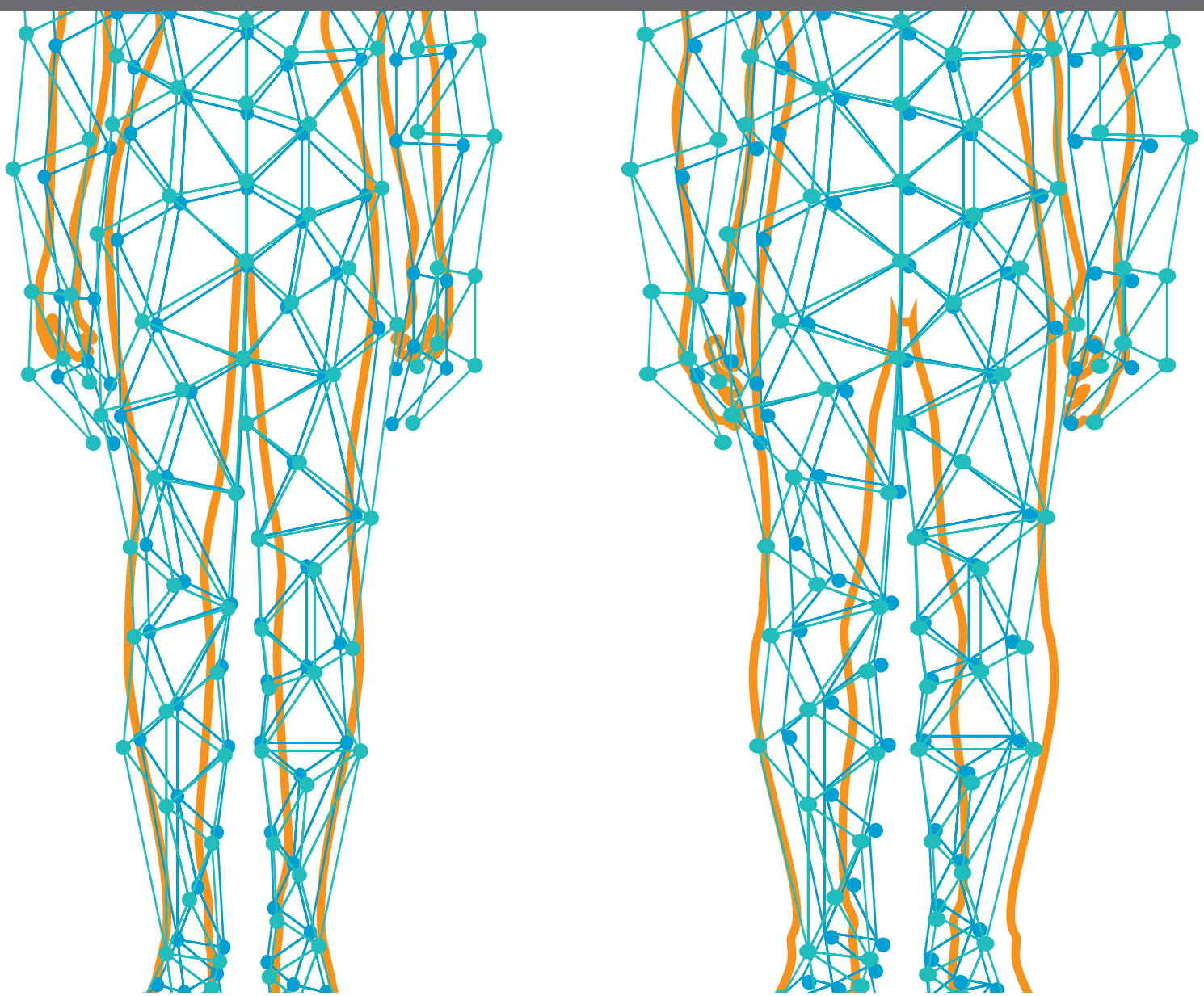
SPOTLIGHT ON THE RELATIONSHIP BETWEEN SEPSIS AND INFECTION: FROM MECHANISMS TO THERAPY

EDITED BY: Alessandro Russo, David Paterson and Matteo Bassetti

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SPOTLIGHT ON THE RELATIONSHIP BETWEEN SEPSIS AND INFECTION: FROM MECHANISMS TO THERAPY

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Table of Contents

06 ***Editorial: Spotlight on the Relationship Between Sepsis and Infection: From Mechanisms to Therapy***
Alessandro Russo, David L. Paterson and Matteo Bassetti

10 ***Challenges in the Management of SARS-CoV2 Infection: The Role of Oral Bacteriotherapy as Complementary Therapeutic Strategy to Avoid the Progression of COVID-19***
Gabriella d'Ettorre, Giancarlo Ceccarelli, Massimiliano Marazzato, Giuseppe Campagna, Claudia Pinacchio, Francesco Alessandri, Franco Ruberto, Giacomo Rossi, Luigi Celani, Carolina Scagnolari, Cristina Mastropietro, Vito Trinchieri, Gregorio Egidio Recchia, Vera Mauro, Guido Antonelli, Francesco Pugliese and Claudio Maria Mastroianni

17 ***Infection, Sepsis and the Inflammatory Response: Mechanisms and Therapy***
Dagan O. Lonsdale, Reya V. Shah and Jeffrey Lipman

23 ***Role of Complement and Histones in Sepsis***
Firas S. Zetoune and Peter A. Ward

29 ***Sphingolipidomics in Translational Sepsis Research—Biomedical Considerations and Perspectives***
Ralf A. Claus and Markus H. Graeler

47 ***Keep Your Friends Close, but Your Enemies Closer: Role of Acid Sphingomyelinase During Infection and Host Response***
Ha-Yeun Chung and Ralf A. Claus

64 ***Antimicrobial Resistance in* Enterobacterales *and Its Contribution to Sepsis in Sub-saharan Africa***
Kathleen Tompkins, Jonathan J. Juliano and David van Duin

72 ***Early Detection of Sepsis With Machine Learning Techniques: A Brief Clinical Perspective***
Daniele Roberto Giacobbe, Alessio Signori, Filippo Del Puente, Sara Mora, Luca Carmisciano, Federica Briano, Antonio Vena, Lorenzo Ball, Chiara Robba, Paolo Pelosi, Mauro Giacomini and Matteo Bassetti

83 ***Immunological Endotyping of Chronic Critical Illness After Severe Sepsis***
Brittany P. Fenner, D. B. Darden, Lauren S. Kelly, Jaimar Rincon, Scott C. Brakenridge, Shawn D. Larson, Frederick A. Moore, Philip A. Efron and Lyle L. Moldawer

98 ***Adjunctive Immunotherapy With Polyclonal Ig-M Enriched Immunoglobulins for Septic Shock: From Bench to Bedside. The Rationale for a Personalized Treatment Protocol***
Stefano Busani, Erika Roat, Martina Tosi, Emanuela Biagioni, Irene Coloretti, Marianna Meschiari, Roberta Gelmini, Lucio Brugioni, Sara De Biasi and Massimo Girardis

106 ***Current Challenges in the Management of Infective Endocarditis***
Guillermo Cuervo, Francesc Escrihuela-Vidal, Carlota Gudiol and Jordi Carratalà

121 ***Dynamic Changes in Microbial Composition During Necrotizing Soft-Tissue Infections in ICU Patients***
Michael Thy, Sébastien Tanaka, Alexy Tran-Dinh, Lara Ribeiro, Brice Lortat-Jacob, Julia Donadio, Nathalie Zappella, Mouna Ben-Rehouma, Parvine Tashk, Aurelie Snauwaert, Enora Atchade, Nathalie Grall and Philippe Montravers

132 ***Management of* Staphylococcus aureus *Bloodstream Infections***
Aurelia Kimmig, Stefan Hagel, Sebastian Weis, Christina Bahrs, Bettina Löffler and Mathias W. Pletz

