.* (2006) 148:19–25. doi: 10.1016/j.lab.2006.03.006
- Podoplelova NA, Sveshnikova AN, Kotova YN, Eckly A, Receveur N, Nechipurenko DY, et al. Coagulation factors bound to procoagulant platelets

- concentrate in cap structures to promote clotting. *Blood*. (2016) 128:1745–55. doi: 10.1182/blood-2016-02-696898
22. Alberio A, Safa O, Clemetson KJ, Esmon CT, Dale GL. Surface expression and functional characterization of alpha-granule factor V in human platelets: effects of ionophore A23187, thrombin, collagen, and convulxin. *Blood*. (2000) 95:1694–702. doi: 10.1182/blood.V95.5.1694.005k24_1694_1702
 23. Heemskerk JW, Mattheij NJ, Cosemans JM. Platelet-based coagulation: different populations, different functions. *J Thromb Haemost*. (2013) 11:2–16. doi: 10.1111/jth.12045
 24. Swieringa F, Spronk HHM, Heemskerk JWM, van der Meijden JPE. Integrating platelet and coagulation activation in fibrin clot formation. *Res Pract Thromb Haemost*. (2018) 2:450–460. doi: 10.1002/rth2.12107
 25. London FS, Marcinkiewicz M, Walsh PN. A subpopulation of platelets responds to thrombin- or SFLLRN-stimulation with binding sites for factor IXa. *J Biol Chem*. (2004) 279:19854–9. doi: 10.1074/jbc.M310624200
 26. Alberio LJ, Clemetson KJ. All platelets are not equal: COAT platelets. *Curr Hematol Rep*. (2004) 3:338–43
 27. Dale GL, Friese P, Batar P, Hamilton SF, Reed GL, Jackson KW, et al. Stimulated platelets use serotonin to enhance their retention of procoagulant proteins on the cell surface. *Nature*. (2002) 415:175–9. doi: 10.1038/415175a
 28. Dale GL. Coated-platelets: an emerging component of the procoagulant response. *J Thromb Haemost*. (2005) 3:2185–92. doi: 10.1111/j.1538-7836.2005.01274.x
 29. Mattheij NJ, Swieringa F, Mastenbroek TG, Berny-Lang MA, May F, Baaten CC, et al. Coated platelets function in platelet-dependent fibrin formation via integrin $\alpha_{IIb}\beta_3$ and transglutaminase factor XIII. *Haematologica*. (2016) 101:427–36. doi: 10.3324/haematol.2015.131441
 30. van der Meijden JPE, Heemskerk MJW. Platelet biology and functions: new concepts and clinical perspectives. *Nat Rev Cardiol*. (2019) 16:166–179. doi: 10.1038/s41569-018-0110-0
 31. Heemskerk JW, Vuisst WM, Feijge MA, Reutelingsperger CP, Lindhout T. Collagen but not fibrinogen surfaces induce bleb formation, exposure of phosphatidylserine, and procoagulant activity of adherent platelets: evidence for regulation by protein tyrosine kinase-dependent Ca^{2+} responses. *Blood*. (1997) 90:2615–25. doi: 10.1182/blood.V90.7.2615.2615_2615_2625
 32. Munnix IC, Kuijpers MJ, Auger J, Thomassen CM, Panizzi P, van MA, et al. Segregation of platelet aggregatory and procoagulant microdomains in thrombus formation: regulation by transient integrin activation. *Arterioscler Thromb Vasc Biol*. (2007) 27:2484–90. doi: 10.1161/ATVBAHA.107.151100
 33. Agbani EO, den Bosch VMT, Brown E, Williams CM, Mattheij NJ, Cosemans JM, et al. Coordinated membrane ballooning and procoagulant spreading in human platelets. *Circulation*. (2015) 132:1414–24. doi: 10.1161/CIRCULATIONAHA.114.015036
 34. Hess MW, Siljander P. Procoagulant platelet balloons: evidence from cryopreparation and electron microscopy. *Histochem Cell Biol*. (2001) 115:439–43. doi: 10.1007/s004180100272
 35. Reddy EC, Wang H, Christensen H, McMillan-Ward E, Israels SJ, Bang KWA, et al. Analysis of procoagulant phosphatidylserine-exposing platelets by imaging flow cytometry. *Res Pract Thromb Haemost*. (2018) 2:736–750. doi: 10.1002/rth2.12144
 36. Kulkarni S, Jackson SP. Platelet factor XIII and calpain negatively regulate integrin $\alpha_{IIb}\beta_3$ adhesive function and thrombus growth. *J Biol Chem*. (2004) 279:30697–706. doi: 10.1074/jbc.M40359200
 37. Jackson SP, Schoenwaelder SM. Procoagulant platelets: are they necrotic? *Blood*. (2010) 116:2011–8. doi: 10.1182/blood-2010-01-261669
 38. Hua VM, Abeynake L, Glaros E, Campbell H, Pasalic L, Hogg PJ, et al. Necrotic platelets provide a procoagulant surface during thrombosis. *Blood*. (2015) 126:2852–62. doi: 10.1182/blood-2015-08-663005
 39. Hua VM, Chen VM. Procoagulant platelets and the pathways leading to cell death. *Semin Thromb Hemost*. (2015) 41:405–12. doi: 10.1055/s-0034-1544002
 40. Mazepa M, Hoffman M, Monroe D. Superactivated platelets: thrombus regulators, thrombin generators, and potential clinical targets. *Arterioscler Thromb Vasc Biol*. (2013) 33:1747–52. doi: 10.1161/ATVBAHA.113.301790
 41. Abaeva AA, Canault M, Kotova YN, Obyednny SI, Yakimenko AO, Podoplelova NA, et al. Procoagulant platelets form an α -granule protein-covered “cap” on their surface that promotes their attachment to aggregates. *J Biol Chem*. (2013) 288:29621–32. doi: 10.1074/jbc.M113.474163
 42. Storrie B. A tip of the cap to procoagulant platelets. *Blood*. (2016) 128:1668–9. doi: 10.1182/blood-2016-08-730622
 43. Agbani EO, Poole AW. Procoagulant platelets: generation, function, and therapeutic targeting in thrombosis. *Blood*. (2017) 130:2171–2179. doi: 10.1182/blood-2017-05-787259
 44. Segawa K, Kurata S, Nagata S. Human Type IV P-type ATPases that work as plasma membrane phospholipid flippases and their regulation by caspase and calcium. *J Biol Chem*. (2016) 291:762–72. doi: 10.1074/jbc.M115.690727
 45. Belezney Z, Zachowski A, Devaux PF, Navazo MP, Ott P. ATP-dependent aminophospholipid translocation in erythrocyte vesicles: stoichiometry of transport. *Biochemistry*. (1993) 32:3146–52. doi: 10.1021/bi00063a029
 46. Bitbol M, Fellmann P, Zachowski P, Devaux PF. Ion regulation of phosphatidylserine and phosphatidylethanolamine outside-inside translocation in human erythrocytes. *Biochim Biophys Acta*. (1987) 904:268–82. doi: 10.1016/0005-2736(87)90376-2
 47. Suzuki J, Umeda M, Sims PJ, Nagata S. Calcium-dependent phospholipid scrambling by TMEM16F. *Nature*. (2010) 468:834–8. doi: 10.1038/nature09583
 48. Lhermusier T, Chap H, Payraastre B. Platelet membrane phospholipid asymmetry: from the characterization of a scramblase activity to the identification of an essential protein mutated in Scott syndrome. *J Thromb Haemost*. (2011) 9:1883–91. doi: 10.1111/j.1538-7836.2011.04478.x
 49. Kunzelmann K, Nilius B, Owsianik G, Schreiber R, Ousingsawat J, Sirianant L, et al. Molecular functions of anoctamin 6 (TMEM16F): a chloride channel, cation channel, or phospholipid scramblase? *Pflugers Arch*. (2014) 466:407–14. doi: 10.1007/s00424-013-1305-1
 50. Watanabe R, Sakuragi T, Noji H, Nagata S. Single-molecule analysis of phospholipid scrambling by TMEM16F. *Proc Natl Acad Sci USA*. (2018) 115:3066–71. doi: 10.1073/pnas.1717956115
 51. Le T, Jia Z, Le SC, Zhang Y, Chen J, Yang H. An inner activation gate controls TMEM16F phospholipid scrambling. *Nat Commun*. (2019) 10:1846. doi: 10.1038/s41467-019-09778-7
 52. Schoenwaelder SM, Yuan Y, Josefsson EC, White MJ, Yao Y, Mason KD, et al. Two distinct pathways regulate platelet phosphatidylserine exposure and procoagulant function. *Blood*. (2009) 114:663–6. doi: 10.1182/blood-2009-01-200345
 53. Zwaal RF, Comfurius P, Bevers EM. Surface exposure of phosphatidylserine in pathological cells. *Cell Mol Life Sci*. (2005) 62:971–88. doi: 10.1007/s00018-005-4527-3
 54. Yang M, Kholmukhamedov A, Schulte ML, Cooley BC, Scoggins NO, Wood JP, et al. Platelet CD36 signaling through ERK5 promotes caspase-dependent procoagulant activity and fibrin deposition *in vivo*. *Blood Adv*. (2018) 2:2848–61. doi: 10.1182/bloodadvances.2018025411
 55. Abbasian N, Millington-Burgess SL, Chabra S, Malcor JD, Harper MT. Supramaximal calcium signaling triggers procoagulant platelet formation. *Blood Adv*. (2020) 4:154–64. doi: 10.1182/bloodadvances.2019000182
 56. Wolfs JL, Comfurius P, Rasmussen JT, Keuren JF, Lindhout T, Zwaal RF, et al. Activated scramblase and inhibited aminophospholipid translocase cause phosphatidylserine exposure in a distinct platelet fraction. *Cell Mol Life Sci*. (2005) 62:1514–25. doi: 10.1007/s00018-005-5099-y
 57. Leytin V, Allen DJ, Mykhaylov S, Mis L, Lyubimov EV, Garvey B, et al. Pathologic high shear stress induces apoptosis events in human platelets. *Biochem Biophys Res Commun*. (2004) 320:303–10. doi: 10.1016/j.bbrc.2004.05.166
 58. Pang A, Cui Y, Chen Y, Cheng N, Delaney MK, Gu M, et al. Shear-induced integrin signaling in platelet phosphatidylserine exposure, microvesicle release, and coagulation. *Blood*. (2018) 132:533–43. doi: 10.1182/blood-2017-05-785253
 59. Harper MT, Molkenin JD, Poole AW. Protein kinase C α enhances sodium-calcium exchange during store-operated calcium entry in mouse platelets. *Cell Calcium*. (2010) 48:333–40. doi: 10.1016/j.ceca.2010.10.006
 60. Alberio L, Ravanat C, Hechler B, Mangin PH, Lanza F, Gachet C. Delayed-onset of procoagulant signalling revealed by kinetic analysis of COAT platelet formation. *Thromb Haemost*. (2017) 117:1101–14. doi: 10.1160/TH16-09-0711
 61. Harper MT, Londoño JE, Quick K, Londoño JC, Flockner V, Philipp SE, et al. Transient receptor potential channels function as a coincidence signal

- detector mediating phosphatidylserine exposure. *Sci Signal.* (2013) 6:ra50. doi: 10.1126/scisignal.2003701
62. Croce K, Flaumenhaft R, Rivers M, Furie B, Furie BC, Herman IM, et al. Inhibition of calpain blocks platelet secretion, aggregation, and spreading. *J Biol Chem.* (1999) 274:36321–7. doi: 10.1074/jbc.274.51.36321
 63. Schoenwaelder SM, Yuan Y, Jackson SP. Calpain regulation of integrin α IIb β 3 signaling in human platelets. *Platelets.* (2000) 11:189–98. doi: 10.1080/09537100050057620
 64. Fox JE. Cytoskeletal proteins and platelet signaling. *Thromb Haemost.* (2001) 86:198–213. doi: 10.1055/s-0037-1616218
 65. Mattheij NJ, Gilio K, van Kruchten R, Jobe SM, Wieschhaus AJ, Chishti AH, et al. Dual mechanism of integrin α IIb β 3 closure in procoagulant platelets. *J Biol Chem.* (2013) 288:13325–36. doi: 10.1074/jbc.M112.428359
 66. Nechipurenko DY, Receveur N, Yakimenko AO, Shepelyuk TO, Yakusheva AA, Kerimov RR, et al. Clot contraction drives the translocation of procoagulant platelets to thrombus surface. *Arterioscler Thromb Vasc Biol.* (2019) 39:37–47. doi: 10.1161/ATVBAHA.118.311390
 67. Baines CP, Kaiser RA, Purcell NH, Blair NS, Osinska H, Hambleton MA, et al. Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature.* (2005) 434:658–62. doi: 10.1038/nature03434
 68. Basso E, Fante L, Fowlkes J, Petronilli V, Forte MA, Bernardi P. Properties of the permeability transition pore in mitochondria devoid of Cyclophilin D. *J Biol Chem.* (2005) 280:18558–61. doi: 10.1074/jbc.C500089200
 69. Jobe SM, Wilson KM, Leo L, Raimondi A, Molkentin JD, Lentz SR, et al. Critical role for the mitochondrial permeability transition pore and Cyclophilin D in platelet activation and thrombosis. *Blood.* (2008) 111:1257–65. doi: 10.1182/blood-2007-05-092684
 70. Choo HJ, Saafir TB, Mkumba L, Wagner MB, Jobe SM. Mitochondrial calcium and reactive oxygen species regulate agonist-initiated platelet phosphatidylserine exposure. *Arterioscler Thromb Vasc Biol.* (2012) 32:2946–55. doi: 10.1161/ATVBAHA.112.300433
 71. Remenyi G, Szasz R, Friese P, Dale GL. Role of mitochondrial permeability transition pore in coated-platelet formation. *Arterioscler Thromb Vasc Biol.* (2005) 25:467–71. doi: 10.1161/01.ATV.0000152726.49229.bf
 72. Petit PX, Zamzami N, Vayssi  re JL, Mignotte B, Kroemer G, Castedo M. Implication of mitochondria in apoptosis. *Mol Cell Biochem.* (1997) 174:185–8. doi: 10.1007/978-1-4615-6111-8_28
 73. Halestrap AP, McStay GP, Clarke SJ. The permeability transition pore complex: another view. *Biochimie.* (2002) 84:153–66. doi: 10.1016/S0300-9084(02)01375-5
 74. Leytin V, Freedman J. Platelet apoptosis in stored platelet concentrates and other models. *Transfus Apher Sci.* (2003) 28:285–95. doi: 10.1016/S1473-0502(03)00048-X
 75. Leytin V, Allen DJ, Mykhaylov S, Lyubimov E, Freedman J. Thrombin-triggered platelet apoptosis. *J Thromb Haemost.* (2006) 4:2656–63. doi: 10.1111/j.1538-7836.2006.02200.x
 76. Leung R, Gwozdz AM, Wang H, Bang KW, Packham MA, Freedman J, et al. Persistence of procoagulant surface expression on activated human platelets: involvement of apoptosis and aminophospholipid translocase activity. *J Thromb Haemost.* (2007) 5:560–70. doi: 10.1111/j.1538-7836.2007.02354.x
 77. Rand ML, Wang H, Bang KW, Poon KS, Packham MA, Freedman J. Procoagulant surface exposure and apoptosis in rabbit platelets: association with shortened survival and steady-state senescence. *J Thromb Haemost.* (2004) 2:651–9. doi: 10.1111/j.1538-7836.2004.00670.x
 78. van Kruchten R, Mattheij NJ, Saunders C, Feijge MA, Swieringa F, Wolfs JL, et al. Both TMEM16F-dependent and TMEM16F-independent pathways contribute to phosphatidylserine exposure in platelet apoptosis and platelet activation. *Blood.* (2013) 121:1850–7. doi: 10.1182/blood-2012-09-454314
 79. Leytin V, Allen DJ, Mutlu A, Gylkhandanyan AV, Mykhaylov S, Freedman J. Mitochondrial control of platelet apoptosis: effect of cyclosporin A, an inhibitor of the mitochondrial permeability transition pore. *Lab Invest.* (2009) 89:374–84. doi: 10.1038/labinvest.2009.13
 80. Topalov NN, Yakimenko AO, Canault M, Artemenko EO, Zakharova NV, Abaeva AA, et al. Two types of procoagulant platelets are formed upon physiological activation and are controlled by integrin α IIb β 3. *Arterioscler Thromb Vasc Biol.* (2012) 32:2475–83. doi: 10.1161/ATVBAHA.112.253765
 81. Choo HJ, Kholmukhamedov A, Zhou C, Jobe S. Inner mitochondrial membrane disruption links apoptotic and agonist-initiated phosphatidylserine externalization in platelets. *Arterioscler Thromb Vasc Biol.* (2017) 37:1503–12. doi: 10.1161/ATVBAHA.117.309473
 82. Wolf BB, Goldstein JC, Stennicke HR, Beere H, Amarante-Mendes GP, Salvesen GS, et al. Calpain functions in a caspase-independent manner to promote apoptosis-like events during platelet activation. *Blood.* (1999) 94:1683–92. doi: 10.1182/blood.V94.5.1683
 83. Baaten FCCM, Swieringa F, Misztal T, Mastenbroek TG, Feijge HMA, Bock PE, et al. Platelet heterogeneity in activation-induced glycoprotein shedding: functional effects. *Blood Adv.* (2018) 2:2320–31. doi: 10.1182/bloodadvances.2017011544
 84. Gerrard JM, White JG, Rao GH. Effects of the ionophore A23187 on the blood platelets II. Influence on ultrastructure. *Am J Pathol.* (1974) 77:151–66.
 85. Agbani EO, Hers I, Poole AW. Temporal contribution of the platelet body and balloon to thrombin generation. *Haematologica.* (2017) 102:e379–81. doi: 10.3324/haematol.2017.166819
 86. Gasecka A, Nieuwland R, Siljander PR-M. Platelet-derived extracellular vesicles. In: A. Michelson, M. Cattaneo, A. Frelinger and P. Newman editors. *Platelets* 4th ed. London: Academic Press Platelets (2019). p. 401–16. doi: 10.1016/B978-0-12-813456-6.00022-9
 87. Sims PJ, Wiedmer T., Esmon CT, Weiss HJ, Shattil SJ. Assembly of the platelet prothrombinase complex is linked to vesiculation of the platelet plasma membrane. studies in scott syndrome: an isolated defect in platelet procoagulant activity. *J Biol Chem.* (1989) 264:17049–57.
 88. Weiss HJ. Scott syndrome: a disorder of platelet coagulant activity. *Semin Hematol.* (1994) 31:312–9.
 89. Boilard E, Ducheze AC, Brisson A. The diversity of platelet microparticles. *Curr Opin Hematol.* (2015) 22:437–44. doi: 10.1097/MOH.0000000000000166
 90. Ponomareva AA, Nevzorova TA, Mordakhanova ER, Andrianova IA, Rauova L, Litvinov RI, et al. Intracellular origin and ultrastructure of platelet-derived microparticles. *J Thromb Haemost.* (2017) 15:1655–67. doi: 10.1111/jth.13745
 91. Boilard E, Nigrovic PA, Larabee K, Watts GF, Coblyn JS, Weinblatt ME, et al. Platelets amplify inflammation in arthritis via collagen-dependent microparticle production. *Science.* (2010) 327:580–3. doi: 10.1126/science.1181928
 92. Rand ML, Wang H, Bang KW, Packham MA, Freedman J. Rapid clearance of procoagulant platelet-derived microparticles from the circulation of rabbits. *J Thromb Haemost.* (2006) 4:1621–3. doi: 10.1111/j.1538-7836.2006.02011.x
 93. S  dergren AL, Ramstr  m S. Platelet subpopulations remain despite strong dual agonist stimulation and can be characterised using a novel six-colour flow cytometry protocol. *Sci Rep.* (2018) 8:1441. doi: 10.1038/s41598-017-19126-8
 94. Rand ML, Wang H, Bang KW, Packham MA, Freedman J. Persistence of phosphatidylserine exposure on activated platelets *in vivo* in rabbits. *Thromb Haemost.* (2007) 98:477–8. doi: 10.1160/TH06-12-0702
 95. Segawa K, Suzuki J, Nagata S. Constitutive exposure of phosphatidylserine on viable cells. *Proc Natl Acad Sci USA.* (2011) 108:19246–51. doi: 10.1073/pnas.1114799108
 96. Dillon SR, Mancini M, Rosen A, Schlissel MS, Annexin V binds to viable B cells and colocalizes with a marker of lipid rafts upon B cell receptor activation. *J Immunol.* (2000) 164:1322–32. doi: 10.4049/jimmunol.164.3.1322
 97. Fager AM, Wood JP, Bouchard BA, Feng P, Tracy PB. Properties of procoagulant platelets: defining and characterizing the subpopulation binding a functional prothrombinase. *Arterioscler Thromb Vasc Biol.* (2010) 30:2400–7. doi: 10.1161/ATVBAHA.110.216531
 98. Reddy EC, Wang H, Bang AKW, Packham MA, Rand ML. Young steady-state rabbit platelets do not have an enhanced capacity to expose procoagulant phosphatidylserine. *Platelets.* (2018) 29:27–33. doi: 10.1080/09537104.2017.1295434