140 ***Extensively Drug-Resistant Hypervirulent* Klebsiella pneumoniae *From a Series of Neonatal Sepsis in a Tertiary Care Hospital, India***
Tuhina Banerjee, Jayalaxmi Wangkheimayum, Swati Sharma, Ashok Kumar and Amitabha Bhattacharjee

147 ***Rapid Antimicrobial Susceptibility Testing Methods for Blood Cultures and Their Clinical Impact***
Ritu Banerjee and Romney Humphries

156 ***Genetic Basis of Antimicrobial Resistant Gram-Negative Bacteria Isolated From Bloodstream in Brazil***
Melise Chaves Silveira, Cláudio Marcos Rocha-de-Souza, Ivson Cassiano de Oliveira Santos, Leilane da Silva Pontes, Thamirys Rachel Tavares e Oliveira, Camila Bastos Tavares-Teixeira, Nataly de Almeida Cossatis, Natacha Ferreira Pereira, Orlando Carlos da Conceição-Neto, Bianca Santos da Costa, Daiana Cristina Silva Rodrigues, Rodolpho Mattos Albano, Fabrício Alves Barbosa da Silva, Elizabeth Andrade Marques, Robson Souza Leão and Ana Paula D'Alincourt Carvalho-Assef

165 ***Accuracy and Prognosis Value of the Sequential Organ Failure Assessment Score Combined With C-Reactive Protein in Patients With Complicated Infective Endocarditis***
Yaowang Lin, Shaohong Dong, Jie Yuan, Danqing Yu, Weijie Bei, Ruimian Chen and Haiyan Qin

173 ***Understanding and Managing Sepsis in Patients With Cancer in the Era of Antimicrobial Resistance***
Carlota Gudiol, Adaia Albasanz-Puig, Guillermo Cuervo and Jordi Carratalà

188 ***D-Dimer as Biomarker for Early Prediction of Clinical Outcomes in Patients With Severe Invasive Infections Due to* Streptococcus Pneumoniae *and* Neisseria Meningitidis**
Simone Meini, Emanuela Sozio, Giacomo Bertolino, Francesco Sbrana, Andrea Ripoli, Carlo Pallotto, Bruno Viaggi, Roberto Andreini, Vittorio Attanasio, Carolina Rescigno, Luigi Atripaldi, Silvia Leonardi, Mariano Bernardo and Carlo Tascini

200 ***Minireview on Novel Anti-infectious Treatment Options and Optimized Drug Regimens for Sepsis***
Maya Hites

208 ***Related Factors to Streptococcus pneumoniae Invasive Infection and Clinical Manifestations: The Potential Role of Nasopharyngeal Microbiome***
Beatriz Dietl, Desirée Henares, Lucía Boix-Palop, Carmen Muñoz-Almagro, Javier Garau and Esther Calbo

218 ***Novel Cephalosporins in Septic Subjects and Severe Infections: Present Findings and Future Perspective***
Silvia Corcione, Tommaso Lupia and Francesco Giuseppe De Rosa

227 ***Prognostic Significance of the Fission1/Parkin Ratio for Sepsis: A Prospective Cohort Study***
Wei Huang, Xiaoting Wang, Hongmin Zhang, Guangjian Wang and Dawei Liu

236 ***Sepsis—Pathophysiology and Therapeutic Concepts***
Dominik Jarczak, Stefan Kluge and Axel Nierhaus

258 ***Clinical Profile, Prognostic Factors, and Outcome Prediction in Hospitalized Patients With Bloodstream Infection: Results From a 10-Year Prospective Multicenter Study***
Longyang Jin, Chunjiang Zhao, Henan Li, Ruobing Wang, Qi Wang and Hui Wang

266 ***Neonatal Sepsis: The Impact of Carbapenem-Resistant and Hypervirulent* Klebsiella pneumoniae**
Subhankar Mukherjee, Shravani Mitra, Shanta Dutta and Sulagna Basu

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EDITORIAL
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Editorial: Spotlight on the Relationship Between Sepsis and Infection: From Mechanisms to Therapy

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Editorial on the Research Topic

Spotlight on the Relationship Between Sepsis and Infection: From Mechanisms to Therapy

INTRODUCTION

The dysregulated host response to infection leading to sepsis and septic shock is a life-threatening event that, despite advances in organ support and antimicrobial therapy, is associated with a mortality rate of >30% (1). Despite the implementation of international guidelines supporting early-goal-directed therapy, recent randomized trials have demonstrated that these interventions do not improve the survival of septic patients. This evidence warrants an urgent clarification of the molecular mechanisms underlying clinical responses in patients with sepsis or septic shock. Therefore, there is an urgent global need to improve the prevention, recognition, diagnosis, and management of sepsis. The key to improving these processes lies in acquiring in-depth knowledge of the intricate interplay between host defense, infection, and pathogen virulence as well as the timing and type of interventions that are most effective according to the personal characteristics of individual patients. In recent years, infections due to multidrug-resistant (MDR) Gram-negative and Gram-positive pathogens, have been increasingly observed among critically ill patients admitted to the intensive care unit, but also in medicine wards. In this scenario, a predominant role of fungal etiology was associated with the development of severe infections (2).

The management of critically ill patients includes early diagnosis and immediate administration of antimicrobials (3–5). Previous observations about septic patients highlighted the crucial role of timely empirical antimicrobial treatment and the importance of a definitive anti-infective therapy with *in vitro* activity against the microbial isolates, emphasizing also the importance of adequate and early source control of infection (6–8). Moreover, the pharmacokinetic and pharmacodynamic properties of antibiotics should be considered due to changes in the clearance and volume of distribution that are frequently observed in critically ill patients, with the potential to influence the concentration of the drug at the site of infections (9, 10).

Recently, new drugs for the treatment of severe infections have been approved but their role in clinical practice needs to be focused (11–15). As mentioned previously, knowledge of mechanisms related to progression from sepsis to septic shock and adequate management of

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patients, including choice and dosages of antimicrobials, prove crucial to improving the outcome of septic patients (16).

In this Research Topic, the authors have provided contributions about crucial aspects of sepsis: (1) the key roles of mechanisms of sepsis susceptibility, clinical presentation, and outcomes; (2) new avenues for healthcare intervention and to accelerate improved treatments for sepsis; (3) a specific focus on MDR etiology and new antimicrobial therapies.

MECHANISMS UNDERLYING SEPSIS

Lonsdale et al. discussed the pathophysiology underlying sepsis and inflammatory response, reviewing the current management strategies. In the review by Jarczak et al., the authors provided an overview of sepsis immune pathophysiology, to update the choice of therapeutic approaches targeting different immunological mechanisms in the course of sepsis and septic shock, and to call for a paradigm shift from the pathogen to the host response as a potentially more promising approach.

Fenner et al. analyzed a new syndrome, chronic critical illness, that includes sepsis patients who survive the early "cytokine or genomic storm," but fail to fully recover, and progress into a persistent state of manageable organ injury requiring prolonged intensive care. Despite being a common outcome, there are no therapeutic interventions other than supportive therapies for this common disorder. Only through an improved understanding of the immunological endotype rational therapeutic interventions could be designed.

Chung and Claus focused their review on the role of acid sphingomyelinase (ASM) that when secreted can hydrolyze sphingomyelin present at the outer leaflet of membranes to ceramide. During an episode of sepsis, a broad panel of cells, tissue, and organ response is controlled by stress-induced ceramide generation. There is a broad understanding of the potentially harmful effects of ceramide generation during sepsis and there is a wide range panel of well-established and approved drugs with effectiveness for ASM inhibition, indicating the need for more systematic studies for detailed examination of an unattended or an intended inhibition of ASM during sepsis to improve patient outcomes.

Zetoune and Ward highlighted the role of complement activation products (especially C5a anaphylatoxin and its receptors C5aR1 and C5aR2) on the adverse effects of sepsis. During sepsis, the appearance of these complement products is followed by the appearance of extracellular histones in plasma, which have powerful proinflammatory and prothrombotic activities that cause cell injury and multiorgan dysfunction in septic mice, but also in septic humans. Histone appearance in plasma is related to complement activation and the appearance of C5a and its interaction with its receptors. Neutralization of C5a with antibody or absence of C5aR1 blocks the appearance of extracellular histones and cell and organ failure in sepsis, with survival rates in septic mice greatly improved after blockade of C5a with antibody.

RISK FACTORS FOR SEPSIS AND SEPTIC SHOCK

Jin et al. described the clinical and microbiological characteristics and mortality predictive factors in patients with bloodstream infections (BSI). A higher mortality rate was significantly associated with older age, cancer, sepsis diagnosis, intensive care unit (ICU) admission, and prolonged hospital stay prior to BSI onset.

Banerjee et al. highlighted the emergence and burden of XDR hypervirulent isolates of *K. pneumoniae*, causing neonatal sepsis in a tertiary care hospital in India. In another study, Silveira et al. analyzed the genetic basis of antimicrobial resistant Gram-negative bacteria isolated from bloodstreams in Brazil. These data may help physicians manage patients with BSI or other nosocomial infections caused by these pathogens. The study by Mukherjee et al. discusses the current understanding of neonatal sepsis caused by carbapenem-resistant *Klebsiella pneumoniae* (CRKP), including hypervirulent *Klebsiella pneumoniae* in neonates, with a focus on strategies to effectively identify and treat these organisms.

Tompkins et al. reported data about antibiotic resistant Enterobacterales in Sub-Saharan Africa. Genes causing antibiotic resistance are easily spread between the environment and humans and infections due to drug resistant organisms contributing to sepsis mortality *via* delayed time to appropriate antimicrobial therapy. Additionally, second or third-line antibiotics are often not available or are prohibitively expensive in resource-constrained settings, leading to limited treatment options. Lack of access to water and sanitation facilities, unregulated use of antibiotics, and malnutrition are contributors to high rates of antibiotic resistance in the region. The authors concluded that improvements in the monitoring of drug resistant infections and antibiotic stewardship are needed to preserve the efficacy of antibiotics for the future.

Finally, Gudiol et al. reviewed the mechanisms involved in the development of sepsis and septic shock in patients with cancer, focusing on the risk factors associated with a worse prognosis, the impact of adequate initial empirical antibiotic therapy, and the optimal management of sepsis in this special population.

ADVANCES IN DIAGNOSIS

Giacobbe et al. focused on the role of machine learning techniques to early detect sepsis. A rigorous multidisciplinary approach to enrich our understanding in the application of machine learning techniques for the early recognition of sepsis may show potential to augment medical decision-making when facing this heterogeneous and complex syndrome.

Banerjee and Humphries described new and emerging phenotypic and genotypic antimicrobial susceptibility testing methods and summarized the evidence that implementation of these methods can impact clinical outcomes of patients with BSI.

In patients with complicated infective endocarditis (IE) Lin et al. observed that a high SOFA score, combined with increased CRP levels, was associated with in-hospital mortality. Moreover,

the SOFA score predicted long-term mortality in complicated IE. In the review by Cuervo et al., the authors report on current challenges in the management of IE that require the close collaboration of multidisciplinary endocarditis teams.

In the study by Meini et al., D-dimer resulted in a useful tool to stratify the risk of in-hospital mortality and complications in patients with invasive infections due to *Neisseria meningitidis*, while for *Streptococcus pneumoniae* the mortality rate was irrespective by D-dimer values.

Thy et al. analyzed the microbiological characteristics of surgical samples obtained during initial surgery, compared with those obtained during the first reoperation. The authors concluded that the emergence of microorganisms, including MDR bacteria, is frequently noted in necrotizing skin and soft tissue infections, however without affecting mortality.

Interestingly, Huang et al. discussed the role of Fission1 (Fis1) and parkin as key proteins related to mitochondrial fission and mitophagy, respectively. In this study the Fis1/parkin ratio resulted in being valuable for risk stratification in patients with sepsis and is associated with poor clinical outcomes for sepsis in the ICU.

Claus and Graeler reviewed the role of mass spectrometry of sphingolipids and related species ("sphingolipidomics") to investigate the cellular and compartment-specific response to stress in sepsis. The authors concluded that this method is on the rise and the ability to integrate multiple datasets from diverse classes of biomolecules by mass spectrometry measurements and metabolomics will be crucial to fostering our understanding of human health as well as response to disease and treatment.

Finally, the potential role of nasopharyngeal microbiome (NP) in *Streptococcus pneumoniae* invasive infection (IPD) was reviewed by Dietl et al. Although NP microbiome in patients with IPD has not been properly characterized yet, there seem to be discordant results between pediatric and adult populations; then, new longitudinal studies with a larger number of participants and a homogeneous system to collect samples should help to elucidate the potential role of the previously observed microbial species in adults and their relationship with increasing or reducing risk for the development of respiratory infections, especially IPD.

MANAGEMENT, ANTIMICROBIALS, AND ADJUNCTIVE THERAPIES

Kimmig et al. review and discuss current diagnostic and therapeutic key elements and open questions for the management of Staph*ylococcus aureus* BSI.

Hites discussed the different anti-infectious treatments currently available and suggestions on how to deliver optimized dosage regimens to septic patients, with a particular emphasis on newly available anti-infectious therapies. In the review by Corcione et al., the authors focused on the role of novel cephalosporins in septic subjects and severe infections, reporting present findings and future perspectives.

Busani et al. reported on how adjuvant therapies can help physicians to modulate the immune system, summarizing the rationale for using immunoglobulins as an adjunctive treatment.

The study of d'Ettorre et al. reports on a specific bacterial formulation that showed a significant ameliorating impact on the clinical conditions of patients with SARS-CoV-2 infection, focusing on the role of complementary therapeutic strategy to avoid the progression of COVID-19.

CONCLUSION

In this Research Topic, the authors focused on crucial aspects of sepsis, including the mechanisms underlying sepsis, risk factors for the development of sepsis and septic shock, advances in diagnosis, management, and therapy of these severe infections, and the role of adjunctive therapies for the treatment of septic patients. The management of septic patients includes early diagnosis and immediate administration of antimicrobials through the identification of risk factors and mechanisms underlying the development of sepsis and septic shock. A timely empirical antimicrobial treatment and the importance of definitive anti-infective therapy with *in vitro* activity against the microbial isolates are crucial to improve survival, emphasizing the importance of an adequate and early source control of infection. Moreover, the pharmacokinetic and pharmacodynamic properties of antibiotics should be considered because of changes in clearance and volume of distribution that are frequently observed in critically ill patients, with the potential to influence the concentration of the drug at the site of infections.

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AR planned the editorial. DP and MB performed revision of the text. All authors contributed to the article and approved the submitted version.

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Challenges in the Management of SARS-CoV2 Infection: The Role of Oral Bacteriotherapy as Complementary Therapeutic Strategy to Avoid the Progression of COVID-19

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Background: Gastrointestinal disorders are frequent in COVID-19 and SARS-CoV-2 has been hypothesized to impact on host microbial flora and gut inflammation, infecting intestinal epithelial cells. Since there are currently no coded therapies or guidelines for treatment of COVID-19, this study aimed to evaluate the possible role of a specific oral bacteriotherapy as complementary therapeutic strategy to avoid the progression of COVID-19.

Methods: We provide a report of 70 patients positive for COVID-19, hospitalized between March 9th and April 4th, 2020. All the patients had fever, required non-invasive oxygen therapy and presented a CT lung involvement on imaging more than 50%. Forty-two patients received hydroxychloroquine, antibiotics, and tocilizumab, alone or in combination. A second group of 28 subjects received the same therapy added with oral bacteriotherapy, using a multistrain formulation.