99. Baaten CCMJ, Ten Cate H, van der Meijden JPE, Heemskerk JWM. Platelet populations and priming in hematological diseases. *Blood Rev.* (2017) 31:389–99. doi: 10.1016/j.blre.2017.07.004
100. Furihata K, Clemetson KJ, Deguchi H, Kunicki TJ. Variation in human platelet glycoprotein VI content modulates glycoprotein VI-specific prothrombinase activity. *Arterioscler Thromb Vasc Biol.* (2001) 21:1857–63. doi: 10.1161/hq1001.096643
101. Leytin V. Apoptosis in the anucleate platelet. *Blood Rev.* (2012) 26:51–63. doi: 10.1016/j.blre.2011.10.002
102. Winkler J, Rand ML, Schmugge M, Speer O. Omi/HtrA2 and XIAP are components of platelet apoptosis signalling. *Thromb Haemost.* (2013) 109:532–9. doi: 10.1160/TH12-06-0404
103. Gyulkhanyan AV, Mutlu A, Allen DJ, Freedman J, Leytin V. BH3-mimetic ABT-737 induces strong mitochondrial membrane depolarization in platelets but only weakly stimulates apoptotic morphological changes, platelet shrinkage and microparticle formation. *Thromb Res.* (2014) 133:73–9. doi: 10.1016/j.thromres.2013.10.041
104. Gyulkhanyan AV, Mutlu A, Freedman J, Leytin V. Mitochondrial permeability transition pore (MPTP)-dependent and -independent pathways of mitochondrial membrane depolarization, cell shrinkage and microparticle formation during platelet apoptosis. *Br J Haematol.* (2015) 169:142–5. doi: 10.1111/bjh.13180
105. Vogler M, Hamali HA, Sun XM, Bampton ET, Dinsdale D, Snowden RT, et al. BCL2/BCL-X(L) inhibition induces apoptosis, disrupts cellular calcium homeostasis, and prevents platelet activation. *Blood.* (2011) 117:7145–54. doi: 10.1182/blood-2011-03-344812
106. Schoenwaelder SM, Jarman KE, Gardiner EE, Hua M, Qiao J, White MJ, et al. Bcl-xL-inhibitory BH3 mimetics can induce a transient thrombocytopenia that undermines the hemostatic function of platelets. *Blood.* (2011) 118:1663–74. doi: 10.1182/blood-2011-04-347849
107. Wei H, Harper MT. ABT-737 triggers caspase-dependent inhibition of platelet procoagulant extracellular vesicle release during apoptosis and secondary necrosis *in vitro*. *Thromb Haemost.* (2019) 19:1665–74. doi: 10.1055/s-0039-1693694
108. Segawa K, Nagata S. An apoptotic “eat me” signal: phosphatidylserine exposure. *Trends Cell Biol.* (2015) 25:639–50. doi: 10.1016/j.tcb.2015.08.003
109. Mason KD, Carpinelli MR, Fletcher JL, Collinge JE, Hilton AA, Ellis S, et al. Programmed anuclear cell death delimits platelet life span. *Cell.* (2007) 128:1173–86. doi: 10.1016/j.cell.2007.01.037
110. Kile BT. The role of the intrinsic apoptosis pathway in platelet life and death. *J Thromb Haemost.* (2009) 7(Suppl.1):214–7. doi: 10.1111/j.1538-7836.2009.03366.x
111. Zhang H, Nimmer PM, Tahir SK, Chen J, Fryer RM, Hahn KR, et al. Bcl-2 family proteins are essential for platelet survival. *Cell Death Differ.* (2007) 14:943–51. doi: 10.1038/sj.cdd.4402081
112. Weiss HJ, Vivic WJ, Lages BA, Rogers J. Isolated deficiency of platelet procoagulant activity. *Am J Med.* (1979) 67:206–13. doi: 10.1016/0002-9343(79)90392-9
113. Zwaal RF, Comfurius P, Bevers EM. Scott syndrome, a bleeding disorder caused by defective scrambling of membrane phospholipids. *Biochim Biophys Acta.* (2004) 1636:119–28. doi: 10.1016/j.bbali.2003.07.003
114. Castoldi E, Collins PW, Williamson PL, Bevers EM. Compound heterozygosity for 2 novel TMEM16F mutations in a patient with Scott syndrome. *Blood.* (2011) 117:4399–40. doi: 10.1182/blood-2011-01-332502
115. Boisseau P, Bene MC, Besnard T, Pachcek S, Giraud M, Talarmain P, et al. A new mutation of ANO6 in two familial cases of Scott syndrome. *Br J Haematol.* (2018) 180:750–2. doi: 10.1111/bjh.14439
116. Brooks MB, Catalfamo JL, MacNguyen R, Tim D, Fancher S, McCardle JA. A TMEM16F point mutation causes an absence of canine platelet TMEM16F and ineffective activation and death-induced phospholipid scrambling. *J Thromb Haemost.* (2015) 13:2240–52. doi: 10.1111/jth.13157
117. Flores-Nascimento MC, Orsi FL, Yokoyama AP, Pereira FG, Lorand-Metze I, De EV, et al. Diagnosis of Scott syndrome in patient with bleeding disorder of unknown cause. *Blood Coagul Fibrinolysis.* (2012) 23:75–7. doi: 10.1097/MBC.0b013e32834d0c81
118. Millington-Burgess SL, Harper MT. Gene of the issue. *Platelets.* (2019) 1–4. doi: 10.1080/09537104.2019.1693039
119. Yang H, Kim A, David T, Palmer D, Jin T, Tien J, et al. TMEM16F forms a Ca²⁺-activated cation channel required for lipid scrambling in platelets during blood coagulation. *Cell.* (2012) 151:111–22. doi: 10.1016/j.cell.2012.07.036
120. Fujii T, Sakata A, Nishimura S, Eto K, Nagata S. TMEM16F is required for phosphatidylserine exposure and microparticle release in activated mouse platelets. *Proc Natl Acad Sci USA.* (2015) 112:12800–5. doi: 10.1073/pnas.1516594112
121. Mattheij NJ, Braun A, van Kruchten R, Castoldi E, Pircher J, Baaten CC, et al. Survival protein anoctamin-6 controls multiple platelet responses including phospholipid scrambling, swelling, and protein cleavage. *FASEB J.* (2016) 30:727–37. doi: 10.1096/fj.15-280446
122. Baig AA, Haining EJ, Geuss E, Beck S, Swieringa F, Wanitchakool P, et al. Nieswandt: TMEM16F-mediated platelet membrane phospholipid scrambling is critical for hemostasis and thrombosis but not thromboinflammation in mice—brief report. *Arterioscler Thromb Vasc Biol.* (2016) 36:2152–7. doi: 10.1161/ATVBAHA.116.307727
123. Solari FA, Mattheij NJ, Burkhardt JM, Swieringa F, Collins PW, Cosemans JM, et al. Combined quantification of the global proteome, phosphoproteome, and proteolytic cleavage to characterize altered platelet functions in the human Scott syndrome. *Mol Cell Proteomics.* (2016) 15:3154–69. doi: 10.1074/mcp.M116.060368
124. Misceo D, Holmgren A, Louch WE, Holme PA, Mizobuchi M, Morales RJ, et al. A dominant STIM1 mutation causes Stormorken syndrome. *Hum Mutat.* (2014) 35:556–64. doi: 10.1002/humu.22544
125. Shcherbina A, Rosen FS, Remold-O'Donnell E. Pathological events in platelets of Wiskott-Aldrich syndrome patients. *Br J Haematol.* (1999) 106:875–83. doi: 10.1046/j.1365-2141.1999.01637.x
126. Obyednyy SI, Artemenko EO, Sveshnikova AN, Ignatova AA, Varlamova TV, Gambaryan S, et al. Mechanisms of increased mitochondria-dependent necrosis in Wiskott-Aldrich syndrome platelets. *Haematologica.* (2019) 2018:214460. doi: 10.3324/haematol.2018.214460
127. Rand ML, Wang H, Bang KW, Teitel JM, Blanchette VS, Freedman J, et al. Phosphatidylserine exposure and other apoptotic-like events in Bernard-Soulier syndrome platelets. *Am J Hematol.* (2010) 85:584–92. doi: 10.1002/ajh.21768
128. Catani L, Fagioli ME, Tazzari PL, Ricci F, Curti A, Rovito M, et al. Dendritic cells of immune thrombocytopenic purpura (ITP) show increased capacity to present apoptotic platelets to T lymphocytes. *Exp Hematol.* (2006) 34:879–87. doi: 10.1016/j.exphem.2006.03.009
129. Winkler J, Kroiss S, Rand ML, Azzouzi I, Annie Bang KW, Speer O, et al. Platelet apoptosis in paediatric immune thrombocytopenia is ameliorated by intravenous immunoglobulin. *Br J Haematol.* (2012) 156:508–15. doi: 10.1111/j.1365-2141.2011.08973.x
130. Vulliamy P, Gillespie S, Armstrong PC, Allan HE, Warner TD, Brohi K. Histone H4 induces platelet ballooning and microparticle release during trauma hemorrhage. *Proc Natl Acad Sci USA.* (2019) 116:17444–9. doi: 10.1073/pnas.1904978116
131. Pasalic L, Wing-Lun E, Lau JK, Campbell H, Pennings GJ, Lau E, et al. Novel assay demonstrates that coronary artery disease patients have heightened procoagulant platelet response. *J Thromb Haemost.* (2018) 16:1198–210. doi: 10.1111/jth.14008
132. Agbani EO, Williams CM, Hers I, Poole AW. Membrane ballooning in aggregated platelets is synchronised and mediates a surge in microvesiculation. *Sci Rep.* (2017) 7:2770. doi: 10.1038/s41598-017-02933-4
133. Prodan CI, Stoner JA, Cowan LD, Dale GL. Higher coated-platelet levels are associated with stroke recurrence following non-lacunar brain infarction. *J Cereb Blood Flow Metab.* (2013) 33:287–92. doi: 10.1038/jcbfm.2012.168
134. Kirkpatrick AC, Tafur AJ, Vincent AS, Dale GL, Prodan CI. Coated-platelets improve prediction of stroke and transient ischemic attack in asymptomatic internal carotid artery stenosis. *Stroke.* (2014) 45:2995–3001. doi: 10.1161/STROKEAHA.114.006492
135. Tan CW, Wong WH, Campbell H, Cheong MA, Cheng J, Tohidi-Esfahani I, et al. Novel assay demonstrates increased procoagulant platelet response in essential thrombocythemia segregating with JAK2V617F mutation status and thrombosis. *Res Pract Thromb Haemost.* (2019) 3(Suppl.1):168–9. doi: 10.1002/rth2.12227

136. Kirkpatrick AC, Vincent AS, Dale GL, Prodan CI. Increased platelet procoagulant potential predicts recurrent stroke and TIA after lacunar infarction. *J Thromb Haemost.* (2019). doi: 10.1111/jth.14714. [Epub ahead of print].
137. Yuan Y, Alwis I, Wu LMC, Kaplan Z, Ashworth K, Bark D, et al. Neutrophil macroaggregates promote widespread pulmonary thrombosis after gut ischemia. *Sci Transl Med.* (2017) 9:aam5861. doi: 10.1126/scitranslmed.aam5861
138. Kuypers FA, Larkin SK, Emeis JJ, Allison AC. Interaction of an annexin V homodimer (Diannexin) with phosphatidylserine on cell surfaces and consequent antithrombotic activity. *Thromb Haemost.* (2007) 97:478–86. doi: 10.1160/TH06-08-0436
139. Rand ML, Wang H, Pluthero FG, Stafford AR, Ni R, Vaezzadeh N, et al. Diannexin, an annexin A5 homodimer, binds phosphatidylserine with high affinity and is a potent inhibitor of platelet-mediated events during thrombus formation. *J Thromb Haemost.* (2012) 10:1109–19. doi: 10.1111/j.1538-7836.2012.04716.x
140. Galán AM, van Heerde WL, Escolar G, Ordinas A, Sixma J, de Groot PG. Antithrombotic action of annexin V proved as efficient as direct inhibition of tissue factor or thrombin. *Eur J Clin Invest.* (2006) 36:633–9. doi: 10.1111/j.1365-2362.2006.01698.x
141. Römisch J, Seiffge D, Reiner G, Pâques EP, Heimburger N. *In-vivo* antithrombotic potency of placenta protein 4 (annexin V) *Thromb Res.* (1991) 61:93–104. doi: 10.1016/0049-3848(91)90236-P
142. Van Ryn-McKenna J, Merk H, Müller HT, Buchanan MR, Eisert WG. The effects of heparin and annexin V on fibrin accretion after injury in the jugular veins of rabbits. *Thromb Haemost.* (1993) 69:227–30. doi: 10.1055/s-0038-1651585
143. Thiagarajan P, Benedict CR. Inhibition of arterial thrombosis by recombinant annexin V in a rabbit carotid artery injury model. *Circulation.* (1997) 96:2339–47. doi: 10.1161/01.CIR.96.7.2339
144. Kuijpers MJ, Munnix IC, Cosemans JM, Vlijmen BV, Reutelingsperger CP, Egbrink MO, et al. Key role of platelet procoagulant activity in tissue factor- and collagen-dependent thrombus formation in arterioles and venules *in vivo* differential sensitivity to thrombin inhibition. *Microcirculation.* (2008) 15:269–82. doi: 10.1080/10739680701653517
145. Shi J, Pipe SW, Rasmussen JT, Heegaard CW, Gilbert GE. Lactadherin blocks thrombosis and hemostasis *in vivo*: correlation with platelet phosphatidylserine exposure. *J Thromb Haemost.* (2008) 6:1167–74. doi: 10.1111/j.1538-7836.2008.03010.x
146. Millington-Burgess SL, Rahman T, Harper MT. An empirical screen of the effects of polyphenolic compounds on platelet scramblase activity. *Res Pract Thromb Haemost.* (2019) 3(Suppl.1):571
147. Millington-Burgess SL, Rahman T, Harper MT. R5421 is not a selective inhibitor of platelet phosphatidylserine exposure. *Res Pract Thromb Haemost.* (2019) 3(Suppl.1):148–9.
148. Agbani EO, Williams CM, Li Y, van den Bosch MT, Moore SF, Mauroux A, et al. Aquaporin-1 regulates platelet procoagulant membrane dynamics and *in vivo* thrombosis. *JCI Insight.* (2018) 3:99062. doi: 10.1172/jci.insight.99062

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Reddy and Rand. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Platelet Pathogen Reduction Technologies Alter the MicroRNA Profile of Platelet-Derived Microparticles

Idrissa Diallo^{1,2}, Abderrahim Benmoussa^{1,2}, Jonathan Laugier^{1,2}, Abdimajid Osman^{3,4}, Walter E. Hitzler⁵ and Patrick Provost^{1,2*}

¹ Research Center of the CHU de Québec, Québec, QC, Canada, ² Department of Microbiology-Infectious Disease and Immunity, Faculty of Medicine, Université Laval, Québec, QC, Canada, ³ Department of Clinical Chemistry, Linköping University, Linköping, Sweden, ⁴ Department of Biomedical and Clinical Sciences, Linköping University, Linköping, Sweden, ⁵ Transfusion Center, University Medical Center, Johannes Gutenberg University Mainz, Mainz, Germany

OPEN ACCESS

Edited by:

Paul Jurasz,
University of Alberta, Canada

Reviewed by:

Vera Ignjatovic,
Royal Children's Hospital, Australia
Craig N. Jenne,
University of Calgary, Canada
Aneta Radziwon-Balicka,
Nordsjællands Hospital, Denmark

*Correspondence:

Patrick Provost
patrick.provost@
crchudequebec.ulaval.ca

Specialty section:

This article was submitted to
Atherosclerosis and Vascular
Medicine,
a section of the journal
Frontiers in Cardiovascular Medicine

Received: 04 October 2019

Accepted: 21 February 2020

Published: 19 March 2020

Citation:

Diallo I, Benmoussa A, Laugier J, Osman A, Hitzler WE and Provost P (2020) Platelet Pathogen Reduction Technologies Alter the MicroRNA Profile of Platelet-Derived Microparticles. *Front. Cardiovasc. Med.* 7:31. doi: 10.3389/fcvm.2020.00031

Despite improvements in donor screening and increasing efforts to avoid contamination and the spread of pathogens in clinical platelet concentrates (PCs), the risks of transfusion-transmitted infections remain important. Relying on an ultraviolet photo activation system, pathogen reduction technologies (PRTs), such as Intercept and Mirasol, utilize amotosalen, and riboflavin (vitamin B2), respectively, to mediate inactivation of pathogen nucleic acids. Although they are expected to increase the safety and prolong the shelf life of clinical PCs, these PRTs might affect the quality and function of platelets, as recently reported. Upon activation, platelets release microparticles (MPs), which are involved in intercellular communications and regulation of gene expression, thereby mediating critical cellular functions. Here, we have used small RNA sequencing (RNA-Seq) to document the effect of PRT treatment on the microRNA profiles of platelets and derived MPs. PRT treatment did not affect the microRNA profile of platelets. However, we observed a specific loading of certain microRNAs into platelet MPs, which was impaired by treatment with Intercept or its Additive solution (SSP+). Whereas, Intercept had an impact on the microRNA profile of platelet-derived MPs, Mirasol did not impact the microRNA profile of platelets and derived MPs, compared to non-treated control. Considering that platelet MPs are able to transfer their microRNA content to recipient cells, and that this content may exert biological activities, those findings suggest that PRT treatment of clinical PCs may modify the bioactivity of the platelets and MPs to be transfused and argue for further investigations into PRT-induced changes in clinical PC content and function.

Keywords: clinical platelet concentrate, pathogen reduction, mirasol, intercept, extracellular vesicles, small RNA-sequencing, microRNA

INTRODUCTION

Derived from giant megakaryocytes in the hematopoietic bone marrow, platelets are small discoid and anucleate blood elements (1). Platelets have a key hemostatic role and are guardians of the structural integrity of blood vessels (2). Upon activation, platelets release extracellular vesicles (EVs), known as microparticles (MPs) (3), along with other particles, such as free mitochondria and

mito-MPs (4). These MPs can mediate intercellular communications through delivery of bioactive molecules (5). With their rich content in protein-coding messenger RNAs, non-coding RNAs (e.g., microRNAs), cytokines, and lipids, platelet MPs may play an important role in gene regulation and homeostasis (5, 6). We reported previously that MPs released from human platelets can transfer functional microRNAs to human endothelial cells (1) and macrophages (2), in which platelet-derived microRNAs could regulate host cell gene expression.

Transfusion of clinical platelet concentrates (PCs) is required in various pathological conditions (e.g., thrombocytopenia, cancers, transplant surgery) (7) or for prophylactic purposes (8). In contrast to other blood products, clinical PCs, once collected and prepared (e.g., by apheresis or from buffy coats) are stored at room temperature under gentle agitation. This almost systematic procedure limits platelet storage to 5–7 days (9).