Results: The two cohorts of patients were comparable for age, sex, laboratory values, concomitant pathologies, and the modality of oxygen support. Within 72 h, nearly all patients treated with bacteriotherapy showed remission of diarrhea and other symptoms as compared to less than half of the not supplemented group. The estimated risk of developing respiratory failure was eight-fold lower in patients receiving oral bacteriotherapy. Both the prevalence of patients transferred to ICU and mortality were higher among the patients not treated with oral bacteriotherapy.

Conclusions: A specific bacterial formulation showed a significant ameliorating impact on the clinical conditions of patients positive for SARS-CoV-2 infection. These results also stress the importance of the gut-lung axis in controlling the COVID-19 disease.

Keywords: COVID-19, SARS-CoV-2, bacteriotherapy, probiotic, lactobacillus, gut-lung axis, gut

INTRODUCTION

Understanding the invasive process of SARS-COV-2 is essential. We know that the entry points for the virus into the body, such as ACE2 receptors, are enzymes that are linked to intestinal cells. Coronaviruses constantly change their binding patterns as they evolve, and the potential target in the lungs also varies, but not in the small intestine, where it remains constant. The cells of the intestinal mucosa (enterocytes) could, therefore, be a reservoir for coronaviruses (1). In the acute phase, only 10% of coronavirus disease 19 (COVID-19) patients present virus cDNA in the blood, but almost 50% of them excrete it in the stools. The infectious form of the virus was even identified several times, suggesting that the orofecal route is a mode of contamination (1). The gut involvement might explain the wide variation in viral load from one test to another in the same person as if the virus were hiding there (2). Chinese researchers have investigated changes in the microbiota in the patients who have died for COVID-19 infection. The sequencing of their microbiota revealed a significant decrease in *bifidobacteria* and *lactobacilli*, the main families of symbiotic bacteria, as well as an increase in opportunistic bacteria such as *Corynebacterium* or *Ruthenibacterium* (1). Intestinal dysbiosis has a long-reaching immune impact on the pulmonary immune system (3), and hence might be an additional risk for respiratory distress induced by COVID-19. In this context, the use of oral bacteriotherapy might be an option. Some strains of *lactobacilli* and *bifidobacteria* have a protective role against influenza virus, rhinovirus, respiratory syncytial virus, adenovirus, and pneumovirus (4, 5). We report here our observation on patients supplemented with oral bacteriotherapy in addition to the current anti-COVID-19 treatment (hydroxychloroquine, azithromycin, tocilizumab). The comparison group was COVID-19 positive subjects not treated with oral bacteriotherapy, hospitalized in the same clinic at the same time. Our results stress the importance of the gut-lung axis in the control of the COVID-19 illness (6, 7).

STUDY POPULATION, SETTINGS AND DATA COLLECTION

The patients evaluated in this study were hospitalized at the Department of Infectious Diseases, Policlinico Umberto I, "Sapienza" University of Rome, Italy, between March 9th, 2020 and April 4th, 2020 (sub-intensive care unit for COVID-19). Ethical approval was obtained from Ethics Committee of Policlinico Umberto I (approval number/ID Prot. 109/20209). All the patients were staying at home before their referral to the Emergency Department, and from there to our Department. Oropharyngeal and nasopharyngeal swabs for diagnosis of COVID-19 were performed in duplicate for SARS-CoV-2 E and S gene by a reverse transcriptase polymerase chain reaction (RT-PCR). All the patients were positive for COVID-19 and met the following clinical criteria: Fever: $> 37.5°C$, need of non-invasive oxygen therapy, and CT lung involvement on imaging more than 50%. They were diagnosed with symptomatic COVID-19 disease state, which, however, at the time of evaluation did not require endotracheal intubation and invasive mechanical ventilation. Oxygen therapy was delivered via Venturi mask in spontaneous breathing patients; if hypoxemia persisted continuous positive airway pressure (CPAP) was applied. Dyspnea was defined as "a subjective experience of breathing discomfort that consists of qualitatively distinct sensations that vary in intensity" (8). Acute diarrhea was defined as a stool with increased water content, volume, or frequency that lasts <14 days (9). High-resolution CT scan was used to identify lung involvement according to the official diagnosis and treatment protocol (6th edition) declared by the National Health Commission of China. Typical CT findings of COVID-19 are (1) ground-glass opacities, (2) consolidation, (3) reticular pattern, (4) crazy paving pattern (10). Patients with severe acute hypoxemia due to COVID-19 pneumonia and in need for invasive mechanical ventilation were referred to the Intensive Care Unit (ICU) of Policlinico Umberto I. Since there are, currently, no coded therapies or guidelines for the medical treatment of COVID-19, the patients were treated with hydroxychloroquine (HCQ) 200 mg bid, antibiotics (ABX) (azithromycin 500 mg) and Tocilizumab (TCZ) dosage is 8 mg/kg (up to a maximum of 800 mg per dose) with an interval of 12 h for two times, eventually plus oxygen. In addition to the above treatments, randomly chosen patients initiated oral bacteriotherapy on March 13th. For each patient, the Charlson comorbidity index (11), the oxygen-support requirement, as well as, laboratory values comprising alanine aminotransferase (ALT), aspartate aminotransferase (ALT), hemoglobin (Hb), pH, hydrogen carbonate (HCO_3^-), Lactic acid and arterial carbon anhydride pressure ($PaCO_2$) were determined at baseline. The observed partial pressure of arterial oxygen (PaO_2), the fraction of inspired oxygen FiO_2, the disappearance of symptoms associated to COVID-19, adverse events, and the number of patients transferred to ICU were collected at 24 h, 48 h, 72 h, and 7 days from the start of oral bacteriotherapy and hospitalization for all the patients independently from the treatments. Patients were considered

Abbreviations: ABX, antibiotics; ALT, alanine aminotransferase; ALT, aspartate aminotransferase; CI, confidence interval; COVID-19, coronavIrus disease 19; CPAP, continuous positive airway pressure; ECMO, extracorporeal membrane oxygenation; FDR, False Discovery Rate; GLA, gut lung axis; Hb, hemoglobin; HCQ, hydroxychloroquine; HIV, Human immunodeficiency virus; HO-1, Heme oxygenase-1; ICU, Intensive Care Unit; IRQ, interquartile range; Nrf2, nuclear factor erythroid 2p45-related factor 2; OB–, oral bacteriotherapy not administered group; OB+, oral bacteriotherapy administered group; ROS, reactive oxygen species; SCFA, short chain fatty acids; TCZ, Tocilizumab.

positive for respiratory failure when the determined PaO_2/FiO_2 ratio was <300. Since this is a retrospective real-life emergency data collection, some laboratory data were unavailable. In particular, in the case of significant clinical and respiratory gas exchange improvement, sometimes the clinician has not repeated the follow up blood gas analysis, considering it an unnecessary painful invasive procedure.

Oral Bacteriotherapy

The formulation administered in this study contained: *Streptococcus thermophilus* DSM 32345, *L.acidophilus* DSM 32241, *L. helveticus* DSM 32242, *L. paracasei* DSM 32243, *L. plantarum* DSM 32244, *L. brevis* DSM 27961, *B. lactis* DSM 32246, *B. lactis* DSM 32247. Ormendes SA, Lausanne, Switzerland which gifted the product Sivomixx® (SivoBiome® in USA) is responsible for the standardization of the product in terms of enzymatic content, biochemical and immunological profile. The oral bacteriotherapy involved the use of 2,400 billion bacteria per day. The formulation was administered in three equal doses per day.

Statistical Analysis

No sample-size calculations were performed. The categorical variables were compared using the χ^2 test and showed as absolute frequencies and percentage. The Shapiro–Wilk test was used to test the normality of distribution of continuous variables. When they were not normally distributed, logarithmic transformation was performed in accordance to BoxCox transformation with $-0.25 \leq \lambda \leq 0.25$. For normally distributed continuous variables, mean values between two groups were compared by Student's *t*-test and showed as mean ± SD (Standard Deviation); for data not normally distributed, the Mann–Whitney test was used and indicated as median (25th−75th). The longitudinal analysis of data relative to respiratory failure in relation to the "not treated vs. treated" group was performed by a General Linear Mixed Model with the GLIMMIX procedure considering the binary as distribution and logit as link function. The Benjamini–Hochberg False Discovery Rate (FDR) correction was used to account for multiple hypothesis testing when necessary. A $p < 0.05$ was considered statistically significant. All statistical analyses were performed by using SAS v.9.4 and JMP v. 14 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Data relative to 70 subjects positive to the SARS-CoV-2 test (median age, 59 years, interquartile range, 50–70) were collected during period March 9th–April 4th, 2020. None of the patients had recently traveled to China or South Korea or Iran. Patients were unable to confirm if they had contact with persons infected with COVID-19 and were also not accurate in recalling the exact duration of the symptoms before hospital admission. The proportion of females (29, 41.4%) was lower respect to the percentage of males (41, 58.6%). Symptomatology of patients at admission was: fever (66, 94.3%), cough (54, 77.1%), dyspnea (44, 62.9%), headache (11, 15.7%), asthenia (15, 21.4%), myalgia (4, 5.7%), diarrhea (33, 47.1%), while 56 (80.0%), presented comorbidities in a range of 1 to 6. A group of 28 subjects received Oral Bacteriotherapy (OB+), while another group of 42 individuals not supplemented with oral bacteriotherapy (OB–) was the comparison group. **Table 1** shows the characteristics of patients at admittance. No statistically significant differences were observed between the OB+ group and the OB– one respect to sex, age, AST, ALT, Hb, Body Mass Index (BMI), and Charlson comorbidity index at baseline. No significant differences between groups were also found for respiratory parameters as well as for the proportion of subjects presenting diarrhea, fever, cough, dyspnea, headache, asthenia, and myalgia. Furthermore, all patients had clinical and radiological signs compatible with COVID-19 pneumonia and needed respiratory assistance in the hospital setting but not resuscitation support. The two groups of patients were homogeneous respect to the proportion of subjects needing non-invasive oxygen support delivered via Venturi mask in spontaneous breathing or by continuous positive airway pressure (CPAP).

TABLE 1 | Characteristics of the groups of patients obtained on the base of bacteriotherapy administration.

	OB+ (*N* = 28)		OB– (*N* = 42)		
Characteristics	**Obs.**	**Values**	**Obs.**	**Values**	***p*-value**
Age* (year)	28	59 ± 14.4	42	60.5 ± 14.2	0.66
Gender male	28	17 (60.7)	42	24 (57.1)	0.77
BMI* (kg/m^2)	27	24.7 ± 3.4	42	23.4 ± 3.5	0.13
ALT*(IU per liter)	27	22.7 ± 10.5	42	30.5 ± 22.2	0.16
AST*(IU per liter)	27	37 ± 33.5	42	40.5 ± 33	0.56
Hb (g/dL)	27	13 (12–14)	42	13 (12–14)	0.58
Charlson index	28	2 (1–3)	41	2 (1–3)	0.64
Symptoms					
Fever	28	27 (96.4)	42	39 (92.9)	0.53
Cough	28	22 (78.6)	42	32 (76.2)	0.82
Dyspnea	28	20 (71.4)	42	24 (57.1)	0.23
Asthenia	28	6 (21.4)	42	9 (21.4)	1.0
Headache	28	3 (10.7)	42	8 (19.0)	0.35
Myalgia	28	2 (7.1)	42	2 (4.8)	0.67
Diarrhea	28	14 (50.0)	42	19 (45.2)	0.70
Respiratory parameters					
FiO_2 (%)	27	31 (21–43)	39	21 (21–35)	0.17
SO_2 (%)	24	99 (97–99)	36	99 (97–100)	0.89
pH	26	7.5 (7.4–7.5)	35	7.5 (7.4–7.5)	0.71
HCO_3^- (mmol/L)	26	25.47 ± 3.37	33	25.58 ± 2.74	0.89
Lactic acid* (mmol/L)	26	0.9 ± 0.4	34	0.9 ± 0.3	0.74
pO_2 (mmHg)	27	89 (76–103)	39	86 (77–97)	0.73
pCO_2 (mmHg)	26	36 (32–39)	36	36 (34–39)	0.40
Oxygen support					
Venturi mask	28	25 (89.3)	42	38 (90.5)	0.87
CPAP	28	3 (10.7)	42	3 (7.1)	0.60

*The p-values are relative to the chi-square test and Mann–Whitney and when less of 0.05 these comparisons showed no significant differences. Obs., number of available observations for each group. *data log_{10}-transformed. OB– (oral bacteriotherapy not administered group), OB+ (oral bacteriotherapy administered group).*

Notably, at admittance, a significantly higher proportion of patients with respiratory failure was present in the group treated with oral bacteriotherapy respect to the OB– one (OB– 11/42, 26.2%; OB+ 14/28, 50%; $p = 0.042$). Therefore, all enrolled patients were classifiable in stage III (Severe pneumonia–Severe COVID-19) of the syndromic classification proposed by the Italian Society of Anesthesia and Resuscitation (SIAARTI) (12).

For what concerns drug therapies, both groups did not differ for number, type, and combinations of administered drugs during the period of hospitalization (**Table 2**). The median time from diagnosis to the start of oral bacteriotherapy administration was 1 day (min 0–max 2) and duration of treatment was 14 days for all patients.

The oral bacterial administration was associated with the disappearance of diarrhea in all the patients within 7 days. Interestingly, a large proportion of OB+ subjects (6/14, 42.9%) solved diarrhea within 24 h and almost the totality (13/14, 92.9%) within 3 days (**Figure 1A**). Also, the other signs and symptoms–fever, asthenia, headache, myalgia, and dyspnea–considered cumulatively, presented a similar trend, more evident from the second day of bacteriotherapy (**Figure 1B**). Notably, less than half of patients not treated with bacteriotherapy experienced the disappearance of diarrhea or other symptoms within 7 days. For what concerns the respiratory outcome, by applying the General Linear Mixed model with the GLIMMIX procedure, we observed a significant difference in the evolution of respiratory outcome between the OB+ group and the OB– one ($p < 0.001$). After 7 days of treatment, the calculated model showed an 8-fold significantly decreased risk to evolve a respiratory failure, with the need of resuscitation support i.e., in need for prone ventilation or extracorporeal membrane oxygenation (ECMO) for patients administered with bacteriotherapy respect to the OB– individuals (**Figure 2**).

QT interval prolongation, hepatic and renal abnormalities, and immunosuppression were monitored carefully, also for the propensity of the QT interval to increase in patients treated with azithromycin. No adverse events were recorded. Patients treated with Tocilizumab reported a sense of asthenia after the administration of the drug and a reduction of the blood pressure, which did not require any medical treatment. As of April 4th, 2020, although not statistically significant, the OB– group showed a higher prevalence of patients transferred to the ICU for mechanical ventilation (OB– vs. OB+; 2/42, 4.8% vs. 0/28, 0.0%) or coming to a lethal outcome (OB– vs. OB+; 4/42, 9.5% vs. 0/28, 0.0%). The observed prevalence of patients with lethal outcome within the control group was in line with that (mean ± SD 9.4 ± 1.7%;) recorded in Italy in the period March 9th–April 4th, 2020 (13). All the patients also treated with bacteriotherapy survived the COVID-19 illness, and none required invasive mechanical ventilation and ICU admission.

TABLE 2 | Drug therapies administered to the groups of subjects as determined by the administration of bacteriotherapy.