To increase their shelf life, these clinical PCs go through a series of routine tests and are sometimes treated with pathogen reduction technologies (PRTs) to eliminate the potential risks of viral, bacterial, fungal, or protozoal type contamination (10). The main PRTs available on the market are based on a photochemical principle (excitation at a given wavelength) to cross-link the pathogens' nucleic acids. The pathogen inhibitor Mirasol uses riboflavin (vitamin B2), a natural photochemical compound that binds to nucleic acids after UV exposure. This is followed by oxidation of guanine bases leading to single-strand breaks (11, 12). On the same principle, Intercept, along with its Additive solution, uses the properties of amotosalen to irreversibly block the replication of DNA and RNA, thus preventing the proliferation of pathogens (11, 13, 14). There are other pathogen inhibitors under development, such as Theraflex, which does not use photochemical compounds, but only ultraviolet C (UVC) irradiation (15).

However, these technologies, which are essentially based on photochemistry, do not receive unanimous agreement regarding their innocuousness on platelets and derived MPs, especially on their repertoire of functional nucleic acids (16). Although PRTs are convenient to reduce the risk of transfusion-transmitted infections, they can affect platelets and derived MPs' properties and, therefore, influence their functions. In fact, PRTs affect the metabolic (pH, sugars, nucleosides) and physiological parameters of platelets (e.g., increased platelet activation, reduced aggregation in response to agonists such as collagen, ADP, and thrombin, and increased risk of bleeding, as reported in several clinical cases) (17–20). However, less is known about their effects on the generation, content, and bioactivity of platelet MPs.

The advent of PRTs predated the discovery of the rich and functional nucleic acid content (e.g., messenger RNA, transfer RNA, microRNA, and long non-coding RNA) of platelets and derived MPs. Their effects on the profile of mRNAs and, in particular, microRNAs are yet to be unveiled. We have previously reported that PRT treatment of clinical PCs altered the level of some platelet microRNAs (21), suggesting that it might also alter the microRNA content of MPs derived from PRT-treated platelets. In this study, we have used small RNA sequencing (RNA-Seq) to characterize the microRNA profile of platelets,

treated or not with pathogen inhibitors Mirasol or Intercept, as well as that of the MPs derived from these platelets.

METHODS

Samples

The study was designed as previously described (19, 21). Briefly, clinical PCs were prepared from blood by apheresis using a standard blood bank protocol (19). PCs were subjected to one of the following four conditions: (1) Control (platelets stored in donor plasma); (2) Mirasol [platelets stored in donor plasma and treated with riboflavin and ultraviolet B (UVB) light]; (3) Additive solution [platelets stored in 65% storage solutions for platelets (SSP+; MacoPharma) and 35% donor plasma]; or (4) Intercept [platelets stored in SSP+ (Additive solution) and treated with amotosalen and UVA light]. For each group, 6 PC samples were pooled ($n = 6$ PCs per treatment, 24 samples in total, 4 pools). All PR treatments were performed according to the standard blood bank procedures or the manufacturer's instructions without modification.

Platelet Storage and MPs Isolation

Clinical PCs, treated or not with PRTs, or with the Additive solution, were stored under blood bank conditions (at room temperature under gentle rocking) for 7 days.

For platelet analysis, platelets were isolated from the PCs, as previously described (19), using anti-CD45 magnetic beads to minimize leukocyte co-isolation (<1 leukocyte per 3.2 million platelets; $<0.03\%$ of the platelet RNA preparations) (22, 23).

For MP analysis, platelets were sedimented at 1,000 g for 10 min. Platelet-free supernatant (PFS) was further spun a 18,000 g for 90 min to isolate platelet MPs.

RNA Isolation and Sequencing

Considering the role of MPs in intercellular communications, we analyzed the microRNA content of platelet MPs by RNA-Seq, as we did for platelets.

RNA Isolation

Total RNA from platelets or MPs was isolated using Trizol LS (Life Technologies—Ambion, Thermo-Fisher Scientific) and suspended in DEPC-treated DNase-RNase-free water (Invitrogen) prior to RNA purification and subsequent on-column DNase treatment using RNeasy mini-kit (Qiagen) following the manufacturer's protocol. Total RNA was then shipped on dry ice to the sequencing platform (ArrayStar).

Library Preparation

The purity, quality, and concentration of total RNA samples were determined using NanoDrop ND-1000 (Thermo Scientific) and 2100 Bioanalyzer (Agilent). Total RNA of each sample was used to prepare the microRNA sequencing library, which included the following steps: (1) 3'-adapter ligation, (2) 5'-adapter ligation, (3) cDNA synthesis, (4) PCR amplification, and (5) size selection of PCR amplified fragments of ~ 130 – 150 base pairs (corresponding to small RNAs of ~ 15 – 35 nucleotides). The complete libraries were quantified by Agilent 2100 Bioanalyzer.

Small RNA Sequencing

The samples were diluted to a final concentration of 8 pM and denatured as single-stranded DNA prior to cluster generation performed on an Illumina cBot using a TruSeq Rapid SR cluster kit (#GD-402-4001, Illumina). The clusters were then sequenced for 51 cycles on an Illumina HiSeq 2000 using TruSeq Rapid SBS Kits (#FC-402-4002, Illumina), as per the manufacturer's instructions.

Bioinformatics Analysis

The clean reads that passed the quality filter were processed to remove the adaptor sequence to generate the trimmed reads. All analyses displayed here were obtained from ArrayStar standard analysis pipeline and refined using R (Free Software Foundation). MicroRNA read counts were normalized as read counts per million microRNA alignments (RPM). Sequences known to be contaminant confounders from RNA isolation procedures were discarded before analysis.

Illustrations

Figures displayed in this manuscript were generated using R (Free Software Foundation), Inkscape software (Free Software Foundation) and/or Prism 8 (GraphPad Software, Inc.).

RESULTS

PRTs Have No Effect on the Most Abundant microRNAs in Platelets

We first analyzed, by RNA-Seq, the profile of microRNAs in platelets either not treated (Control) or treated with Mirasol, Additive solution, or Intercept. MicroRNA profiling and clustering data suggest that treatment of platelets with Mirasol, Additive solution, or Intercept altered the global profile of platelet microRNAs (**Figure 1A**). However, when looking at the 20 most abundant platelet microRNAs, we obtained similar profiles between the four experimental conditions (**Figure 1B**).

Certain MicroRNAs Might Be Specifically Loaded Into MPs

We observed that the 20 most abundant microRNAs, in both platelets (**Figure 1B**) and derived MPs (**Figure 2A**), comprised approximately two-thirds of all sequences. Fourteen of the 20 most abundant microRNAs in platelets were found among the 20 most abundant microRNAs in platelet MPs, suggesting that the microRNA profile of platelet MPs slightly differs from that of the platelets from which they derive (**Figure 2A** vs. **Figure 1B**).

Platelet MPs (780 unique sequences; **Figure 2A**) appeared to contain a more diverse array of microRNAs than platelets (544 unique sequences; **Figure 1B**), with the difference concerning mainly the least abundant sequences.

A correlation of the platelet and derived MP microRNAs suggests that some microRNAs may be specifically loaded in platelet MPs (**Figure 2B**). Among the top 20 microRNAs above 1,000 reads per million (RPM), hsa-miR-454-3p, hsa-miR-16-5p, and hsa-miR-20a-5p are the three microRNAs most specifically enriched in platelets (versus MPs) (**Figure 2C**). For platelet MPs,

the top three specific microRNAs were hsa-miR-320b, hsa-miR-320a, and hsa-miR-4433b-3p (**Figure 2D**).

microRNA Loading Into MPs Is Impacted by Intercept Treatment of Platelets

Linear regression analyses unveiled that Mirasol treatment of platelets had no effect on the microRNA content of the released MPs (**Figure 3A**). Whereas, the exposure of platelets to the vehicle of Intercept, Additive solution, slightly disturbed the MP microRNA correlation with MPs released from control platelets (**Figure 3B**), the treatment of platelets with Intercept had more profound effects (**Figure 3C**). A closer examination of **Figure 3C** revealed two groups of microRNAs: those that are enriched (the steepest slope) and those that are impoverished (the smoothest slope) in MPs released from Intercept-treated platelets. These results suggest that Intercept may interfere with the natural process underlying MP formation and released from platelets, leading to the production of MPs with a different content and, possibly, different bioactivity.

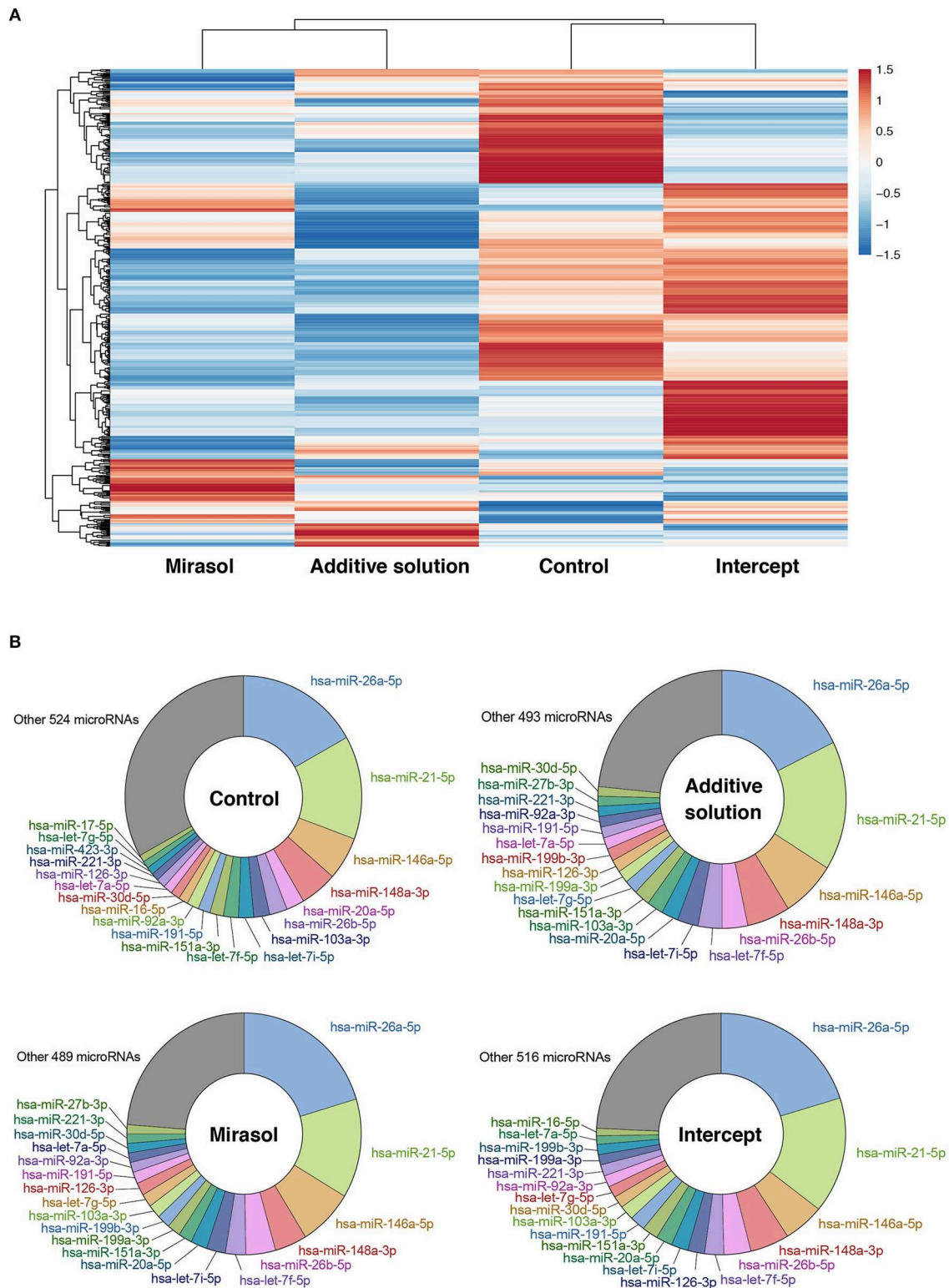
We observed the same trend when focusing our analysis to the 20 most abundant microRNAs (**Figure 3D** and **Tables 1–3**). Mirasol treatment led to a feeble decrease in the level of certain microRNAs, such as hsa-miR-22-3p or hsa-miR-423-5p, in MPs compared to control MPs (**Figure 3D** and **Table 1**). On the contrary, the Additive solution decreased the level of certain highly abundant microRNAs, such as hsa-miR-151a-3p, hsa-miR-320a, or hsa-miR-221-3p, while increasing specifically the level of hsa-miR-26a-5p (**Figure 3D** and **Table 2**). Intercept treatment led to a more pronounced alteration of MP microRNA content, with a decrease in hsa-miR-185-5p levels and an increased abundance of hsa-let7i-5p (up to 3 fold) and hsa-miR-451a (up to 6.3 fold), compared to its vehicle (Additive solution) (**Figure 3D** and **Table 3**).

Similar trends were observed when analyzing the data of the other, less abundant microRNAs (not in the top 20, but >1,000 RPM). The effect of Mirasol was very limited, consisting mainly in downregulation of some microRNAs (**Table 1**). As above, the Additive solution modified (decreased or increased) the level of several other MP microRNAs of lower abundance (**Table 2**). Similarly, Intercept downregulated some MP microRNAs, such as hsa-miR-151a-3p (0.3-fold change vs. control MPs), while increasing the level of other MP microRNAs, such as hsa-miR-144-3p (13.7-fold change vs. control MPs) (**Table 3**).

DISCUSSION

Overall, our results indicate that Mirasol treatment of platelets had no marked effect on the microRNA profile of derived MPs, while Intercept exposure induced a more pronounced alteration in microRNA levels in platelet-derived MPs, an effect to which its Additive solution vehicle likely contributes.

Storage and quality control of PCs is a major challenge in blood banks, for which several checkpoints are set up from blood/platelet collection to PC transfusion. Based on the use of photochemical products designed to interfere with the genetic material of pathogens, through chemically induced cross-linking



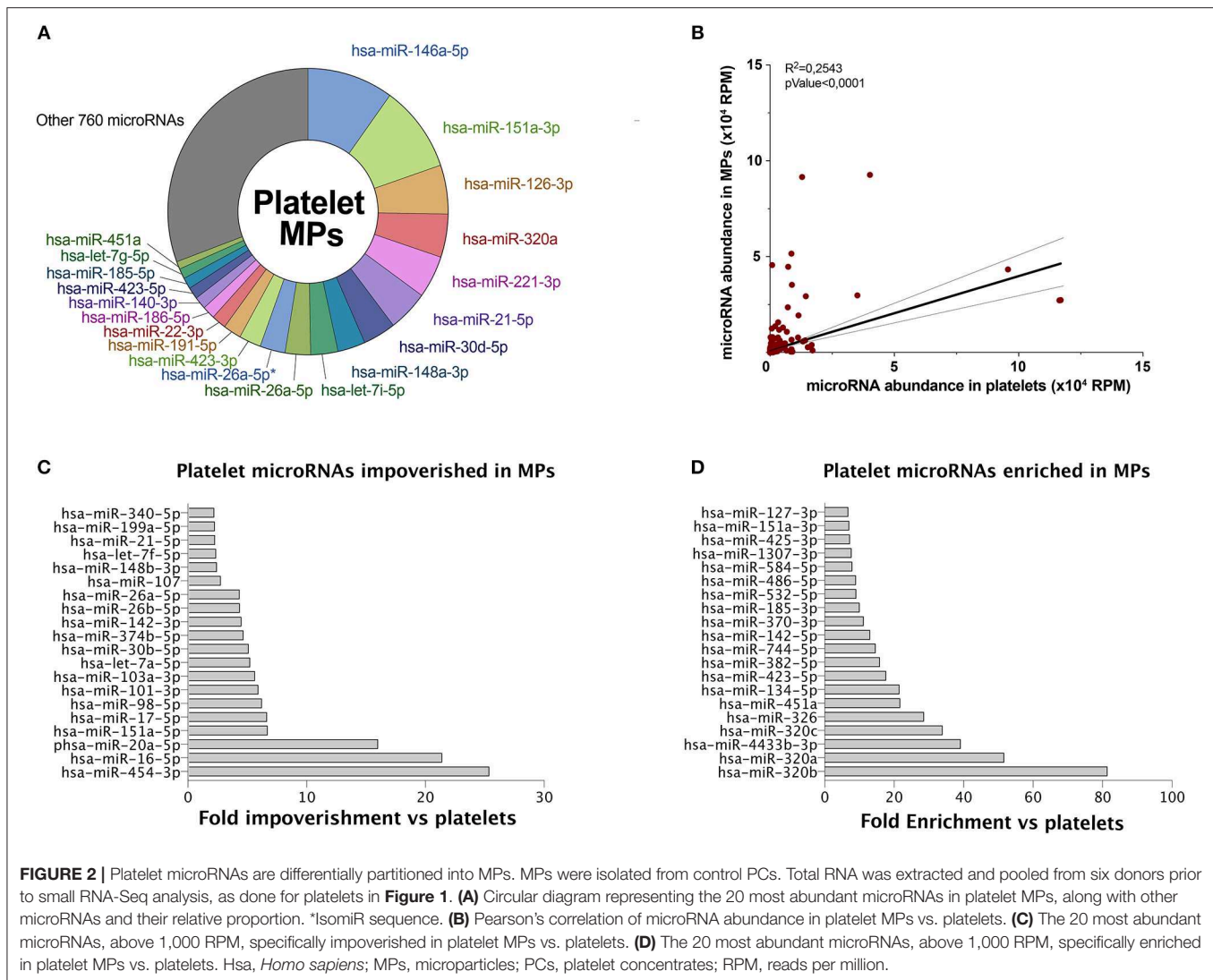


FIGURE 2 | Platelet microRNAs are differentially partitioned into MPs. MPs were isolated from control PCs. Total RNA was extracted and pooled from six donors prior to small RNA-Seq analysis, as done for platelets in **Figure 1**. **(A)** Circular diagram representing the 20 most abundant microRNAs in platelet MPs, along with other microRNAs and their relative proportion. *IsomiR sequence. **(B)** Pearson's correlation of microRNA abundance in platelet MPs vs. platelets. **(C)** The 20 most abundant microRNAs, above 1,000 RPM, specifically impoverished in platelet MPs vs. platelets. **(D)** The 20 most abundant microRNAs, above 1,000 RPM, specifically enriched in platelet MPs vs. platelets. Hsa, *Homo sapiens*; MPs, microparticles; PCs, platelet concentrates; RPM, reads per million.

of nucleic acids, PRTs had revolutionized the control of PC safety and prolonged their shelf life. These technologies, however, were developed before we learned about the existence of functional nucleic acids (e.g., microRNAs) in human platelets (22, 24). Indeed, platelets carry all the components of the transcription (25) and translation (26) machinery of classical cells. They also contain a wide range of non-coding RNAs, including microRNAs (25, 27). Therefore, we cannot exclude the possibility that PRTs might impact the functional activity and/or level of nucleic acids contained in platelets or its derived MPs.

In this study, we expanded our previous qPCR analyses of selected microRNAs (21) by performing small RNA-Seq microRNA analyses of platelets, treated or not with PRT. These analyses corroborated our previous findings that Intercept may alter microRNA levels in exposed platelets (21), in addition to modulating the platelet mRNA transcriptome (19), which suggest that these consequences might be related.