	OB+ ($N = 28$)		OB– ($N = 42$)		
	Obs.	**Values**	**Obs.**	**Values**	***p*-value**
Administered drugs–no. (%)					
HCQ	28	25 (89.3)	42	40 (95.2)	0.34
TCZ	28	7 (25)	42	16 (38.1)	0.25
ABX	28	11 (39.3)	42	21 (50)	0.38
Administered drugs–no. (%)					0.16
None	28	2 (7.1)	42	1 (2.4)	
One drug	28	13 (46.4)	42	11 (26.2)	
Two drugs	28	9 (32.1)	42	24 (57.1)	
Three drugs	28	4 (14.3)	42	6 (14.3)	
Combinations of drugs–no. (%)					
HCQ/TCZ/ABX	28	4 (14.3)	42	6 (14.3)	1.0
HCQ/TCZ	28	2 (7.1)	42	9 (21.4)	0.11
HCQ/ABX	28	6 (21.4)	42	15 (35.7)	0.20
STCZ/ABX	28	1 (3.6)	42	0 (0)	0.22

A $p \leq 0.05$ was considered statistically significant. ABX, antibiotics; HCQ, hydroxychloroquine; IRQ, interquartile range; Obs., number of available observations for each group; TCZ, Tocilizumab.

DISCUSSION

This report comes from doctors in the "trenches" during the Italian war against the COVID-19 infection. The urgency of the COVID-19 pandemic shifted the balance between waiting for evidence before deciding whether to administer therapy or creating evidence during routine patient care, in favor of the second choice. The United States Food and Drug Administration (FDA) has permitted an emergency-use authorization to prescribe the hydroxychloroquine (14). The WHO, CDC, and FDA have not taken a position on the use of Tocilizumab in COVID-19, even though China's National Health Commission recommends it for use in COVID-19 patients but only if elevated IL-6 levels are present (15). Also, Italian clinicians are utilizing a variety of empirical approaches to managing COVID-19, in a "learn while doing" method (16). There is an urgent need to determine which interventions against COVID-19 are the best, but in the absence of clinical trials to guide the management, not collecting the data from the use of off-label therapies it is a missed opportunity. Here we report a "snapshot" on 70 patients hospitalized at the Department of Infectious Diseases between March 9th and April 4th, 2020. A group of patients has been treated with hydroxychloroquine, Tocilizumab, and antibiotics alone or in combination, while, a second group of subjects were administered with oral bacteriotherapy in addition to the standard drug therapy. Results evidenced a worse survival, as well as, a higher risk of transfer to an intensive resuscitation for the patient not supplemented with bacteriotherapy respect to the supplemented one. Also, the estimated risk to develop respiratory failure during COVID-19 course was more than eight times lower in the group treated with oral bacteriotherapy respect to the not treated one. As for the other signs and symptoms associated with COVID-19, i.e., diarrhea, fever, cough, dyspnea, asthenia, myalgia a

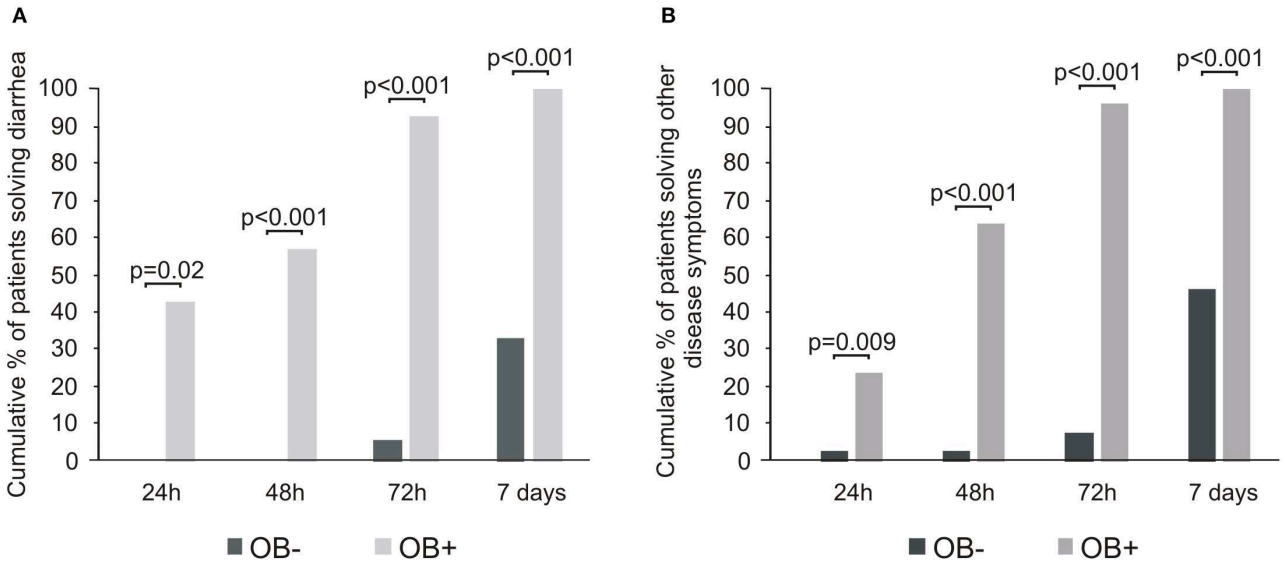


FIGURE 1 | Color-coded barplots based on probiotic administration showing the disappearance of diarrhea **(A)** as well as other symptoms **(B)** at different time points. The Benjamini Hochberg FDR correction was used to account for multiple hypothesis testing. Statistical significance between the group at alpha level 0.05 was also reported.

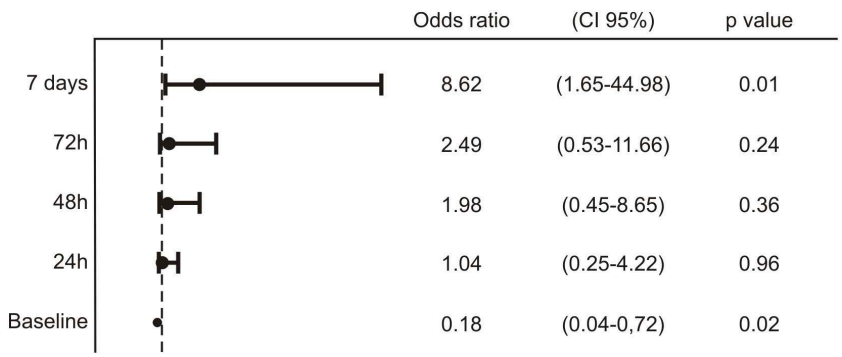


FIGURE 2 | Analysis of the longitudinal data for the respiration variable in relation to the "OB– vs. OB+" group performed by GLIMMIX. For each time point, the odds ratio, the confidence interval 95% and the statistical significance were reported.

significant improvement is already evident as early as after 24–48 h after the start of the bacteriotherapy. There are potential anatomical communications and complex pathways involving the gut-lung axis (GLA) (5). The mesenteric lymphatic system is the pathway between the lungs and the intestine, through which intact bacteria, their fragments or metabolites can cross the intestinal barrier to reach systemic circulation and influence the pulmonary immune response (17–19). Intestinal metabolites significantly affect not only local intestinal immunity but also other organs through the lymphatic and circulatory system. For example, short chain fatty acids (SCFA) produced primarily by bacterial fermentation of dietary fiber, act in the lungs as signaling to attenuate inflammatory and allergic responses (20, 21). Mice with SCFA receptor deficiency show increased inflammatory responses in experimental models of asthma (19). Human cells possess antioxidative defense systems for their protection against reactive oxygen species (ROS) generated by viruses; however, viral infections often inhibit such a response (22). There is no reason to believe that that this is not true also for COVID-19 (23). We hypothesized that in patients infected by COVID-19, a bacterial formulation with the "appropriate" biochemical and immunological profile might trigger several protective biological functions. The bacterial strains present in the product we administered enhance the production of both the *nuclear factor erythroid 2p45-related factor 2* (Nrf2) and its target *Heme oxygenase-1* (HO-1) (24). These molecules exert antiviral activity through a reduction of oxidative stress. Nrf2 and HO-1 have significant antiviral activity against a wide variety of viruses, including Human immunodeficiency virus (HIV), influenza virus, respiratory syncytial virus, dengue virus, and Ebola virus among others (25–29). Notably, beneficial properties of HO-1 expression have been reported for viruses

that produce lung disease. Mice that overexpress HO-1 in the lungs display less inflammatory cell infiltration into the lungs and decreased apoptosis of respiratory epithelial cells, as compared to control mice. Therefore, HO-1 expression prevents an exacerbated immune response in this tissue and subsequent damage (26). The collection of clinical data, examination, and nursing of COVID-19 patients is challenging for the risk of virus transmission. The COVID-19 is present in the stools, even in discharged patients, with potential recurrence and transmission of the virus (30, 31). Our initiative aimed to modulate the gut-lung axis, facilitate patient management and possibly determine the outcome of lung infection. Oral bacteriotherapy has shown a statistically significant impact on the clinical conditions of COVID-19 patients. Having considered the different outcomes and unethical to deprive a percentage of COVID-19 patients of the chance to get oral bacteriotherapy, we did not include more patients or extended the time of observation. Pending the results of confirmatory clinical trials, this report is aimed at providing an interim suggestion for improving the management of the COVID-19 illness, keeping in mind that different bacterial preparations may have quite different outcomes (32).

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Policlinico Umberto I. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Gd'E, GCe, and MM contributed substantially to the conception and design of the study and interpretation and wrote the manuscript. GCa, CP, FA, FR, GR, LC, CS, CM, VT, GER, and VM contributed substantially to the the acquisition of data and the analysis. GA, FP, and CMM drafted or provided critical revision of the article and provided final approval of the version to publish. GR contributed substantially to data interpretation.

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REVIEW
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Infection, Sepsis and the Inflammatory Response: Mechanisms and Therapy

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Sepsis secondary to bacterial infection remains a significant cause of morbidity and mortality globally. Recent decades have seen the evolution of international collaborations to improve care for these patients and identify areas for research. In this article we discuss the pathophysiology underlying the condition, review the current recommended management strategies, discuss areas of controversy, and highlight the need for ongoing research, particularly in diagnostics.

Keywords: infection, intensive & critical care, antibiotics, sepsis, Infectious disease

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INTRODUCTION

The global burden of bacterial infection is significant. In the UK, beta-lactams and macrolide antibiotics account for 25 million prescriptions annually, and global antibiotic prescriptions are in the billions (1, 2). Associated healthcare costs are considerable, accounting for over $30 billion (∼8%) of US healthcare spending (3). At the severe end of the spectrum, sepsis is a common reason for admission to intensive care, accounting for as much as 30% of admissions to adult (4–6) and 12% of admissions to pediatric units (7, 8). Up to 70% of intensive care patients will receive at least one course of antibiotics during their stay, regardless of age (9, 10). Mortality from severe infection in adults and children remains as high as 25–30% (8, 11). In neonates, infection remains one of the most common causes of death worldwide (12–14). Understanding and managing infection is therefore a core skill for physicians and healthcare professionals. In this article, we will explore the pathology, diagnosis, and management of infection and sepsis, discuss some of the complexities and challenges.

INFECTION, SEPSIS, AND THE INFLAMMATORY RESPONSE

The human response to an infection follows recognition of infectious pathogens by immune cells via pattern recognition receptors. The resulting cellular activation leads to the production of pro- (particularly IL-1 and TNFα) and anti-inflammatory mediators and the subsequent recruitment and activation of other immune cells (e.g., polymorphonucleocytes and B-cells) (15). In the normal host response to infection, equilibrium is reached between pro- and anti-inflammatory processes, bactericidal activity is maximized, alongside necessary phagocytosis, and repair of damaged tissue. In sepsis, this equilibrium is lost. Localized tissue response to infection becomes systemic and the inflammatory process becomes deleterious in its own right. Sepsis being formally defined as "life-threatening

organ dysfunction caused by a dysregulated host response to infection" (16). Effects are seen across organ and tissue types. Endothelial damage results in loss of normal homeostasis, leading to fluid leak, and tissue oedema. In the vasculature there is vasodilatation mediated through several mechanisms such as the effect of acidosis on vascular smooth muscle, induction of nitric oxide by inflammatory mediators, and adrenal insufficiency. The consequent drop in systemic vascular resistance may be compensated by increased cardiac output or compounded by myocardial depression–leading to hypotension and reduced tissue perfusion. In the lungs, fluid leak may impair gas exchange and progress to acute respiratory distress syndrome. Acute kidney injury may result from direct cytokine effects as well as damage to the microvasculature or reduced tissue perfusion and hepatic effects may further impair a dysregulated coagulation system (15, 17). In the brain, inflammatory mediators, hypoxia, and hypotension may all contribute to the evolution of encephalopathy. Blood dyscrasias are also common, with mechanisms not fully understood. It may be that the expansion in haematopoietic stem cells secondary to demand and the inflammatory state may actually lead to the production of dysfunctional stem cells, paradoxically leading to production of less mature cells like neutrophils (and subsequent neutropenia) (18).

The dysregulated immune response to infection is unpredictable, leading to a wide spectrum of clinical presentations, and time-course for the disease. Multiple attempts have been made to describe the clinical effects in a simplified manner that allows early recognition of the septic patient. Perhaps most notably with the concept of the systemic inflammatory response syndrome (19). The use of clinical signs (fever or hypothermia, tachycardia, tachypnoea) and readily available investigations (high or low white cell count) to identify patients with this complex physiology in as simple a manner as possible is attractive. However, the lack of specificity of the SIRS criteria to identify sepsis specifically and the prevalence of cases of sepsis that do not meet these criteria make it an imperfect diagnostic tool (20–22). This lack of specificity may well-contribute to the high prevalence of antibiotic prescriptions in hospitals and the ICU (9, 10).

DIAGNOSING SEPSIS

In an attempt to address some of the limitations of the SIRS criteria and accompanying definitions of sepsis, a new consensus definition for sepsis was proposed in 2016—life threatening organ dysfunction caused by infection (16). Organ dysfunction was defined as an increase in sequential organ failure assessment (SOFA) score of 2 or more points. Whilst this definition may increase the sensitivity of diagnosing sepsis and identify those with the greatest mortality risk, it remains non-specific and has not been universally adopted (23, 24). Sepsis remains a challenge to diagnose. The search for a specific biomarker in sepsis is yet to yield success, despite hundreds of potential molecules being identified (25–27). Blood cultures are often cited as a gold standard method of identifying pathogens, but the test remains flawed in its utility in managing the early stages of sepsis given the long turn-around time and frequency of contaminated and false-positive/negative samples (28). Clinicians must therefore use signs, symptoms and supportive investigations to identify or suspect infection. This may be straightforward in the coughing, hypoxic patient with lobar consolidation identified on a chest radiograph or challenging in an elderly patient for whom dementia contributes to non-specific symptoms. Supportive signs and investigations like temperature, white cell count and inflammatory biomarkers like C-reactive protein or procalcitonin may be raised in any stress response and early antibiotics, whilst important for treatment, may impair the identification of a pathogen from samples cultured after administration.

THERAPIES

The decision on where to treat a person who has sepsis will largely depend on local hospital resources and the mode and route of presentation. Many cases will present to the emergency department directly but management of cases identified in the community or in hospital inpatients merit consideration. Regardless of the route to identification of a case, consensus remains that early treatment of the infection and provision of supportive care improves outcome (24, 29, 30).