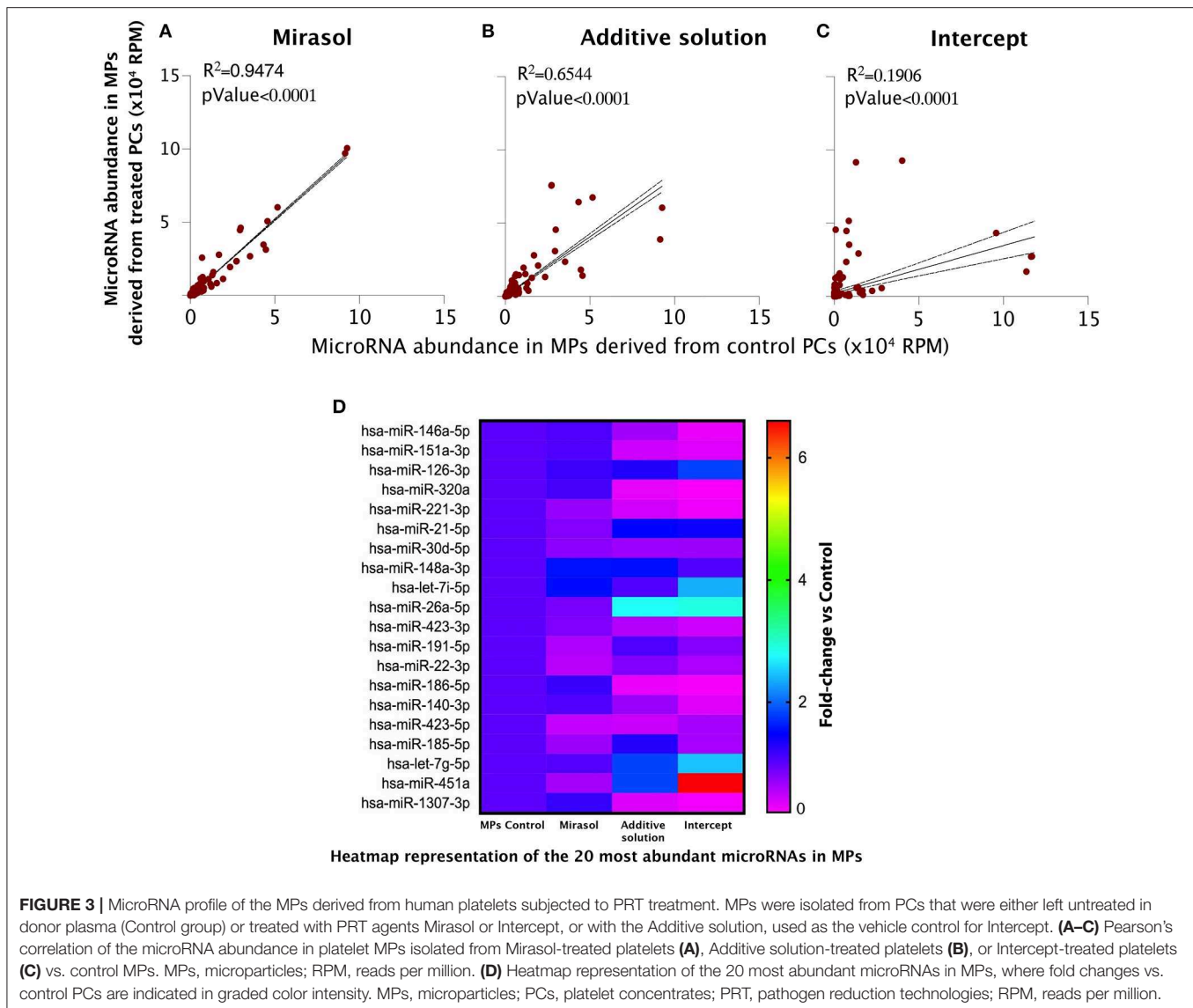
PRTs induced variations on less abundant microRNAs. While such differences might have limited functional consequences

individually, it is important to note that microRNAs generally act in clusters (28) and that changes, even subtle, in the level of several microRNAs might have an impact on platelet function or physiological response to agonists (21).

In the current study, we observed that platelets and their MPs had a different microRNA profile, suggesting a specific microRNA loading into MPs upon platelet activation. This difference, as also suggested by Diehl et al. (29), reflects an active and selective mechanism for packaging platelet microRNAs in MPs, which may confer specific regulatory functions to MPs.

Like outer membrane vesicles from bacteria (30) or other EV-like exosomes in eukaryotes (31), the profile of the genetic material, embedded in EVs, often depends on the physiological context and EV formation mechanisms at play in the cell of origin. It is, therefore, of importance to assess the effects that PRTs can have on the microRNA profile of platelet MPs.

Although we detected important differences in the microRNA repertoire of MPs derived from platelets exposed to Intercept



or its Additive solution vehicle, none of the PRT treatments influenced the profile of microRNA isoforms (also known as isomiRs; **Supplementary File 1**). These observations suggest that PRT may influence microRNA loading into MPs rather than microRNA processing events.

Several studies have reported reduced platelet function upon Intercept treatment (19, 21, 32–34). Intercept has been shown to induce activation of p38, diminish the level of CD42 (also known as GpIb, which, in its complex, allows platelet adhesion and platelet clogging) (35), and deregulate expression of the pro-survival gene Bcl-xl and anti-apoptotic genes (21). In addition, to modulate the platelet mRNA transcriptome (19), Intercept was reported to alter platelet characteristics (mean volume), activation level, and aggregation response to physiological agonists (21). As the loading of genetic material (e.g., microRNAs) and the process of MP formation can be affected upon alteration of these characteristics

and properties, the effect of Intercept on the microRNA profile of platelet MPs may thus be the consequence of all these interferences.

Intercept, in particular, seems to trigger the specific overrepresentation of hsa-miR-451a. This microRNA is known to modulate cytokines, such as Macrophage Migration Inhibitory factor (MIF), which plays essential roles in the immune system and cell growth (36–38). As platelet MPs are internalized and transmit their microRNA content to macrophages (6), the transfer of this specific MP-enriched microRNA may reprogram the macrophage function of the PC recipient.

Other microRNAs, such as miR-26a, are also overrepresented in platelet MPs upon Intercept and Additive solution treatments. MiR-26a is involved in the regulation of numerous target genes (e.g., PTEN, SMAD1, AKT, and MAP3K2/MEKK2) and was found to play a role in normal tissue growth and development (39). The increase in platelet

TABLE 1 | Impact of Mirasol treatment on the microRNA profile of platelet-derived MPs.

Top 20	Fold change	Upregulated	Fold change	Downregulated	Fold change
hsa-miR-146a-5p	1.1	hsa-miR-370-3p	3.7	hsa-miR-423-5p	0.5
hsa-miR-151a-3p	1.1	hsa-miR-127-3p	2.8	hsa-miR-25-3p	0.5
hsa-miR-126-3p	1.2	hsa-miR-185-3p	2.2	hsa-miR-92a-3p	0.4
hsa-miR-320a	1.1	hsa-miR-493-3p	2.3	hsa-miR-142-5p	0.5
hsa-miR-221-3p	0.7	hsa-miR-576-3p	3.0	hsa-miR-27a-3p	0.4
hsa-miR-21-5p	0.8	hsa-miR-379-5p	2.4	hsa-miR-24-3p	0.4
hsa-miR-30d-5p	0.8	hsa-miR-139-3p	2.8	hsa-miR-223-3p	0.4
hsa-miR-148a-3p	1.6	hsa-miR-374a-3p	2.7	hsa-miR-23a-3p	0.3
hsa-let-7i-5p	1.5			hsa-miR-484	0.3
hsa-miR-26a-5p	0.9			hsa-miR-126-5p	0.5
hsa-miR-423-3p	0.8			hsa-miR-128-3p	0.3
hsa-miR-191-5p	0.6			hsa-miR-10a-5p	0.4
hsa-miR-22-3p	0.5			hsa-let-7d-3p	0.4
hsa-miR-186-5p	1.2			hsa-miR-128-3p	0.3
hsa-miR-140-3p	1.1			hsa-miR-30e-3p	0.3
hsa-miR-423-5p	0.5			hsa-miR-30c-5p	0.3
hsa-miR-185-5p	0.7			hsa-miR-16-2-3p	0.5
hsa-let-7g-5p	1.0			hsa-miR-22-5p	0.3
hsa-miR-451a	0.6			hsa-miR-543	0.2
hsa-miR-1307-3p	1.2			hsa-miR-29a-3p	0.4
				hsa-miR-363-3p	0.2
				hsa-miR-374b-5p	0.5
				hsa-miR-10b-5p	0.2
				hsa-miR-323b-3p	0.3
				hsa-miR-122-5p	0.4
				hsa-miR-197-3p	0.5
				hsa-miR-361-5p	0.2
				hsa-miR-150-5p	0.1
				hsa-miR-28-5p	0.4
				hsa-miR-335-3p	0.4
				hsa-miR-335-5p	0.3
				hsa-miR-501-3p	0.5
				hsa-miR-15b-3p	0.4
				hsa-miR-410-3p	0.4
				hsa-miR-338-5p	0.4
				hsa-miR-421	0.4
				hsa-miR-92b-3p	0.2
				hsa-let-7a-3p	0.5

The 20 most abundant microRNAs are displayed, along with the microRNAs that were the most impacted (downregulated or upregulated) by Mirasol treatment. Data are expressed as fold change vs. MPs derived from control platelets.

MP miR-26a levels may thus affect endothelial cell growth, as platelet MPs transmit their microRNA content to such cells (5).

The changes in the level of certain microRNAs in MPs derived from platelets exposed to Intercept or its Additive solution vehicle, may have long-term, cumulative effects upon multiple transfusions.

TABLE 2 | Impact of Additive solution treatment on the microRNA profile of platelet-derived MPs.

Top 20	Fold change	Upregulated	Fold change	Downregulated	Fold change
hsa-miR-146a-5p	0.7	hsa-let-7f-5p	2.4	hsa-miR-151a-3p	0.4
hsa-miR-151a-3p	0.4	hsa-miR-1-3p	2.3	hsa-miR-320a	0.3
hsa-miR-126-3p	1.3	hsa-miR-101-3p	2.1	hsa-miR-221-3p	0.4
hsa-miR-320a	0.3	hsa-miR-107	2.3	hsa-miR-186-5p	0.3
hsa-miR-221-3p	0.4	hsa-miR-142-3p	2.2	hsa-miR-423-5p	0.5
hsa-miR-21-5p	1.5	hsa-miR-143-3p	2.1	hsa-miR-1307-3p	0.4
hsa-miR-30d-5p	0.7	hsa-miR-144-3p	2.7	hsa-miR-320c	0.3
hsa-miR-148a-3p	1.5	hsa-miR-145-5p	3.3	hsa-miR-486-5p	0.5
hsa-let-7i-5p	1.1	hsa-miR-150-5p	2.5	hsa-miR-4433b-3p	0.4
hsa-miR-26a-5p	2.8	hsa-miR-182-5p	2.0	hsa-miR-320b	0.3
hsa-miR-423-3p	0.6	hsa-miR-199b-5p	2.3	hsa-miR-361-3p	0.5
hsa-miR-191-5p	1.1	hsa-miR-23a-3p	2.2	hsa-miR-185-3p	0.2
hsa-miR-22-3p	0.8	hsa-miR-23b-3p	3.7	hsa-miR-326	0.3
hsa-miR-186-5p	0.3	hsa-miR-26a-5p	2.8	hsa-miR-425-3p	0.4
hsa-miR-140-3p	0.7	hsa-miR-26b-5p	2.8	hsa-miR-532-5p	0.4
hsa-miR-423-5p	0.5	hsa-miR-32-5p	2.2	hsa-let-7d-3p	0.5
hsa-miR-185-5p	1.3	hsa-miR-335-3p	2.0	hsa-miR-629-5p	0.5
hsa-let-7g-5p	1.8	hsa-miR-340-3p	2.1	hsa-miR-576-3p	0.4
hsa-miR-451a	1.8	hsa-miR-3613-5p	2.8	hsa-miR-345-5p	0.4
hsa-miR-1307-3p	0.4	hsa-miR-369-3p	3.1	hsa-miR-6852-5p	0.3
		hsa-miR-379-5p	2.4	hsa-miR-1260b	0.4
		hsa-miR-410-3p	2.2	hsa-miR-769-5p	0.3
		hsa-miR-411-5p	3.0	hsa-miR-320c	0.3
		hsa-miR-487b-3p	2.4	hsa-miR-99b-3p	0.4
		hsa-miR-494-3p	2.0	hsa-miR-139-3p	0.1
				hsa-miR-2355-3p	0.3
				hsa-miR-6842-3p	0.4
				hsa-miR-760	0.3
				hsa-miR-501-3p	0.3
				hsa-miR-1908-5p	0.2
				hsa-miR-215-5p	0.4
				hsa-miR-2110	0.3
				hsa-miR-338-5p	0.4
				hsa-miR-574-3p	0.5
				hsa-miR-92b-3p	0.5
				hsa-miR-1250-5p	0.2
				hsa-miR-320d	0.2
				hsa-miR-320d	0.2
				hsa-miR-29c-5p	0.3

The 20 most abundant microRNAs are displayed, along with the microRNAs that were the most impacted (downregulated or upregulated) by Additive solution treatment. Data are expressed as fold change vs. MPs derived from control platelets.

CONCLUSION

In this study, while there was no major effect on platelets, we found that PRT may impact the microRNA profile of platelet-derived MPs and induced overrepresentation of certain microRNAs. As platelet MPs may reprogram the function of macrophages by transferring their content in

TABLE 3 | Impact of Intercept treatment on the microRNA profile of platelet-derived MPs.

Top 20	Fold change	Upregulated	Fold change	Downregulated	Fold change
hsa-miR-146a-5p	0.3	hsa-let-7f-5p	3.3	hsa-miR-151a-3p	0.3
hsa-miR-151a-3p	0.3	hsa-miR-1-3p	2.7	hsa-miR-320a	0.2
hsa-miR-126-3p	1.8	hsa-miR-101-3p	3.1	hsa-miR-221-3p	0.3
hsa-miR-320a	0.2	hsa-miR-142-3p	3.0	hsa-miR-186-5p	0.2
hsa-miR-221-3p	0.3	hsa-miR-144-3p	13.7	hsa-miR-1307-3p	0.3
hsa-miR-21-5p	1.4	hsa-miR-150-5p	2.9	hsa-miR-320c	0.2
hsa-miR-30d-5p	0.7	hsa-miR-182-5p	2.3	hsa-miR-320b	0.2
hsa-miR-148a-3p	1.1	hsa-miR-23a-3p	2.1	hsa-miR-185-3p	0.2
hsa-let-7i-5p	2.4	hsa-miR-23b-3p	3.1	hsa-miR-326	0.2
hsa-miR-26a-5p	2.9	hsa-miR-26a-5p	2.9	hsa-miR-425-3p	0.4
hsa-miR-423-3p	0.4	hsa-miR-26b-5p	2.9	hsa-miR-532-5p	0.5
hsa-miR-191-5p	0.8	hsa-miR-32-5p	3.3	hsa-miR-629-5p	0.4
hsa-miR-22-3p	0.6	hsa-miR-3613-5p	3.5	hsa-miR-576-3p	0.2
hsa-miR-186-5p	0.2	hsa-miR-369-3p	2.5	hsa-miR-345-5p	0.4
hsa-miR-140-3p	0.3	hsa-miR-411-5p	3.7	hsa-miR-6852-5p	0.2
hsa-miR-423-5p	0.6	hsa-let-7a-5p	3.6	hsa-miR-1260b	0.4
hsa-miR-185-5p	0.6	hsa-let-7b-5p	2.4	hsa-miR-769-5p	0.2
hsa-let-7g-5p	2.5	hsa-let-7c-5p	3.2	hsa-miR-320c	0.2
hsa-miR-451a	6.6	hsa-let-7d-5p	4.0	hsa-miR-99b-3p	0.2
hsa-miR-1307-3p	0.3	hsa-let-7g-5p	2.5	hsa-miR-139-3p	0.4
		hsa-let-7i-5p	2.4	hsa-miR-2355-3p	0.2
		hsa-miR-15b-5p	2.2	hsa-miR-6842-3p	0.3
		hsa-miR-16-5p	2.2	hsa-miR-760	0.3
		hsa-miR-183-5p	3.0	hsa-miR-501-3p	0.5
		hsa-miR-363-3p	2.6	hsa-miR-1908-5p	0.3
		hsa-miR-374a-5p	2.3	hsa-miR-2110	0.3
		hsa-miR-451a	6.6	hsa-miR-574-3p	0.2
		hsa-miR-98-5p	4.3	hsa-miR-1250-5p	0.1
				hsa-miR-320d	0.1
				hsa-miR-29c-5p	0.3
				hsa-miR-146a-5p	0.3

The 20 most abundant microRNAs are displayed, along with the microRNAs that were the most impacted (downregulated or upregulated) by Intercept treatment. Data are expressed as fold change vs. MPs derived from control platelets.

functional microRNAs, such as miR-126-3p (6) or miR-223 in endothelial cells (5), treatments that impact microRNA loading into MPs will also impact their bioactivity. The long-term consequences of transfusing modified platelet MPs to patients, and their detrimental/beneficial effects on health remain

REFERENCES

- Thon JN, and Italiano JE. Platelets: production, morphology and ultrastructure. *Handb Exp Pharmacol.* (2012) 210:3–22. doi: 10.1007/978-3-642-29423-5_1
- Ho-Tin-Noé B, Demers M, Wagner DD. How platelets safeguard vascular integrity. *J Thromb Haemost.* (2011) 9(Suppl.1):56–65. doi: 10.1111/j.1538-7836.2011.04317.x
- Boilard E, Blanco P, Nigrovic PA. Platelets: active players in the pathogenesis of arthritis and SLE. *Nat Rev Rheumatol.* (2012) 8:534–42. doi: 10.1038/nrrheum.2012.118
- Boudreau LH, Duchez A-C, Cloutier N, Soulet D, Martin N, Bollinger J, et al. Platelets release mitochondria serving as substrate for bactericidal group IIA-secreted phospholipase A2 to promote inflammation. *Blood.* (2014) 124:2173–83. doi: 10.1182/blood-2014-05-573543
- Laffont B, Corduan A, Plé H, Duchez AC, Cloutier N, Boilard E, et al. (2013). Activated platelets can deliver mRNA regulatory Ago2-microRNA complexes to endothelial cells via microparticles. *Blood.* 122:253–61. doi: 10.1182/blood-2013-03-492801
- Laffont B, Corduan A, Rousseau M, Duchez AC, Lee CHC, Boilard E, et al. Platelet microparticles reprogram macrophage gene expression and function. *Thromb Haemost.* (2016) 115:311–23. doi: 10.1160/th15-05-0389

to be determined, especially in patients receiving PC on a regular basis.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in NCBI under accession number GSE138735.

ETHICS STATEMENT

The study was conducted by following the German pharmaceutical law for assessment of the quality of platelet products produced for routine use in hemotherapy and was approved by the Ethics Committee of the Medical Association of Rheinland-Pfalz (Ethik-Kommission bei der Landesärztekammer Rheinland-Pfalz-EK LÄK RLP). All platelet donors gave written informed consent to participate to the study.

AUTHOR CONTRIBUTIONS

WH and PP conceived and coordinated the study, and designed and planned the experiments. WH coordinated the sampling and treatment of the platelet concentrates. ID led the project, performed some of the experiments, and wrote the first draft of the manuscript. AB, AO, and JL performed some experiments and/or analysis. PP reviewed and finalized the manuscript. All authors have reviewed and approved the final manuscript before submission.

FUNDING

AB received Ph.D. studentship award no. 262093 from the *Fonds de Recherche du Québec—Santé* (FRQ-S). This work was supported by Grant Nos. 286,777 and 327,364 from the Canadian Blood Services (CBS)/Canadian Institutes of Health Research (CIHR) Partnership–Blood Utilization and Conservation Initiative, via Health Canada (to PP). The views expressed herein do not necessarily represent the view of the federal government.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcvm.2020.00031/full#supplementary-material>

7. Wandt H, Schäfer-Eckart K, Greinacher A. Platelet transfusion in hematology, oncology and surgery. *Dtsch Arztebl Int.* (2014) 111:809–15. doi: 10.3238/arztebl.2014.0809
8. Crighton GL, Estcourt LJ, Wood EM, Trivella M, Doree C, Stanworth S. A therapeutic-only versus prophylactic platelet transfusion strategy for preventing bleeding in patients with haematological disorders after myelosuppressive chemotherapy or stem cell transplantation. *Cochrane Database Syst Rev.* (2015) 11:CD010981. doi: 10.1002/14651858.CD010981.pub2
9. Slichter SJ, Corson J, Jones MK, Christoffel T, Pellham E, Bailey SL, et al. Exploratory studies of extended storage of apheresis platelets in a platelet additive solution (PAS). *Blood.* (2014) 123:271–80. doi: 10.1182/blood-2013-05-501247
10. Mundt JM, Rouse L, Van den Bossche J, Goodrich RP. Chemical and biological mechanisms of pathogen reduction technologies. *Photochem Photobiol.* (2014) 90:957–64. doi: 10.1111/php.12311
11. Kwon SY, Kim IS, Bae JE, Kang JW, Cho YJ, Cho NS, et al. Pathogen inactivation efficacy of Mirasol PRT System and Intercept Blood System for non-leucoreduced platelet-rich plasma-derived platelets suspended in plasma. *Vox Sang.* (2014) 107:254–60. doi: 10.1111/vox.12158
12. Marschner S, Goodrich R. Pathogen reduction technology treatment of platelets, plasma and whole blood using riboflavin and UV light. *Transfus Med Hemother.* (2011) 38:8–18. doi: 10.1159/000324160
13. Infanti L, Stebler C, Job S, Ruesch M, Gratwohl A, Irsch J, et al. Pathogen-inactivation of platelet components with the INTERCEPT blood system™: a cohort study. *Transfus Apher Sci.* (2011) 45:175–81. doi: 10.1016/j.transci.2011.07.013
14. Irsch J, Lin L. Pathogen inactivation of platelet and plasma blood components for transfusion using the INTERCEPT blood system™. *Transfus Med Hemother.* (2011) 38:19–31. doi: 10.1159/000323937
15. Sandgren P, Tolksdorf F, Struff WG, Gulliksson H. *In vitro* effects on platelets irradiated with short-wave ultraviolet light without any additional photoactive reagent using the THERAFLEX UV-Platelets method. *Vox Sang.* (2011) 101:35–43. doi: 10.1111/j.1423-0410.2010.01454.x
16. Magron A, Laugier J, Provost P, Boilard E. Pathogen reduction technologies: the pros and cons for platelet transfusion. *Platelets.* (2018) 29:2–8. doi: 10.1080/09537104.2017.1306046
17. Janetzko K, Cazenave J-P, Klüter H, Kientz D, Michel M, Beris P, et al. Therapeutic efficacy and safety of photochemically treated apheresis platelets processed with an optimized integrated set. *Transfusion.* (2005) 45:1443–52. doi: 10.1111/j.1537-2995.2005.00550.x
18. Kerkhoffs J-LH, van Putten WLJ, Novotny VMJ, Te Boekhorst PAW, Schipperus MR, Zwaginga JJ, et al. Clinical effectiveness of leucoreduced, pooled donor platelet concentrates, stored in plasma or additive solution with and without pathogen reduction. *Br J Haematol.* (2010) 150:209–17. doi: 10.1111/j.1365-2141.2010.08227.x
19. Osman A, Hitzler WE, Ameer A, Provost P. Differential expression analysis by RNA-seq reveals perturbations in the platelet mRNA transcriptome triggered by pathogen reduction systems. *PLoS ONE.* (2015) 10:e0133070. doi: 10.1371/journal.pone.0133070
20. Stivala S, Gobbato S, Infanti L, Reiner MF, Bonetti N, Meyer SC, et al. Amotosalen/ultraviolet A pathogen inactivation technology reduces platelet activatability, induces apoptosis and accelerates clearance. *Haematologica.* (2017) 102:1650–60. doi: 10.3324/haematol.2017.164137
21. Osman A, Hitzler WE, Meyer CU, Landry P, Corduan A, Laffont B, et al. Effects of pathogen reduction systems on platelet microRNAs, mRNAs, activation, and function. *Platelets.* (2015) 26:154–63. doi: 10.3109/09537104.2014.898178
22. Landry P, Plante I, Ouellet DL, Perron MP, Rousseau G, Provost P. Existence of a microRNA pathway in anucleate platelets. *Nat Struct Mol Biol.* (2009) 16:961–6. doi: 10.1038/nsmb.1651
23. Teruel-Montoya R, Kong X, Abraham S, Ma L, Kunapuli SP, Holinstat M, et al. MicroRNA expression differences in human hematopoietic cell lineages enable regulated transgene expression. *PLoS ONE.* (2014) 9:e102259. doi: 10.1371/journal.pone.0102259
24. Zimmerman GA, Weyrich AS. Signal-dependent protein synthesis by activated platelets: new pathways to altered phenotype and function. *Arterioscler Thromb Vasc Biol.* (2008) 28:s17–24. doi: 10.1161/ATVBAHA.107.160218
25. Schubert S, Weyrich AS, Rowley JW. A tour through the transcriptional landscape of platelets. *Blood.* (2014) 124:493–502. doi: 10.1182/blood-2014-04-512756
26. Weyrich AS, Dixon DA, Pabla R, Elstad MR, McIntyre TM, Prescott SM, et al. Signal-dependent translation of a regulatory protein, Bcl-3, in activated human platelets. *Proc Natl Acad Sci USA.* (1998) 95:5556–61. doi: 10.1073/pnas.95.10.5556
27. Plé H, Landry P, Benham A, Coarfa C, Gunaratne PH, Provost P. The repertoire and features of human platelet microRNAs. *PLoS ONE.* (2012) 7:e50746. doi: 10.1371/journal.pone.0050746
28. Yu J, Wang F, Yang GH, Wang FL, Ma YN, Du ZW, et al. Human microRNA clusters: genomic organization and expression profile in leukemia cell lines. *Biochem Biophys Res Commun.* (2006) 349:59–68. doi: 10.1016/j.bbrc.2006.07.207
29. Diehl P, Fricke A, Sander L, Stamm J, Bassler N, Htun N, et al. Microparticles: major transport vehicles for distinct microRNAs in circulation. *Cardiovasc Res.* (2012) 93:633–44. doi: 10.1093/cvr/cvs007
30. Ellis TN, Kuehn MJ. Virulence and immunomodulatory roles of bacterial outer membrane vesicles. *Microbiol Mol Biol Rev.* (2010) 74:81–94. doi: 10.1128/MMBR.00031-09
31. Hessvik NP, Llorente A. Current knowledge on exosome biogenesis and release. *Cell Mol Life Sci.* (2018) 75:193–208. doi: 10.1007/s00018-017-2595-9
32. Abonnenc M, Crettaz D, Tacchini P, Di Vincenzo L, Sonogo G, Prudent M, et al. Antioxidant power as a quality control marker for completeness of amotosalen and ultraviolet A photochemical treatments in platelet concentrates and plasma units. *Transfusion.* (2016) 56:1819–27. doi: 10.1111/trf.13638
33. Johnson L, Hyland R, Tan S, Tolksdorf F, Sumian C, Seltsam A, et al. *In vitro* quality of platelets with low plasma carryover treated with ultraviolet C light for pathogen inactivation. *Transfus Med Hemother.* (2016) 43:190–7. doi: 10.1159/000441830
34. Picker SM, Oustianskaia L, Schneider V, Gathof BS. Functional characteristics of apheresis-derived platelets treated with ultraviolet light combined with either amotosalen-HCl (S-59) or riboflavin (vitamin B2) for pathogen-reduction. *Vox Sang.* (2009) 97:26–33. doi: 10.1111/j.1423-0410.2009.01176.x
35. Hechler B, Ravanat C, Gachet C. Amotosalen/UVA pathogen inactivation technology reduces platelet activability, induces apoptosis and accelerates clearance. *Haematologica.* (2017) 102:e502–3. doi: 10.3324/haematol.2017.180539
36. Graham A, Falcone T, Nothnick WB. The expression of microRNA-451 in human endometriotic lesions is inversely related to that of macrophage migration inhibitory factor (MIF) and regulates MIF expression and modulation of epithelial cell survival. *Hum Reprod.* (2015) 30:642–52. doi: 10.1093/humrep/dev005
37. Lolis E, Bucala R. Macrophage migration inhibitory factor. *Expert Opin Ther Targets.* (2003) 7:153–64. doi: 10.1517/14728222.7.2.153
38. Nishihira J. Macrophage migration inhibitory factor (MIF): its essential role in the immune system and cell growth. *J Interferon Cytokine Res.* (2000) 20:751–62. doi: 10.1089/10799900050151012
39. Gao J, Liu QG. The role of miR-26 in tumors and normal tissues (Review). *Oncol Lett.* (2011) 2:1019–23. doi: 10.3892/ol.2011.413

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Diallo, Benmoussa, Laugier, Osman, Hitzler and Provost. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Platelet Measurements and Type 2 Diabetes: Investigations in Two Population-Based Cohorts

Benjamin A. T. Rodriguez and Andrew D. Johnson*

The Framingham Heart Study, Population Sciences Branch, Division of Intramural Research, National Heart Lung and Blood Institute, Framingham, MA, United States

OPEN ACCESS

Edited by:

Marie Lordkipanidzé,
Université de Montréal, Canada

Reviewed by:

Thomas Pierre Lecompte,
Université de Genève, Switzerland
Plinio Cirillo,
University of Naples Federico II, Italy

*Correspondence:

Andrew D. Johnson
johnsonad2@nhlbi.nih.gov

Specialty section:

This article was submitted to
Atherosclerosis and Vascular
Medicine,
a section of the journal
Frontiers in Cardiovascular Medicine

Received: 15 October 2019

Accepted: 04 June 2020

Published: 10 July 2020

Citation:

Rodriguez BAT and Johnson AD
(2020) Platelet Measurements and
Type 2 Diabetes: Investigations in Two
Population-Based Cohorts.
Front. Cardiovasc. Med. 7:118.
doi: 10.3389/fcvm.2020.00118

Type 2 diabetes is a major risk factor for cardiovascular disease. Given the contribution of platelets to atherothrombosis—which in turn is a major contributor to cardiac events, there may be cause to consider platelet function in management of diabetes. Despite the large body of research concerning the role of platelets in cardiovascular complications of type 2 diabetes, evidence from population-based studies of platelet aggregation in diabetes is limited. Mean Platelet Volume (MPV), a cell trait partially associated with markers of platelet activity, is more commonly available. We investigated the association of metabolic syndrome and diabetes with platelet aggregation to three physiological agonists, ADP, collagen, and epinephrine, in the Framingham Heart Study Offspring cohort. We further examined the relationship between MPV measured with Beckman Coulter LH750 instruments and self-reported diabetes as well as MPV and diabetes medication in the UK BioBank cohort, performing the largest such analysis to date. Increased platelet aggregation associated with prevalent diabetes was observed for low concentration epinephrine (0.1 μ M) alone and only in analyses of participants stratified either by male sex and/or having metabolic syndrome. Other agonists and concentrations were not significant for prevalent diabetes, or in opposite direction to the main hypothesis (i.e., they showed lower platelet aggregation associated with diabetes). After a median of 18.1 years follow-up, no platelet aggregation trait was associated with increased risk of diabetes ($n = 344$ cases). As expected, increased MPV was significantly associated with diabetes ($\beta = 0.0976$; $P = 8.62 \times 10^{-33}$). Interestingly, sex-stratified analyses indicated the association of MPV with diabetes is markedly stronger in males ($\beta = 0.1232$; $P = 1.00 \times 10^{-31}$) than females ($\beta = 0.0514$; $P = 7.37 \times 10^{-5}$). Among diabetes medications increased MPV was associated with Insulin ($\beta = 0.1341$; $P = 1.38 \times 10^{-11}$) and decreased MPV with both Metformin ($\beta = 0.0763$; $P = 1.99 \times 10^{-6}$) as well as the sulphonylureas ($\beta = 0.0559$; $P = 0.0034$). Each drug showed the same direction of effect in both sexes, however, the association with MPV was nearly twice as great or more in women compared to men. In conclusion, platelet function as measured by aggregation to ADP, collagen, or epinephrine does not appear to be consistently associated with diabetes, however, MPV is robustly associated suggesting future work may focus on how MPV segments pre-diabetics and diabetics for risk prediction.

Keywords: diabetes mellitus, platelets, platelet aggregation, mean platelet volume (MPV), sex differences, metabolic syndrome

INTRODUCTION

Platelets play a central role in the development of atherothrombosis, a major contributor to cardiovascular events (1). Type 2 diabetes is a major risk factor for cardiovascular disease (2) and a strong predictor of cardiovascular mortality (3). Metabolic syndrome, a cluster of lipid, and nonlipid risk factors of metabolic origin (4), is prevalent and associated with an increased risk for diabetes and CVD in both sexes (5). Multiple aspects of atherothrombosis are suspected to be dysregulated in diabetes, including increased atherosclerosis, chronically activated endothelium, coagulation, and platelet reactivity (6). Platelet activation has been associated with progressive thickening of the carotid artery in diabetic patients (7). Given the contribution of platelets to atherothrombosis there may be cause to consider platelet function in management of diabetes and its macrovascular complications, but this is not part of the current standard of care (8).

Platelets in patients with diabetes are reported to exhibit hyper-reactivity to subthreshold stimuli, and undergo rapid consumption, resulting in accelerated thrombopoiesis of more reactive platelets (9). Reports of increased platelet aggregation to various agonists go back several decades (10, 11); however, studies in diabetics without CVD have produced inconsistent results (12–16). Platelet function in metabolic syndrome is less well-characterized. The two previous studies of platelet aggregation comparing metabolic syndrome patients with healthy controls produced inconsistent results (17, 18). Alterations in several platelet functional measures have been reported in both diabetes and metabolic syndrome: increased platelet P-selectin (17, 19), increased levels of thromboxane metabolites (18, 20), and increased platelet turnover (17, 21). Mean Platelet Volume (MPV), a measurement of platelet size, is partially associated with other established markers of platelet activity, including platelet aggregation (1, 22). Elevated MPV is associated with diabetes (23–27) and has been shown to be an independent prognostic marker of vascular events in diabetics (28) as well as the general population (29).

The majority of previous studies concerning platelet aggregability in diabetes or metabolic syndrome have been of limited sample size and often lacked adjustment for potential confounders known to affect platelet aggregation independent of these conditions, including age and sex. Platelets are significantly more reactive at baseline and after aspirin therapy in women compared to men (30). The relationship of sex with platelet aggregation in diabetes is largely unknown. To this end, we investigated the association of platelet aggregation to three physiological agonists with metabolic syndrome and diabetes in the FHS Offspring cohort, accounting for sex in our models and further stratifying analyses by sex where possible. We further examined the relationship between MPV with self-reported diabetes, accounting for sex, in the UK BioBank cohort, performing the largest such analysis to date. Finally, we examined the relationship between MPV and diabetes medication status, also accounting for sex.

METHODS

Framingham Heart Study (FHS) Cohort, Phenotypes, and Analysis

The FHS is a longitudinal community-based cohort (31). This investigation considered participants in the Framingham Offspring cohort attending the fifth examination cycle (1991–1995) during which platelet function was assayed. Of 3,799 individuals attending the exam, 551 were excluded for one of the following reasons: missing platelet function measures mainly due to the measures being started partway in the exam cycle ($n = 526$), missing current diabetes status ($n = 2$), currently taking an antiplatelet medication other than aspirin ($n = 14$), previous history of leukemia ($n = 3$) or lymphoma ($n = 7$). The Institutional Review Board of Boston University Medical Center (Boston, MA) approved the study protocol, and all participants provided written informed consent.

Platelet aggregation was tested in response to three agonists: ADP (Sigma–Aldrich), epinephrine (Sigma–Aldrich), and collagen (Bio/Data), and arachidonic acid (Bio/Data). Blood samples were collected from the antecubital vein in the morning with participants lying supine after an overnight fast. Blood was drawn into an evacuated collection tube containing 3.8% sodium citrate (Becton Dickinson) and centrifuged at 160 g for 5 min at room temperature to separate platelet rich plasma. Blood was at RT until assay. Platelet rich plasma was not diluted to any specific concentration before assay. A 4-channel light transmission aggregometer (Bio/Data, Horsham, PA) was used to measure platelet aggregation at 37°C and 1,200 rpm. Aggregation was tested with a fixed concentration of arachidonic acid (1.64 mM) and increasing concentrations of ADP (0.05–15 $\mu\text{mol/L}$) and epinephrine (0.01–15 $\mu\text{mol/L}$) (32). As concentrations for epinephrine and ADP were titrated up or down based on individual responsiveness, not all participants were tested at all concentrations. Collagen lag time was measured in response to a single concentration (1.9 $\mu\text{g/mL}$) of calf-derived Type 1 fibrillar collagen. An increase in collagen lag time is indicative of reduced platelet aggregation. For ADP and epinephrine maximal aggregation at each concentration normalized relative to platelet poor plasma was used (scale of 0–100%). All reactions were run the same morning as the blood draw. Aspirin usage was inferred on the basis of the absence of full response to arachidonic acid stimulation. This was done by lab technician opinion after several minutes of aggregation (33).

Because of the variable number of participants tested at a given concentration of agonist, we focused our continuous trait analyses on two concentrations of ADP (1 and 5 μM) and epinephrine (0.1 and 1 μM) which had a sample size >900 at each concentration of agonist. We further characterized the association of prevalent diabetes with platelet aggregation by dichotomous aggregation traits previously applied in the analysis of venous thrombosis and CVD (32, 34). Briefly, ADP hyperreactivity was defined as $\geq 50\%$ maximal aggregation to at least one low concentration (0.05, 0.1, 0.5, and 1 $\mu\text{mol/L}$). Similarly, hyperreactivity to epinephrine required $\geq 50\%$ maximal aggregation to at least one low concentration (0.05, 0.1, 0.5, and 1 $\mu\text{mol/L}$). In contrast, those who failed to

reach 50% maximal aggregation at a higher concentration (5, 10, and 15 $\mu\text{mol/L}$) of ADP or epinephrine were considered hyporesponders. Histograms of maximal percent aggregation at concentrations used to determine hyperreactivity and hyporeactivity are shown for ADP in **Supplementary Figure 1** and for epinephrine in **Supplementary Figure 2**.

Criteria for diabetes mellitus were a fasting glucose level of ≥ 126 mg/dL, non-fasting glucose level ≥ 200 mg/dL, or use of medications to treat hyperglycemia. No specific steps were taken to exclude type 1 diabetes. Metabolic syndrome status was determined by the unified criteria of the International Diabetes Federation Task Force on Epidemiology and Prevention, National Heart, Lung, and Blood Institute, American Heart Association, World Heart Federation, International Atherosclerosis Society, and International Association for the Study of Obesity (35). The presence of any three of five risk factors constitutes a diagnosis of metabolic syndrome. The risk factors and their cut points are: elevated waist girth (male, ≥ 94 cm; female, ≥ 80 cm), elevated triglycerides (≥ 150 mg/dL), reduced HDL (male, <40 mg/dL; female, <50 mg/dL), elevated blood pressure (systolic ≥ 130 and/or diastolic ≥ 85 mm Hg), elevated fasting glucose (≥ 100 mg/dL) (35).

In this study, there were 1,143 distinct family clusters that contributed data. For a reference point, only 45 families out of 1,143 had family size >10 members, so the vast majority of families in the Gen2 study reflect sibships and close cousins. In the FHS cohort multivariable linear mixed-effect models correcting for nonindependence of families were used to determine the association of continuous platelet aggregation traits with metabolic syndrome and/or prevalent diabetes. Similarly, the association of incident diabetes with continuous traits was determined by Cox mixed-effect models. Duration of follow-up was calculated as days between date of baseline examination with no diabetes (Exam 5) and date of the examination where the participant first met the criteria for diabetes (up to Exam 9), ranging from 2.5 to 22.5 years. The association of prevalent diabetes with dichotomized platelet aggregation traits was assessed by multivariable logistic regression analyses. All models adjusted for age at baseline, sex, and aspirin usage at baseline (as determined by low PRP platelet aggregation to 5mg/mL arachidonic acid). A two-tailed $P < 0.05$ was considered nominally significant. Statistical analyses were performed with R, version 3.41. For additional sensitivity analyses, we stratified participants based on aspirin usage (yes/no), or history of cardiovascular disease (yes/no) as previously defined. Main models for prevalent diabetes were already adjusted for age at baseline, sex, and aspirin usage. We ran additional sensitivity models adding history of CVD as a further covariate.

UK Biobank Cohort, Phenotypes, and Analysis

The UK Biobank is a large population-based prospective study ($n = 500,000$), established to allow investigations of the genetic and nongenetic determinants of the diseases of middle and old age (36). IRB approval was granted by the

North West Haydock Research Ethics Committee (16/NW/0274) and this project approved by application to the UK Biobank. Participants were assessed between 2006 and 2010 in 22 locations throughout the UK. The assessment visit comprised electronic signed consent, a self-completed touch-screen questionnaire, a computer-assisted interview, physical, and functional measures, as well as collection of blood, urine, and saliva (36). Blood samples from participants were collected into 4mL EDTA vacutainers by vacuum draw at assessment centers and stored at 4°C. Samples were transported overnight to the UK Biocenter (Stockport, UK) in temperature-controlled containers where full blood cell counts were measured using four Beckman Coulter LH700 Series hematology analyzers (37). The blood cell count values were QC'd centrally by the UK BioBank before release. We applied exclusion criteria that included (A) missing values for any of the following: MPV measurement, blood pressure, BMI, self-report of diabetes diagnosed by a physician, use of anti-platelet, blood pressure, or hyperlipidemia medication; (B) self-report of any of the following conditions: pregnancy, cancer drug treatments, malignant lymph nodes, bone metastases, leukemia, lymphoma, myelodysplasia, multiple myeloma; (C) ICD9 and/or ICD10 codes for any of the following conditions: HIV, neoplasms of bone, polycythemia vera, myelodysplastic syndrome, red cell aplasia, anemias, coagulation defects, other diseases of the blood, diseases of the liver, abnormal findings on blood tests, splenectomy, and hematopoietic stem cell transplantation. A total of 36,297 participants were removed after applying the exclusion criteria. Among those remaining samples with MPV measures, we used the UK BioBank code (2443) indicating participant self-report of diabetes having been diagnosed by a physician. Use of anti-platelet (aspirin, clopidogrel, and dipyridamole), blood pressure, hyperlipidemia, or diabetes medications (insulin, metformin, and sulphonylureas) was also based on self-report. We defined hypertension as a systolic blood pressure ≥ 130 mm Hg, a diastolic blood pressure ≥ 85 mm Hg, or use of any antihypertensive medication. The association of MPV with self-reported diabetes or with diabetes medications among self-reported diabetics was assessed by multivariable linear analyses. No specific steps were taken to exclude type 1 diabetes. All models adjusted for age at baseline, sex, BMI, hypertension, antiplatelet medication, and hyperlipidemia medication. Secondary analyses were performed using interaction terms to assess effect modification by sex. A two-tailed $P < 0.05$ was considered nominally significant. Statistical analyses were performed with R, version 3.41.

RESULTS

Framingham Heart Study

The study sample consisted of 3,244 FHS participants from the Offspring cohort who attended Exam 5 (here treated as baseline) and underwent platelet function testing. Platelet aggregation data from different agonists were available for epinephrine ($n = 3,086$), ADP ($n = 3,215$), and collagen ($n = 3,146$). This is the largest population-based study of platelet aggregation by LTA, the gold standard assay in the field, published to date, and to our knowledge the second largest ($n = 244$ here

vs. $n = 257$ previously) such cross-sectional study conducted in diabetics (38). We believe this is the largest prospective study of platelet aggregation and diabetes ($n = 344$ cases). Descriptive statistics for the population demographics at the baseline examination are shown according to diabetes status in **Table 1**. The average participant age was 55.0 years (SD, 9.9 years) and the sample was 52.8% women ($n = 1,715$). At the baseline exam 41% ($n = 131$) met the criteria for metabolic syndrome (≥ 3 of 5 traits) and 7.5% ($n = 244$) were classified as having diabetes. In linear mixed effects models examining either sex or age separately along with family structure but no other covariates, female sex was strongly associated with higher platelet reactivity for all tests, and weaker associations were observed with older age being associated with increased platelet reactivity (except for collagen lag time; **Supplementary Table 1**). Hence all models, except those sex stratified, adjusted for both age and sex.

We first investigated the relationship between platelet reactivity and metabolic syndrome at the baseline examination. Results of the linear mixed-effect models are presented in **Table 2**. Reduced platelet aggregation in response to 5 μM ADP ($\beta = 3.07$; $P = 1.7 \times 10^{-5}$), 0.1 μM ($\beta = 2.92$; $P = 0.032$), and 1 μM epinephrine ($\beta = 3.11$; $P = 0.011$) as well as increased collagen lag time ($\beta = 2.07$; $P = 0.015$) were associated with metabolic syndrome after adjusting

for age, sex, and aspirin therapy. Analyses stratified by sex showed aggregation was uniformly reduced across agonists and concentrations in females (four of five traits $P < 0.05$), but not in males (one of five traits $P < 0.05$). Further, the magnitudes of the associations with metabolic syndrome were greater in females compared to males (**Table 2**). The same patterns emerge when diabetics with metabolic syndrome are excluded from the analyses (**Supplementary Table 2**), suggesting the association is a pre-diabetic phenomenon. In the absence of metabolic syndrome, by contrast, female sex (compared to male sex) is associated with strong, uniform increases in aggregation across agonists and concentrations (five of five traits $P < 0.05$; **Supplementary Table 3**).

We next investigated the relationship between platelet reactivity and prevalent diabetes (including those with metabolic syndrome). Results of the linear mixed-effect models are presented in **Table 3**. After adjusting for age, sex, and aspirin therapy we observed that reduced platelet aggregation in response to 5 μM ADP ($\beta = 4.55$; $P = 7.5 \times 10^{-4}$), 1 μM epinephrine ($\beta = 7.89$; $P = 7.0 \times 10^{-4}$) as well as increased collagen lag time ($\beta = 8.04$; $P = 1.8 \times 10^{-7}$) were associated with diabetes status. These are the same three traits and directions of effect as we observed in metabolic syndrome (**Table 2**). There was a trend toward increased aggregation to 0.1 μM epinephrine. Stratifying the analysis of 0.1 μM epinephrine by sex, it was evident the increased reactivity to epinephrine associated with diabetes was limited to males ($\beta = 9.75$; $P = 0.006$) and absent in females ($\beta = 0.41$; $P = 0.928$). The association in response to 1 μM ADP differed between the sexes with aggregation increasing in males and decreasing in females, but the models did not reach statistical significance. As previously observed in the models of metabolic syndrome, platelet aggregation measures in females again showed stronger negative associations with diabetes compared to males (**Table 3**). The interpretation of significance and directions of effects were not altered if, instead of adjusting for aspirin use, we stratified to non-aspirin or aspirin takers (**Supplementary Table 4**). Likewise, if analyses were adjusted for or stratified based on prior CVD history they remained largely unchanged (**Supplementary Table 5**).

We explored the relationship between aggregation and diabetes further in multivariable analyses accounting for metabolic syndrome. Results of the linear mixed-effect models are presented in **Table 4**. There was a stronger association with increased aggregation to 0.1 μM epinephrine ($\beta = 6.84$; $P = 0.022$) compared to the diabetes model without metabolic syndrome as a covariate (**Table 3**). Reduced aggregation in response to 5 μM ADP ($\beta = 3.42$; $P = 0.013$) and 1 μM epinephrine ($\beta = 6.88$; $P = 0.004$), as well as increased collagen lag time ($\beta = 7.58$; $P = 1.7 \times 10^{-6}$) remained significantly associated with diabetes status. Stratifying the multivariable analyses by sex again showed increased reactivity to 0.1 μM epinephrine was associated with diabetes in males alone ($\beta = 10.06$; $P = 0.006$). Females, by contrast, once again showed stronger negative associations of platelet aggregation measures with diabetes compared to males (**Table 4**). In this multivariable model the repressive effect of metabolic syndrome on three measures: 0.1 μM ($\beta = 5.26$)

TABLE 1 | Baseline characteristics of Framingham participants.

Baseline characteristics	Diabetic ($n = 244$)		Not diabetic ($n = 3,004$)		P-value
	Mean (n)	SD (%)	Mean (n)	SD (%)	
Female (%)	94	38.5	1,621	54	4.68E-06
Age, year	60.3	8.7	54.6	9.9	2.20E-19
BMI, kg/m ²	31.16	5.75	27.14	4.84	3.95E-22
Metabolic syndrome	217	88.9	1,114	37.1	4.96E-56
Fasting glucose, mg/dL	178.74	62.69	95.25	9.7	1.86E-55
HDL, mg/dL	40.72	12.19	50.57	15.18	5.60E-27
LDL, mg/dL	123.43	33.001	126.78	32.81	0.146
TRIG, mg/dL	225.53	156.16	143.05	101.46	1.98E-14
SBP, mmHg	139.39	19.72	125.39	18.71	1.20E-22
DBP, mmHg	74.21	10.02	78.02	10.25	5.37E-08
Waist girth, in	41.25	5.33	36.31	5.51	5.99E-34
Hypertension medication	87	41.8	481	16.9	1.31E-19
Lipid medication	34	16.3	177	6.2	2.88E-08
Aspirin usage	70	28.7	440	14.6	9.62E-09
Diabetes medication	117	48	0	0	n.a.
Insulin, n (%)	39	15.9	n.a.	n.a.	n.a.
Oral hypoglycemic, n (%)*	88	36.1	n.a.	n.a.	n.a.

BMI indicates body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure; n, sample size; SD, standard deviation; P-value, univariate T-test P-value. *sub-drug not specified but in the time period of this Exam from January 1991 to June 1995 TZDs had not been released and metformin was only released in 1995 so all or nearly all of these participants were likely treated with sulfonylurea drugs.

TABLE 2 | Results of linear mixed-effect models for association of metabolic syndrome with platelet aggregation and stratified by sex.

Phenotype	Beta	SE	P	N	Males				Females			
					Beta	SE	P	N	Beta	SE	P	N
1 μ M ADP	0.05	0.78	9.46E-01	2,999	1.04	0.93	2.63E-01	1,353	-1.14	1.22	3.52E-01	1,646
5 μ M ADP	-3.07	0.71	1.70E-05	2,069	-2.66	1.05	1.16E-02	1,051	-3.77	0.95	6.78E-05	1,018
0.1 μ M EPI	-2.92	1.37	3.26E-02	1,560	0.03	1.89	9.87E-01	609	-5.18	1.90	6.38E-03	951
1 μ M EPI	-3.11	1.23	1.15E-02	2,485	-1.66	1.84	3.67E-01	1,134	-4.78	1.65	3.67E-03	1,351
COLLAGEN	2.07	0.85	1.54E-02	3,144	1.07	1.25	3.96E-01	1,473	3.47	1.16	2.82E-03	1,671

Sample size (N) at different concentrations vary due to concentration titrating scheme originally applied in FHS LTA data acquisition. ADP and EPI values are % Maximal Aggregation whereas COLLAGEN is Lag time. Main model adjusts for age, sex, aspirin usage. Sex stratified model adjusts for age, aspirin usage. SE, standard error; P, P-value.

TABLE 3 | Results of linear mixed-effect models for association of prevalent diabetes with platelet aggregation and stratified by sex.

Phenotype	Beta	SE	P	N	Males				Females			
					Beta	SE	P	N	Beta	SE	P	N
1 μ M ADP	0.01	1.45	9.93E-01	2,998	2.58	1.60	1.06E-01	1,352	-4.02	2.48	1.06E-01	1,646
5 μ M ADP	-4.55	1.35	7.51E-04	2,068	-3.91	1.92	4.17E-02	1,050	-5.70	1.88	2.48E-03	1,018
0.1 μ M EPI	5.27	2.93	7.23E-02	1,560	9.75	3.58	6.40E-03	609	0.41	4.55	9.28E-01	951
1 μ M EPI	-7.89	2.33	7.04E-04	2,485	-5.44	3.36	1.05E-01	1,134	-10.98	3.23	6.72E-04	1,351
COLLAGEN	8.04	1.54	1.81E-07	3,144	6.35	2.08	2.29E-03	1,473	10.85	2.34	3.50E-06	1,671

Sample size (N) at different concentrations vary due to concentration titrating scheme originally applied in FHS LTA data acquisition. ADP and EPI values are % Maximal Aggregation whereas COLLAGEN is Lag time. Main model adjusts for age, sex, aspirin usage. Sex stratified models adjusts for age, aspirin usage. SE, standard error; P, P-value.

TABLE 4 | Results of multivariable linear mixed-effect models for association of prevalent diabetes with platelet aggregation including metabolic syndrome status and stratified by sex.

Phenotype	Beta	SE	P	N	Males				Females			
					Beta	SE	P	N	Beta	SE	P	N
1 μ M ADP	-0.01	1.50	9.94E-01	2,998	2.31	1.64	1.58E-01	1,352	-3.66	2.58	1.56E-01	1,646
5 μ M ADP	-3.42	1.38	1.34E-02	2,068	-3.10	1.95	1.13E-01	1,050	-3.89	1.95	4.65E-02	1,018
0.1 μ M EPI	6.84	2.99	2.21E-02	1,560	10.06	3.65	5.77E-03	609	3.66	4.68	4.34E-01	951
1 μ M EPI	-6.88	2.40	4.08E-03	2,485	-5.01	3.43	1.44E-01	1,134	-9.08	3.36	6.97E-03	1,351
COLLAGEN	7.58	1.59	1.75E-06	3,144	6.26	2.13	3.30E-03	1,473	9.65	2.43	7.24E-05	1,671

Sample size (N) at different concentrations vary due to concentration titrating scheme originally applied in FHS LTA data acquisition. ADP and EPI values are % Maximal Aggregation whereas COLLAGEN is Lag time. Main model adjusts for age, sex, aspirin usage, and metabolic syndrome status. Sex stratified models adjusts for age, aspirin usage, metabolic syndrome status. SE, standard error; P, P-value.

and 1 μ M epinephrine ($\beta = 3.48$) as well as 5 μ M ADP ($\beta = 3.48$) remained significant in females independent of diabetes whereas in males only 0.1 μ M epinephrine ($\beta = 2.37$) remained significant (data not shown).

To further investigate this bias in the influence of metabolic syndrome on platelet reactivity in diabetes, we performed analyses stratifying on metabolic syndrome status and then on metabolic syndrome as well as sex. Results of the linear mixed-effect models are presented in **Table 5**. In the absence of metabolic syndrome, no platelet aggregation measure reached statistical significance in models combining sexes. Analyses of individuals lacking metabolic syndrome stratified by sex indicated that increased aggregation to 1 μ M ADP was observed exclusively in males ($\beta = 7.81$; $P = 0.033$) and

reduced aggregation to 5 μ M ADP trended exclusively in females ($\beta = 18.40$; $P = 0.058$). For individuals with metabolic syndrome a similar complex pattern also emerges where the association with diabetes in response to epinephrine differed by concentration on account of sex. Increased aggregation to low concentration epinephrine was observed exclusively in males ($\beta = 10.99$; $P = 0.007$) and reduced aggregation to high concentration epinephrine exclusively in females ($\beta = 8.50$; $P = 0.012$).

Next we investigated whether platelet aggregation measures were associated with incident diabetes. During follow-up (median, 18.1 years), we observed 344 incident diabetes cases. Results of the Cox mixed-effect hazard models are presented in **Table 6**. After adjusting for age, sex, and aspirin usage,

TABLE 5 | Results of linear mixed-effect models for association of prevalent diabetes with platelet aggregation stratified by metabolic syndrome status and by sex.

	Phenotype	Beta	SE	P	N	Males				Females			
						Beta	SE	P	N	Beta	SE	P	N
MS	1 μ M ADP	−0.95	1.57	5.44E-01	1,218	1.47	1.90	4.40E-01	627	−3.18	2.50	2.04E-01	591
Yes	5 μ M ADP	−3.70	1.48	1.22E-02	821	−3.97	2.10	5.90E-02	464	−3.60	2.02	7.45E-02	357
	0.1 μ M EPI	7.32	2.99	1.42E-02	609	10.99	4.10	7.38E-03	288	3.80	4.32	3.79E-01	321
	1 μ M EPI	−7.23	2.52	4.15E-03	1,003	−5.70	3.72	1.25E-01	518	−8.50	3.39	1.22E-02	485
	COLLAGEN	7.84	1.83	1.78E-05	1,297	6.20	2.44	1.11E-02	702	10.00	2.75	2.70E-04	595
	1 μ M ADP	6.97	4.40	1.13E-01	1,780	7.81	3.67	3.32E-02	725	−18.81	16.43	2.52E-01	1,055
MS	5 μ M ADP	−2.95	3.69	4.23E-01	1,247	−1.15	4.42	7.94E-01	586	−18.40	9.70	5.79E-02	661
No	0.1 μ M EPI	4.62	9.32	6.20E-01	951	3.72	8.13	6.47E-01	321	n.a.	n.a.	n.a.	^a 630
	1 μ M EPI	−7.45	7.30	3.08E-01	1,482	−4.75	8.38	5.71E-01	616	−24.38	19.52	2.12E-01	866
	COLLAGEN	7.52	4.28	7.87E-02	1,847	6.93	4.83	1.52E-01	771	8.09	14.77	5.84E-01	1,076

Sample size (N) at different concentrations vary due to concentration titrating scheme originally applied in FHS LTA data acquisition. ADP and EPI values are % Maximal Aggregation whereas COLLAGEN is Lag time. MS, Metabolic Syndrome. Main models according to MS status adjust for age, sex, aspirin usage. Sex stratified models adjusts for age, aspirin usage.

^aNo female diabetics without metabolic syndrome were in the 0.1 μ M EPI concentration testing sample. SE, standard error; P, P-value.

TABLE 6 | Results of cox mixed-effect hazard models for association of platelet aggregation with incident diabetes and stratified by sex.

Phenotype	Incident diabetes				Males				Females			
	HR	95% CI	P	Num	HR	95% CI	P	Num	HR	95% CI	P	Num
1 μ M ADP	0.999	0.993–1.005	0.757	310/2,763	1.003	0.993–1.013	0.528	165/1,219	0.997	0.989–1.005	0.429	145/1,544
5 μ M ADP	0.990	0.981–0.999	0.021	222/1,914	0.998	0.987–1.009	0.673	132/961	0.974	0.959–0.988	3.32E-04	90/953
0.1 μ M EPI	0.995	0.988–1.002	0.156	153/1,466	0.995	0.983–1.007	0.417	72/560	0.995	0.986–1.004	0.254	81/906
1 μ M EPI	0.999	0.995–1.004	0.760	259/2,296	1.000	0.995–1.006	0.883	143/1,034	0.998	0.991–1.005	0.559	116/1,262
COLLAGEN	1.002	0.997–1.007	0.461	333/2,884	0.997	0.990–1.003	0.332	186/1,316	1.008	1.001–1.015	0.020	147/1,568

Sample size (N) at different concentrations vary due to concentration titrating scheme originally applied in FHS LTA data acquisition. ADP and EPI values are % Maximal Aggregation whereas COLLAGEN is Lag time. Main model adjusts for age, sex, aspirin usage. Sex stratified models adjust for age, aspirin usage. Num indicates Events/Total at risk. HR, hazard ratio; CI, confidence interval; P, P-value.

aggregation to 5 μ M ADP was associated with a minor reduction in incident diabetes. Sex stratified analyses indicated the reduced risk was limited to females (HR = 0.974 [95%CI, 0.959–0.988] $P = 3.3 \times 10^{-4}$). Increased collagen lag time was also associated with a minor reduction in risk among females. These sex-specific risk reductions remained significant in multivariable models accounting for metabolic syndrome (Supplementary Table 6). We next asked whether there are sex differences in diabetes risk associated with metabolic syndrome. Remarkably, the incident diabetes hazard ratio for women with metabolic syndrome was nearly three times that for men, (HR = 14.17 [95%CI, 9.13–22.01] $P = 2.0 \times 10^{-33}$) vs. (HR = 5.46 [95%CI, 3.82–7.80] $P = 2.1 \times 10^{-21}$). Thus, we stratified the platelet aggregation analyses of incident diabetes by both sex and metabolic syndrome status. We observed the sex-specific risk reductions in incident diabetes with ADP and collagen were limited to females with metabolic syndrome (Supplementary Table 7).

We broadened our investigation of platelet reactivity in incident diabetes by considering dichotomous platelet aggregation traits utilizing information available from multiple platelet agonist concentrations, as previously applied to assess risk of venous thrombosis as well as cardiovascular disease risk in the FHS cohort (32, 34). Hyperreactivity to ADP was observed at baseline in 278/3,215 (8.6%); and to epinephrine, in 314/3,086

(10.2%). Hyporeactivity to ADP was seen in 100/3,215 (3.1%); and to epinephrine, in 499/3,086 (16.2%). Results of the Cox mixed-effect hazard models for incident diabetes are presented in **Supplementary Table 8**. No hyper/hypo platelet reactivity traits were associated with incident diabetes.

UK BioBank

Increased MPV is partially associated with other more established markers of increased platelet reactivity, including platelet aggregation (1). We investigated sex differences in the relationship of MPV with diabetes and medications used to manage the disease in the UK BioBank cohort. After applying exclusion criteria, the study sample consisted of 463,703 UKBB participants. Diabetes status is based on self-report of having diabetes diagnosed by a physician. Descriptive statistics for the population demographics are shown according to diabetes status in **Table 7**. Sex stratified descriptive statistics for platelet cell counts and MPV are presented in **Supplementary Tables 9, 10**, respectively. Platelet count and MPV were strongly inversely correlated (Pearson's correlation, $r = -0.46$, $P < 2.2E-16$), as is typically observed in population studies of both indices. The average participant age was 56.3 years (SD, 8.1 years), the sample was 54.8% women ($n = 242,203$), and 4.9% ($n = 22,701$) were diabetics.

TABLE 7 | Baseline characteristics of UK BioBank participants.

Baseline characteristics	Diabetic (<i>n</i> = 22,701)		Not diabetic (<i>n</i> = 441,002)		<i>P</i> -value
	Mean (<i>n</i>)	SD (%)	Mean (<i>n</i>)	SD (%)	
MPV, fL	9.46	1.12	9.33	1.08	6.9E-66
Age, year	59.39	7.25	56.18	8.11	<2.2E-16
Female, <i>n</i> (%)	8,483	39.5	233,720	55.6	<2.2E-16
BMI, kg/m ²	31.28	5.9	27.17	4.61	<2.2E-16
SBP, mm Hg	143.22	18.55	139.41	19.69	1.4E-181
DBP, mm Hg	81.48	10.29	82.24	10.69	1.7E-25
Hypertension, <i>n</i> (%)	16,703	78.9	285,278	69.4	1.6E-190
BP medication, <i>n</i> (%)	14,060	61.9	77,802	17.6	<2.2E-16
Hyperlipidemia medication, <i>n</i> (%)	16,646	73.3	59,900	13.6	<2.2E-16
Anti-platelet medication, <i>n</i> (%)	11,779	18.7	10,922	2.7	<2.2E-16
Diabetes medication, <i>n</i> (%)	15,847	69.8			
Metformin, <i>n</i> (%)	12,475	54.9			
Insulin, <i>n</i> (%)	4,336	19.1			
Sulphonylurea, <i>n</i> (%)	4,723	20.8			

BMI indicates body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure; BP, blood pressure; *n*, sample size; SD, standard deviation; *P*-value, univariate *T*-test *P*-value. Medication usage is based on self-report.

We first investigated the association between diabetes and MPV. Results of the multivariable linear model are presented in **Table 8**. Diabetes was associated with increased MPV ($\beta = 0.0976$; $P = 8.62 \times 10^{-33}$) after adjusting for age, sex, BMI, hypertension, as well as anti-platelet and cholesterol medication. We observed a strong interaction between sex and diabetes status ($P = 3.17 \times 10^{-11}$). Stratifying the analysis of diabetics by sex, we observed the association with increased MPV is markedly stronger in males ($\beta = 0.1232$; $P = 1.00 \times 10^{-31}$) than females ($\beta = 0.0514$; $P = 7.37 \times 10^{-5}$).

In order to obtain interpretable risk estimates based on MPV, we subsequently ran multivariable logistic model associations of MPV with diabetes. MPV was associated with an increase in prevalent diabetes (OR = 1.08 [95%CI, 1.07–1.10] $P = 3.3 \times 10^{-4}$) after adjusting for age, sex, BMI, hypertension, as well as anti-platelet and cholesterol medication. A significant interaction with MPV was found for sex ($P = 3.17 \times 10^{-11}$) (**Table 9**). As demonstrated in sex-stratified analyses, the magnitude of the association was greater for men (OR = 1.11 [95%CI, 1.09–1.13] $P = 3.3 \times 10^{-4}$) than women (OR = 1.05 [95%CI, 1.02–1.07] $P = 4.3 \times 10^{-5}$).

We next investigated the relationship between diabetic drug therapies and MPV. More than two-thirds (69.8%, $n = 15,847$) reported taking medication to manage their diabetes, the most common were Metformin (54.9%, $n = 12,475$), Insulin (19.1%, $n = 4,336$), and the sulphonylureas (20.8%, $n = 4,723$). Results of the multivariable linear models adjusting for age, sex, BMI, hypertension, as well as anti-platelet and cholesterol medication are presented in **Table 10**. Increased MPV was associated with Insulin ($\beta = 0.1341$; $P = 1.38 \times 10^{-11}$) whereas decreased MPV was associated with both Metformin ($\beta =$

0.0763 ; $P = 1.99 \times 10^{-6}$) as well as sulphonylureas ($\beta = 0.0559$; $P = 0.0034$). Drug-sex interactions were significant for Metformin and Insulin, but not sulphonylureas. In sex-stratified analyses each drug showed the same direction of effect in both sexes, however, the magnitude of the association with MPV was nearly twice as great or more in females compared to males (**Table 10**). We investigated whether there were sex differences in diabetes medication rates that could be impacting the associations we observed with MPV. If a bias were to follow the trend from the association of MPV with diabetes (**Table 9**), we reasoned Insulin-dependent diabetes rates may be much higher in male cases whereas females may more often be taking Metformin and sulphonylureas. We did not observe this pattern in medication usage. The difference in Insulin-dependent diabetes rates between males (18.9%, $n = 2,580$) and females (19.4%, $n = 1,756$) was not statistically significant (χ^2 test, $P = 0.32$). A greater proportion of males (56.9%, $n = 7,772$) than females (52.0%, $n = 4,703$) reported taking Metformin (χ^2 test, $P = 5.92 \times 10^{-13}$). Finally, a greater proportion of males (23.4%, $n = 3,192$) than females (16.9%, $n = 1,531$) reported taking sulphonylureas (χ^2 test, $P = 1.88 \times 10^{-31}$).

DISCUSSION

We do not observe a clear and consistent pattern of association between ADP, collagen, or epinephrine measures of platelet function by LTA with metabolic syndrome or diabetes. This is consistent with what we observed prior for VTE (34), but contrasts with arterial disease where there was a strong association of ADP-driven hyper-reactivity with future thrombosis (32). Despite the large body of research concerning the role of platelets in diabetes, evidence from population-based studies of platelet aggregation in diabetes is limited. Study samples are often selected on the basis of comorbidities including CAD (13, 38) or disease severity, such as patients hospitalized for inadequate glycemic control (20). Our study results did not change appreciably if we adjusted for, or stratified, diabetes associations on aspirin use, or on CVD status. Many clinical studies were limited to one sex (10, 15) and the majority have not accounted for sex as a confounding factor. While women in general possess greater platelet reactivity (30), our results do suggest that platelet reactivity associated with both metabolic syndrome and diabetes is reduced in women compared to men. Recently, there was a study of 81 diabetic men and 533 non-diabetic men with platelet aggregation assays of ADP, AA, collagen, thrombin receptor activating peptide (TRAP) and protease-activated receptor 4 agonist peptide (PAR-4AP) that also reported negative findings in regards to platelet function measurements and diabetes status, whether in aspirin or non-aspirin takers (39).

MPV has been suggested as a potential surrogate metric for other established markers of platelet activity including platelet aggregation, though evidence has been mixed (22). Since platelet aggregation has only been measured in cohorts of several thousands and often in clinical centers primarily focused on platelet and bleeding disorders, utilizing MPV

TABLE 8 | Results of multivariable linear model for association of self-reported diabetes with MPV and stratified by sex.

Phenotype	All (n = 440,393)			Males (n = 198,969)			Females (n = 241,424)			<i>P</i> _{inter}
	Beta	SE	<i>P</i>	Beta	SE	<i>P</i>	Beta	SE	<i>P</i>	
MPV	0.0976	0.0082	8.62E-33	0.1232	0.0105	1.00E-31	0.0514	0.0130	7.37E-05	3.17E-11

Main model adjusts for age, sex, BMI, hypertension, anti-platelet, and cholesterol medication usage. Stratified models adjust for age, BMI, hypertension, anti-platelet and cholesterol medication usage. Medication usage is based on self-report. SE, standard error; *P*, *P*-value; *P*_{inter}, *P*-value for interaction between diabetes status and sex.

TABLE 9 | Results of multivariable logistic regression model for association of MPV with self-reported diabetes and stratified by sex.

Phenotype	All (n = 440,393)			Males (n = 198,969)			Females (n = 241,424)			<i>P</i> _{inter}
	OR	95% CI	<i>P</i>	OR	95% CI	<i>P</i>	OR	95% CI	<i>P</i>	
MPV	1.08	1.07–1.10	6.49E-30	1.11	1.09–1.13	3.16E-30	1.05	1.02–1.07	4.28E-05	6.80E-05

Main model adjusts for age, sex, BMI, hypertension, anti-platelet, and cholesterol medication usage. Stratified models adjust for age, BMI, hypertension, anti-platelet, and cholesterol medication usage. Medication usage is based on self-report. OR indicates odds ratio; CI, confidence interval; *P*, *P*-value; *P*_{inter}, *P*-value for interaction between MPV and sex.

TABLE 10 | Results of multivariable linear models for association of diabetes medication usage with MPV and stratified by sex.

Medication	All diabetics (n = 21,314)			Male (n = 12,896)			Female (n = 8,418)			<i>P</i> _{inter}
	Beta	SE	<i>P</i>	Beta	SE	<i>P</i>	Beta	SE	<i>P</i>	
Insulin	0.1341	0.0198	1.38E-11	0.1058	0.0257	3.75E-05	0.1782	0.0313	1.27E-08	2.81E-06
Metformin	−0.0763	0.0160	1.99E-06	−0.0504	0.0206	1.44E-02	−0.1205	0.0257	2.75E-06	6.42E-06
Sulphonylureas	−0.0559	0.0191	3.41E-03	−0.0391	0.0236	9.71E-02	−0.0888	0.0327	6.64E-03	0.077

Main model adjusts for age, sex, BMI, hypertension, anti-platelet, and cholesterol medication usage. Stratified models adjust for age, BMI, hypertension, anti-platelet, and cholesterol medication usage. Medication usage is based on self-report. SE, standard error; *P*, *P*-value; *P*_{inter}, *P*-value for interaction between medication and sex.

in research measurements has the potential to access much larger populations and clinical cohort samples. Utilizing data from the UK BioBank we conducted the largest multivariable analysis of MPV and diabetes to date. While the effect size is modest, MPV appears to be robustly associated with diabetes and differs in magnitude according to sex. The effect among females was less than half of that observed in males. In contrast, among non-diabetics MPV is increased in females compared to males (Supplementary Table 10) an observation consistent with sex differences observed in platelet aggregation studies (30). The ability to detect this association with sex in the general population may depend on the sample size. Increased MPV in females is observed in reference measurements of healthy populations including more than 12,000 subjects (40, 41), but not fewer (42, 43).

We observed strong associations between MPV and several medications used to manage diabetes, including insulin, metformin, and sulphonylureas. In each case, there is evidence for a biological basis to the association. This is the first study to show an association between insulin therapy and MPV in diabetics. Two previous studies (with fewer than 350 insulin-dependent diabetics in each) did not (23, 27). Insulin has been shown to reduce platelet aggregation in healthy subjects both *in vitro* and *in vivo* (44). Insulin inhibits Ca²⁺ mobilization in platelets by interfering with P2Y₁₂-mediated cAMP suppression (45). The platelets of diabetics have decreased sensitivity to insulin, potentially leading to reduced P2Y₁₂ inhibition and increased platelet reactivity (46). The strong increase in MPV

associated with insulin therapy may be explained by the fact that patients with insulin-treated diabetes are likely to be at a more advanced stage of insulin resistance (47). This may further explain increased MPV in females because women are more intrinsically insulin resistant than men (48).

Metformin has been shown to reduce platelet aggregation to ADP in insulin-dependent diabetics (49). This may be related to the drug's protective effect on mitochondrial function. Hyperglycemia induces mitochondrial hyperpolarization in platelets, resulting in increased reactive oxygen species (ROS) generation and subsequent activation (50). Metformin inhibits mitochondrial complex I and thereby reduces activated platelet-induced mitochondrial hyperpolarization, ROS overload, and inhibits mitochondrial DNA release (51). Metformin therapy reduces plasma levels of interleukin-6 (IL-6), an inflammatory cytokine associated with insulin resistance and diabetes risk (52). IL-6 functions in a paracrine signaling loop, stimulating thrombopoietin (TPO) in the liver which increases platelet production in the bone marrow (53). Metformin inhibits hepatic IL-6 signaling *in vivo* (54). While metformin might act to inhibit IL-6/TPO and platelet generation, it is unclear to us how this might lead to a concomitant decrease in the average size of platelets, given that PLT and MPV are typically strongly inversely correlated. However, one indication that there could be a causal effect of metformin on reducing platelet volumes comes from a study of 60 newly diagnosed diabetics treated with metformin monotherapy. After 6 months from baseline, the metformin-treated individuals had a significant reduction in MPV (55).

Inhibition of platelet aggregation by sulphonylureas has also been demonstrated *in vitro* (56, 57). However, a clinical trial of Gliclazide failed to show an effect on platelet aggregation in either insulin- or non-insulin-treated diabetics (58). For the most commonly prescribed sulphonylurea, Gliclazide, the mechanism may involve inhibition of activated glycogen synthetase, activation of adenylate cyclase, modulation of arachidonic acid release from platelet membranes, stimulation of prostacyclin production, and inhibition of the pro-aggregating action of thromboxane A2 (59). Gliclazide therapy has also been shown to reduce IL-6 levels in diabetics presenting with poor glucose control (60), and similar to metformin we speculate that changes in the IL-6/TPO axis could mediate feedback effects on platelet generation, turnover and the average size of platelets.

Sex differences with respect to platelet reactivity were apparent in the FHS as well as UK BioBank cohorts. In the former, platelet aggregation associated with both metabolic syndrome and diabetes was reduced in women compared to men. In the latter, the increase in MPV associated with diabetes among women was less than half of that observed in men. Further, the association of diabetes medications with MPV was nearly twice as great or more in women compared to men. Women are distinctly different from men with regard to the actions of insulin and the susceptibility to develop insulin resistance (48). Our results indicate it will be important for future studies to consider sex differences and medication status. Diabetes, even at an early stage, attenuates the protective effect of female sex on CVD and increases the risk for CVD in females to a greater extent than in males (61). While the effect size of MPV may be small it may prove a useful variable in prediction models for diabetes, especially given the importance of sex differences in its biological risk factors and pathophysiological mechanisms. The precise mechanisms whereby diabetes status influences MPV remains to be studied further and may include complex interactions between disease progression, sex, medications, genetic alleles influencing MPV or diabetes, and effects on platelet generation and turnover.

Limitations

Though LTA is the gold standard, it is still an *in vitro* rather *in vivo* measure of platelet activation. Although, LTA in FHS was performed by lab staff experienced with a high volume of samples, LTA can be subject to technical variability including operator influences that can potentially harm accuracy or generalizability of results (62). We also were limited to those historically measured agonists and concentrations available within FHS. Other pathways of platelet activation (e.g., thrombin) or approaches to platelet function (e.g., whole blood assays, granule release markers, other platelet morphology assays like spreading and imaging-based assays, shear stress flow systems) were not available for analysis in the FHS Offspring cohort at Exam 5. While all regression models accounted for aspirin usage, this was determined by an indirect measure (failure to demonstrate full aggregation response to arachidonic acid). Whether and when participants precisely took aspirin could not be determined. However, a recent study found that arachidonic acid LTA provided the best discrimination of aspirin use in diabetics among nine platelet function tests examined (63).

Both populations we examined are of predominantly European ancestry which could limit the generalizability of the results. Some prior work such as the large NHANES study ($n = 17,969$) suggest that African or Hispanic populations may have higher MPV than European ancestry populations, though the absolute mean difference in NHANES was small (<0.17 femptoliters) (64). There are some limitations unique to the UK BioBank analyses. First, diabetes status is based on self-report, though that of being diagnosed by a physician. We were unable to consider direct measures of several lipid (HDL, triglycerides) and nonlipid (fasting blood glucose) risk factors which would have allowed us to classify participants with metabolic syndrome. However, our models did adjust for BMI, hypertension and indirect evidence of lipid and thrombotic risk, cholesterol lowering and antiplatelet medications, respectively. MPV measurements seem to be effected by pre-analytic factors and analytic factors like timing, temperature, tube type, and anticoagulant, and instrument more so than most other cell count measurements (65–67). A strength of this study is that a single tube type and instrument model were used to measure MPV in a large sample, though this could also limit generalizability to other studies with different instruments or tubes. Due to the size and nature of the study UK BioBank samples were shipped overnight and stored at 4°C. We cannot determine whether these conditions may have impacted diabetes-MPV associations. However, we do not expect storage and transport time to be specifically related to diabetes status, thus, we expect there would be little confounding of our associations. The associations of MPV with Metformin, Insulin, and sulphonylureas are also based on self-report. Retrospective analyses of in the UK have estimated medication adherence to oral hypoglycemics to be 81.6% for monotherapy and 80.8% for dual therapy (68). Adherence to insulin therapy in the UK is estimated to be 70.6% (69).

In conclusion, platelet function as measured by aggregation does not appear to be consistently associated with diabetes. In agreement with prior studies MPV is robustly associated suggesting future work may focus on how MPV segments pre-diabetics and diabetics for risk prediction as well as how this may differ by treatment and sex.

DATA AVAILABILITY STATEMENT

The datasets generated for this study will not be made publicly available. Data from The Framingham Heart Study is available through application on dbGaP (https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000007.v30.p11). Data from UK BioBank is available through application to UK BioBank (<https://www.ukbiobank.ac.uk/researchers/>).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Boston University Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BR conducted all statistical analyses, and BR and AJ wrote the manuscript. Both authors critically edited the manuscript and approved the final submission.

FUNDING

This work was supported by the National Heart, Lung, and Blood Institute (NHLBI) contracts N01-HL 25195, R01-HL-48157, and HHSN268201500001I, and by NHLBI Intramural Research Program funding (BR, AJ).

REFERENCES

- Sharma G, Berger JS. Platelet activity and cardiovascular risk in apparently healthy individuals: a review of the data. *J Thromb Thrombolysis*. (2011) 32:201–8. doi: 10.1007/s11239-011-0590-9
- Fox CS, Golden SH, Anderson C, Bray GA, Burke LE, Boer IHD, et al. Update on prevention of cardiovascular disease in adults with type 2 diabetes mellitus in light of recent evidence. *Circulation*. (2015) 132:691–718. doi: 10.1161/CIR.0000000000000230
- Benjamin EJ, Virani SS, Callaway CW, Chamberlain AM, Chang AR, Cheng S, et al. Heart disease and stroke statistics 2018. Update: a report from the American Heart Association. *Circulation*. (2018) 137:e67–e492. doi: 10.1161/CIR.0000000000000558
- Expert Panel on Detection Evaluation and Treatment of High Blood Cholesterol in Adults. Executive summary of the third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (adult treatment panel III). *JAMA*. (2001). 285:2486–97. doi: 10.1001/jama.285.19.2486
- Wilson PWF, D'Agostino RB, Parise H, Sullivan L, Meigs JB. Metabolic syndrome as a precursor of cardiovascular disease and type 2 diabetes mellitus. *Circulation*. (2005) 112:3066–72. doi: 10.1161/CIRCULATIONAHA.105.539528
- Westein E, Hofer T, Calkin Anna C. Thrombosis in diabetes: a shear flow effect? *Clin Sci*. (2017) 131:1245–60. doi: 10.1042/CS20160391
- Fateh-Moghadam S, Li Z, Ersel S, Reuter T, Htun P, Plöckinger U, et al. Platelet degranulation is associated with progression of intima-media thickness of the common carotid artery in patients with diabetes mellitus type 2. *Arterioscler Thromb Vasc Biol*. (2005) 25:1299–303. doi: 10.1161/01.ATV.0000165699.41301.c5
- Garber AJ, Abrahamson MJ, Barzilay JL, Blonde L, Bloomgarden ZT, Bush MA, et al. Consensus statement by the American association of clinical endocrinologists and American college of endocrinology on the comprehensive type 2 diabetes management algorithm - 2019. Executive summary. *Endocr Pract*. (2019) 25:69–100. doi: 10.4158/CS-2018-0535
- Santilli F, Simeone P, Liani R. The role of platelets in diabetes mellitus. In: Michelson AD, editor. *Platelets, 4th edn*. Cambridge, MA: Elsevier (2019). p. 469–503.
- Colwell JA, Sagel J, Crook L, Chambers A, Laimins M. Correlation of platelet aggregation, plasma factor activity, and megathrombocytes in diabetic subjects with and without vascular disease. *Metabolism*. (1977) 26:279–85. doi: 10.1016/0026-0495(77)90074-9
- Sagel J, Colwell JA, Crook L, Laimins M. Increased platelet aggregation in early diabetes mellitus. *Ann Intern Med*. (1975) 82:733–8. doi: 10.7326/0003-4819-82-6-733
- Al-Sofiani ME, Yanek LR, Faraday N, Kral BG, Mathias R, Becker LC, et al. Diabetes and platelet response to low-dose aspirin. *J Clin Endocrinol Metab*. (2018) 103:4599–608. doi: 10.1210/je.2018-01254
- Angiolillo DJ, Fernandez-Ortiz A, Bernardo E, Ramirez C, Sabaté M, Jimenez-Quevedo P, et al. Platelet function profiles in patients with type 2 diabetes

ACKNOWLEDGMENTS

We thank the FHS (Framingham Heart Study) participants for their ongoing participation and dedication to the study, making this work possible. Research on MPV has been conducted using the UK Biobank Resource.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcvm.2020.00118/full#supplementary-material>

- and coronary artery disease on combined aspirin and clopidogrel treatment. *Diabetes*. (2005) 54:2430–5. doi: 10.2337/diabetes.54.8.2430
- Gresele P, Guglielmini G, De Angelis M, Ciferri S, Ciofetta M, Falcinelli E, et al. Acute, short-term hyperglycemia enhances shear stress-induced platelet activation in patients with type II diabetes mellitus. *J Am Coll Cardiol*. (2003) 41:1013–20. doi: 10.1016/S0735-1097(02)02972-8
- Jones DB, Davis TME, Bown E, Carter RD, Mann JI, Prescott RJ. Determinants of ADP-induced platelet aggregation in diabetes mellitus. *Diabetologia*. (1986) 29:291–4. doi: 10.1007/BF00452065
- Kutti J, Wadenvik H, Henestam B, Stenstrom G. Evaluation of platelet reactivity in diabetes mellitus. *Acta Med Scand*. (1986) 219:195–9. doi: 10.1111/j.0954-6820.1986.tb03298.x
- Vaduganathan M, Alviar CL, Arian ME, Tellez A, Guthikonda S, DeLao T, et al. Platelet reactivity and response to aspirin in subjects with the metabolic syndrome. *Am Heart J*. (2008) 156:1002.e1–e7. doi: 10.1016/j.ahj.2008.08.002
- Vaidya D, Yanek LR, Faraday N, Moy TF, Becker LC, Becker DM. Native platelet aggregation and response to aspirin in persons with the metabolic syndrome and its components. *Metab Syndr Relat Disord*. (2009) 7:289–96. doi: 10.1089/met.2008.0083
- Soma P, Swanepoel AC, du Plooy JN, Mqoco T, Pretorius E. Flow cytometric analysis of platelets type 2 diabetes mellitus reveals 'angry' platelets. *Cardiovasc Diabetol*. (2016). 15:52. doi: 10.1186/s12933-016-0373-x
- Davi G, Catalano I, Aversa M, Notarbartolo A, Strano A, Ciabattini G, Patrono C. Thromboxane biosynthesis and platelet function in type II diabetes mellitus. *N Engl J Med*. (1990) 322:1769–74. doi: 10.1056/NEJM199006213222503
- Rocca B, Santilli F, Pitocco D, Mucci L, Petrucci G, Vitacolonna E, et al. The recovery of platelet cyclooxygenase activity explains interindividual variability in responsiveness to low-dose aspirin in patients with and without diabetes. *J Thromb Haemost*. (2012) 10:1220–30. doi: 10.1111/j.1538-7836.2012.04723.x
- Puurunen M, Johnson AD. Mean platelet volume - a controversial marker of platelets that requires further unpacking. *Thromb Res*. (2017) 153:118–9. doi: 10.1016/j.thromres.2017.03.015
- Papanas N, Symeonidis G, Maltezos E, Mavridis G, Karavageli E, Vosnakidis T, et al. Mean platelet volume in patients with type 2 diabetes mellitus. *Platelets*. (2004) 15:475–8. doi: 10.1080/0953710042000267707
- Demirtunc R, Duman D, Basar M, Bilgi M, Teomete M, Garip T. The relationship between glycemic control and platelet activity in type 2 diabetes mellitus. *J Diabetes Complications*. (2009) 23:89–94. doi: 10.1016/j.jdiacomp.2008.01.006
- Coban E, Bostan F, Ozdogan M. The mean platelet volume in subjects with impaired fasting glucose. *Platelets*. (2006) 17:67–9. doi: 10.1080/09537100500220729
- Shah B, Sha D, Xie D, Mohler ER, Berger JS. The relationship between diabetes, metabolic syndrome, and platelet activity as measured by mean platelet volume. The National Health and Nutrition Examination Survey, 1999–2004. *Diabetes Care*. (2012) 35:1074–8. doi: 10.2337/dc11-1724

27. Verdoia M, Schaffer A, Barbieri L, Cassetti E, Nardin M, Bellomo G, et al. Diabetes, glucose control and mean platelet volume: a single-centre cohort study. *Diabetes Res Clin Pract.* (2014) 104:288–94. doi: 10.1016/j.diabres.2013.12.020
28. Han JY, Choi DH, Choi SW, Kim BB, Ki YJ, Chung JW, et al. Stroke or coronary artery disease prediction from mean platelet volume in patients with type 2 diabetes mellitus. *Platelets.* (2013) 24:401–6. doi: 10.3109/09537104.2012.710858
29. Klovaité J, Benn M, Yazdanyar S, Nordestgaard BG. High platelet volume and increased risk of myocardial infarction: 39,531 participants from the general population. *J Thromb Haemost.* (2011) 9:49–56. doi: 10.1111/j.1538-7836.2010.04110.x
30. Becker DM, Segal J, Vaidya D, Yanek LR, Herrera-Galeano JE, Bray PF, et al. Sex differences in platelet reactivity and response to low-dose aspirin therapy. *JAMA.* (2006) 295:1420–7. doi: 10.1001/jama.295.12.1420
31. Kannel WB, Feinleib M, McNamara PM, Garrison RJ, Castelli WP. An investigation of coronary heart disease in families. The Framingham offspring study. *Am J Epidemiol.* (1979) 110:281–90. doi: 10.1093/oxfordjournals.aje.a112813
32. Puurunen MK, Hwang SJ, Larson MG, Vasan RS, O'Donnell CJ, Tofler G, et al. ADP platelet hyperreactivity predicts cardiovascular disease in the FHS (Framingham Heart Study). *J Am Heart Assoc.* (2018) 7:e008522. doi: 10.1161/JAHA.118.008522
33. Johnson AD, Yanek LR, Chen M-H, Faraday N, Larson MG, Tofler G, et al. Genome-wide meta-analyses identifies seven loci associated with platelet aggregation in response to agonists. *Nat Genet.* (2010) 42:608. doi: 10.1038/ng.604
34. Puurunen MK, Hwang S-J, O'Donnell CJ, Tofler G, Johnson AD. Platelet function as a risk factor for venous thromboembolism in the Framingham Heart Study. *Thromb Res.* (2017) 151:57–62. doi: 10.1016/j.thromres.2017.01.010
35. Alberti KGMM, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, et al. Harmonizing the metabolic syndrome. *Circulation.* (2009) 120:1640–45. doi: 10.1161/CIRCULATIONAHA.109.192644
36. Sudlow C, Gallacher J, Allen N, Beral V, Burton P, Danesh J, et al. UK biobank: an open access resource for identifying the causes of a wide range of complex diseases of middle and old age. *PLoS Med.* (2015) 12:e1001779. doi: 10.1371/journal.pmed.1001779
37. Astle WJ, Elding H, Jiang T, Allen D, Ruklisa D, Mann AL, et al. The allelic landscape of human blood cell trait variation and links to common complex disease. *Cell.* (2016) 167:1415–29. doi: 10.1016/j.cell.2016.10.042
38. Serebruany V, Pokov I, Kuliczowski W, Chesebro J, Badimon J. Baseline platelet activity and response after clopidogrel in 257 diabetics among 822 patients with coronary artery disease. *Thromb Haemost.* (2008) 100:76–82. doi: 10.1160/TH08-03-0136
39. Kring C, Rasmussen LM, Lindholt JS, Diederichsen ACP, Vinholt PJ. Platelet aggregation is not altered among men with diabetes mellitus. *Acta Diabetol.* (2020) 57:389–99. doi: 10.1007/s00592-019-01438-y
40. Lee JS, Kim NY, Na SH, Youn YH, Shin CS. Reference values of neutrophil-lymphocyte ratio, lymphocyte-monocyte ratio, platelet-lymphocyte ratio, and mean platelet volume in healthy adults in South Korea. *Medicine.* (2018) 97:e11138. doi: 10.1097/MD.00000000000011138
41. Zhao F, Yan Z, Meng Z, Li X, Liu M, Ren X, et al. Relationship between mean platelet volume and metabolic syndrome in Chinese patients. *Sci Rep.* (2018). 8:14574. doi: 10.1038/s41598-018-32751-1
42. Hong J, Min Z, Bai-shen P, Jie Z, Ming-ting P, Xian-zhang H, et al. Investigation on reference intervals and regional differences of platelet indices in healthy chinese han adults. *J Clin Lab Anal.* (2015) 29:21–7. doi: 10.1002/jcla.21721
43. Cho SY, Lee HJ, Lee W-I, Suh J-T, Park TS. Large population study for the reference range of mean platelet volume in Korean individuals. (2012) 130:557–8. doi: 10.1016/j.thromres.2012.05.009
44. Trovati M, Anfossi G, Cavalot F, Massucco P, Mularoni E, Emanuelli G. Insulin directly reduces platelet sensitivity to aggregating agents. Studies *in vitro* and *in vivo*. *Diabetes.* (1988) 37:780–6. doi: 10.2337/diabetes.37.6.780
45. Ferreira IA, Eybrechts KL, Mocking AIM, Kroner C, Akkerman J-WN. IRS-1 mediates inhibition of Ca²⁺ mobilization by insulin via the inhibitory G-protein Gi. *J Biol Chem.* (2004) 279:3254–64. doi: 10.1074/jbc.M305474200
46. Ferreira Irlando A, Mocking Astrid IM, Feijge Marion AH, Gorter G, van Haeften Timon W, Heemskerk Johan WM, et al. Platelet inhibition by insulin is absent in type 2 diabetes mellitus. *Arterioscler Thromb Vasc Biol.* (2006) 26:417–22. doi: 10.1161/01.ATV.0000199519.37089.a0
47. Angiolillo DJ, Bernardo E, Ramirez C, Costa MA, Sabaté M, Jimenez-Quevedo P, et al. Insulin therapy is associated with platelet dysfunction in patients with type 2 diabetes mellitus on dual oral antiplatelet treatment. *J Am Coll Cardiol.* (2006) 48:298–304. doi: 10.1016/j.jacc.2006.03.038
48. Mittendorfer B. Insulin resistance: sex matters. *Curr Opin Clin Nutr Metab Care.* (2005) 8:367–72. doi: 10.1097/01.mco.0000172574.64019.98
49. Gin H, Freyburger G, Boisseau M, Aubertin J. Study of the effect of metformin on platelet aggregation in insulin-dependent diabetics. *Diabetes Res Clin Pract.* (1989) 6:61–7. doi: 10.1016/0168-8227(89)90058-2
50. Yamagishi S-i, Edelstein D, Du X-I, Brownlee M. Hyperglycemia potentiates collagen-induced platelet activation through mitochondrial superoxide overproduction. *Diabetes.* (2001) 50:1491. doi: 10.2337/diabetes.50.6.1491
51. Xin G, Wei Z, Ji C, Zheng H, Gu J, Ma L, et al. Metformin uniquely prevents thrombosis by inhibiting platelet activation and mtDNA release. *Sci Rep.* (2016). 6:36222. doi: 10.1038/srep36222
52. Bulcão C, Ribeiro-Filho FF, Sañudo A, Roberta Ferreira SG. Effects of simvastatin and metformin on inflammation and insulin resistance in individuals with mild metabolic syndrome. *Am J Cardiovasc Drugs.* (2007) 7:219–24. doi: 10.2165/00129784-200707030-00007
53. Stone RL, Nick AM, McNeish IA, Balkwill F, Han HD, Bottsford-Miller J, et al. Paraneoplastic thrombocytosis in ovarian cancer. *N Engl J Med.* (2012) 366:610–8. doi: 10.1056/NEJMoa1110352
54. Kim YD, Kim YH, Cho YM, Kim DK, Ahn SW, Lee JM, et al. Metformin ameliorates IL-6-induced hepatic insulin resistance via induction of orphan nuclear receptor small heterodimer partner (SHP) in mouse models. *Diabetologia.* (2012) 55:1482–94. doi: 10.1007/s00125-012-2494-4
55. Dolasik I, Sener SY, Celebi K, Aydin ZM, Korkmaz U, Canturk Z. The effect of metformin on mean platelet volume in diabetic patients. *Platelets.* (2013) 24:118–21. doi: 10.3109/09537104.2012.674165
56. Mizuno A, Yano M, Isobe J, Shima K. Inhibitory action of gliclazide on platelet functions. *Diabetes Res Clin Pract.* (1989) 7:141–7. doi: 10.1016/0168-8227(89)90105-8
57. Siluk D, Kaliszan R, Haber P, Petrusiewicz J, Brzozowski Z, Sut G. Antiaggregatory activity of hypoglycaemic sulphonylureas. *Diabetologia.* (2002) 45:1034–7. doi: 10.1007/s00125-002-0855-0
58. Larkins RG, Jerums G, Taft JL, Godfrey H, Smith IL, Martin TJ. Lack of effect of gliclazide on platelet aggregation in insulin-treated and non-insulin-treated diabetes: a two-year controlled study. *Diabetes Res Clin Pract.* (1988) 4:81–7. doi: 10.1016/S0168-8227(88)80001-9
59. Papazafropoulou A, Papanas N, Pappas S, Maltezos E, Mikhailidis DP. Effects of oral hypoglycemic agents on platelet function. *J Diabetes Complications.* (2015) 29:846–51. doi: 10.1016/j.jdiacomp.2015.04.005
60. Drzewoski J, Zurawska-Klis M. Effect of gliclazide modified release on adiponectin, interleukin-6, and tumor necrosis factor- α plasma levels in individuals with type 2 diabetes mellitus. *Curr Med Res Opin.* (2006) 22:1921–26. doi: 10.1185/030079906X132424
61. Kautzky-Willer A, Harreiter J, Pacini G. Sex and gender differences in risk, pathophysiology and complications of type 2 diabetes mellitus. *Endocr Rev.* (2016) 37:278–316. doi: 10.1210/er.2015-1137
62. Cattaneo M, Cerletti C, Harrison P, Hayward CP, Kenny D, Nugent D, et al. Recommendations for the standardization of light transmission aggregometry: a consensus of the working party from the platelet physiology subcommittee of SSC/ISTH. *J Thromb Haemost.* (2013) 11:1183–89. doi: 10.1111/jth.12231
63. Harrison P, Bethel MA, Kennedy I, Dinsdale R, Coleman R, Holman RR. Comparison of nine platelet function tests used to determine responses to different aspirin dosages in people with type 2 diabetes. *Platelets.* (2019) 30:521–9. doi: 10.1080/09537104.2018.1478402
64. Ball S, Arevalo M, Wongsasak S, Dennis JA, Nugent K. Implications of mean platelet volume in health and disease: a large population study on data from National Health and Nutrition Examination Survey. *Thromb Res.* (2019) 175:90–4. doi: 10.1016/j.thromres.2019.01.011

65. Lance MD, Sloep M, Henskens YM, Marcus MA. Mean platelet volume as a diagnostic marker for cardiovascular disease: drawbacks of preanalytical conditions and measuring techniques. *Clin Appl Thromb Hemost.* (2012) 18:561–8. doi: 10.1177/1076029612458147
66. Latger-Cannard V, Hoarau M, Salignac S, Baumgart D, Nurden P, Lecompte T. Mean platelet volume: comparison of three analysers towards standardization of platelet morphological phenotype. *Int J Lab Hematol.* (2012) 34:300–10. doi: 10.1111/j.1751-553X.2011.01396.x
67. Lippi G, Pavesi F, Pipitone S. Evaluation of mean platelet volume with four hematological analyzers: harmonization is still an unresolved issue. *Blood Coagul Fibrinolysis.* (2015) 26:235–7. doi: 10.1097/MBC.0000000000000220
68. Gordon J, McEwan P, Idris I, Evans M, Puelles J. Treatment choice, medication adherence and glycemic efficacy in people with type 2 diabetes: a UK clinical practice database study. *BMJ Open Diabetes Res Care.* (2018) 6:e000512. doi: 10.1136/bmjdr-2018-000512
69. Donnelly LA, Morris AD, Evans JMM, for the DARTS/MEMO Collaboration. Adherence to insulin and its association with glycaemic control in patients

with type 2 diabetes. *Q J Med.* (2007) 100:345–50. doi: 10.1093/qjmed/hcm031

Disclaimer: The views expressed in this manuscript are those of the authors and do not necessarily represent the views of the National Heart, Lung, and Blood Institute; the National Institutes of Health; or the U.S. Department of Health and Human Services.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Rodríguez and Johnson. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Advantages of publishing in Frontiers



OPEN ACCESS

Articles are free to read
for greatest visibility
and readership



FAST PUBLICATION

Around 90 days
from submission
to decision



HIGH QUALITY PEER-REVIEW

Rigorous, collaborative,
and constructive
peer-review



TRANSPARENT PEER-REVIEW

Editors and reviewers
acknowledged by name
on published articles

Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org

Contact us: frontiersin.org/about/contact



REPRODUCIBILITY OF RESEARCH

Support open data
and methods to enhance
research reproducibility



DIGITAL PUBLISHING

Articles designed
for optimal readership
across devices



FOLLOW US

@frontiersin



IMPACT METRICS

Advanced article metrics
track visibility across
digital media



EXTENSIVE PROMOTION

Marketing
and promotion
of impactful research



LOOP RESEARCH NETWORK

Our network
increases your
article's readership