Antimicrobials

The most recent surviving sepsis guidelines recommend administration of intravenous antimicrobials within 1 h of identification of sepsis (29). This recommendation is supported by multiple observational studies that show an association between delay in antimicrobial administration and increase in mortality (30–32). Choice of antimicrobial should be directed to provide coverage for the most likely pathogen that has caused the septic state. Mirroring the heterogeneity of the clinical presentation of sepsis, the range of possible pathogens is myriad and will be dependent on the patient, their presentation and previous medical history alongside local factors such as antimicrobial resistance patterns. Given this heterogeneity and the fact that pathogens are not usually identified at the time of diagnosis of sepsis, it is not possible to recommend a specific antibiotic or antibiotic combinations. Broad-spectrum coverage is usually therefore necessary, with choice of drug(s) directed at potential site of infection and coverage of risk factors. This may require the use of multiple agents, for example the combination of broad spectrum ß-lactam with vancomycin where MRSA is suspected and addition of antifungals where invasive fungal disease may be present (for example where immunocompromised state exists). Choice of agent should be taken in line with local prescribing guidelines. Dose and mode of antibiotic administration continues to evolve in the management of sepsis. The one-size fits all dosing strategy used in the management of community infection cannot hold in the management of sepsis where profound alterations to antimicrobial pharmacokinetics are likely secondary to both organ dysfunction and changes in volume of distribution from altered volume status (secondary to the disease and fluid resuscitation in its management)

TABLE 1 | Commonly used antibiotics and the pharmacokinetic-pharmacodynamic index associated with therapeutic success.

Antibiotic class	Pharmacokinetic-pharmacodynamic index
Aminoglycosides	C_{MAX}:MIC
β-lactams	T>MIC
Fluroquinolones	C_{MAX}:MIC
Glycopeptides	AUC/MIC
Macrolides	AUC/MIC

Table adapted from Roberts and Lipman (37) and Asin-Prieto et al. (38). C_{MAX}, maximum concentration; MIC, minimum inhibitory concentration of suspected pathogen; T, time; AUC, area under the time-concentration curve.

(29, 33). Whilst organ dysfunction associated with sepsis can lead to accumulation of antimicrobials, this is not universal. Altered antimicrobial pharmacokinetics in sepsis has been shown to reduce plasma concentration to below levels considered therapeutic (34, 35) and failure to achieve therapeutic plasma concentrations has been associated with failure of therapy (36). It is therefore recommended that dose and frequency of administration are considered and individualized from the outset of treatment. These considerations should include the pharmacokinetic-pharmacodynamic relationship between antimicrobial and pathogen (**Table 1**) such that dose and dosing regimen are optimized. For example, the pharmacokinetic-pharmacodynamic index associated with successful bacterial kill of ß-lactams is time with drug concentration above the minimum inhibitory concentration of the target bacteria. Frequent (and adequate) dosing is therefore required to maintain an appropriate concentration time profile for ß-lactams. A phase III clinical trial is ongoing into the utility of continuous infusions (39). Completely individualized treatment courses are difficult to achieve without rapid therapeutic drug monitoring, which is often not routinely available, save perhaps for the monitoring for harm of aminoglycosides. Duration of therapy should minimize exposure to broad-spectrum antibiotics where possible. This should include switching to narrow spectrum agents where pathogens and susceptibility are identified and minimizing the duration of multi-agent regimens by, for example, reducing to single agent cover as shock resolves and there is clinical improvement. Stewardship of antimicrobials in this way will minimize the potential for emergence of resistant pathogens. Indeed, such stewardship should be considered *as* important a place in therapy as initial choice and dose of antibiotics, since infection with multi-drug resistant pathogens is associated with increased hospital length of stay and mortality (40, 41) and alteration in commensal microbiome to resistant pathogens is rapid—as quickly as within 1 day of exposure to imipenem in one study (42).

Source Control

Where sepsis is caused by a focus of infection that is amenable to source control through surgical or other intervention (e.g., removal of infected indwelling catheters), source control should occur as quickly as practical (within 6–12 h) (43–46).

Fluid Therapy

Whilst there are few clinicians who would advocate removing fluids from the armory of sepsis management, the choice, amount and rate of administration of fluid remains controversial and it is an area of need for ongoing research. The principle of restoring a circulating volume to provide adequate tissue perfusion in the vasodilated and fluid shifting septic state is sound, but for some patients with sepsis cardiac output may be elevated and tissue blood flow may be increased and oxygen delivery satisfactory. The one size fits all fluid dose of 30 ml/kg recommended in the most recent surviving sepsis guidelines is, therefore, controversial. Indeed, by the guide's own acknowledgment the evidence to support this recommendation is "low quality" (29) and there is evidence of harm of over resuscitation in some settings (5, 47). A gold standard guide to adequacy of resuscitative volume does not exist. Raised serum lactate, whilst associated with worse outcomes in sepsis, may be elevated in the setting of catecholamines (endogenous or exogenous), hepatic failure, or altered cellular metabolism in sepsis (29, 48). It is therefore an imperfect guide to volume state. Indeed, a recent trial found no difference in mortality outcome between septic patients in whom fluid resuscitation targeted lactate normalization vs. normal capillary refill time (49). It would seem prudent, as with recommendations on antimicrobial and vasopressor administration, that fluid dosing in sepsis should be personalized. This entails the use of small bolus fluid administration (250–500 mL) with frequent reassessment. The perfect assessment modality does not exist, simple measures like capillary refill time or straight leg raise tests may well be as useful as more complex or invasive assessment modalities such as point of care ultrasound or haemodynamic monitoring software.

Crystalloids infusions are favored over albumin and synthetic colloids as the initial fluid of choice, although there is insufficient evidence to guide choice of crystalloid in sepsis. Balanced solutions are often preferred to high chloride (0.9% sodium chloride) infusions but direct head to head trials in sepsis are missing. Albumin may be considered as a fluid choice, but high-quality evidence of benefit over crystalloids is lacking (29, 50, 51).

Target Blood Pressure

A target mean arterial pressure has of 65 mmHg has been advocated in sepsis (29). This target is based on the principal of reduced tissue perfusion with lower pressures. Trials of higher target mean arterial pressure have not shown clinically meaningful benefit and some evidence of harm from adverse drug effects (52–54). However, it may be that a lower target is acceptable. In the recently published "65-trial," a lower target mean arterial pressure of 60 mmHg was tested against the 65 mmHg clinical standard in older (over-65) critically ill patients with vasodilatory shock (55). There was no mortality difference shown between the two targets. The results of this study has been cited as supporting personalisation of pressure targets, accepting lower bound of 60 mmHg in some instances (56), although this has not been adopted in the recent COVID-19 interim guidance issued by the surviving sepsis group (57).

Vasopressors

Norepinephrine is generally accepted as the first-line vasoactive agent in fluid-refractory septic shock in adults (29), with the therapeutic aim of restoring mean arterial pressure and tissue perfusion. Superiority of norepinephrine over dopamine has been demonstrated in systematic reviews, both in terms of survival outcome and reduced adverse effects (58, 59). The surviving sepsis continue to include a recommendation for the use of dopamine as an alternative in select patients with low risk of tachyarrhythmias, although the guidelines do not make recommendations on how to select these patients (29). Vasopressin may be considered as a second line agent in vasodilatory shock that persists despite norepinephrine. There is no mortality benefit of one drug above the other and vasopressin is probably best considered norepinephrine sparing (29, 60, 61). Data guiding the norepinephrine dose at which a second agent should be instituted is lacking. The surviving sepsis authors advocate an upper dose limit of 0.03 units/min of vasopressin, this was the dose used in the intervention arm of the VASST trial (61). The more recent VANISH trial used an upper limit of 0.06 units/min but showed numerically higher incidence of digital and mesenteric ischaemia compared with norepinephrine doses (60). Epinephrine is an alternative second line agent. For the subset of patients with myocardial impairment and low cardiac output secondary to sepsis, the surviving sepsis guidelines suggest considering dobutamine as an inotrope.

Other Potential Therapies

A plethora of additional agents have been advocated as tools in the armory for the treatment of sepsis and shock associated with sepsis. Corticosteroids, immunoglobulins, vitamin C (with or without thiamine) are some more common recent examples. None have been shown to provide meaningful mortality benefit in high quality randomized controlled trials. Corticosteroids retain a place for those with adrenal insufficiency (chronic steroid use, Addison's disease etc.). Some also advocate corticosteroid use (200 mg/day for 7-days) in septic shock refractory to fluid and vasopressor therapy, citing shorter time to resolution of shock and shorter ICU stay (62, 63). There does not appear to be a risk of increased secondary infection with this approach, although hyperglycaemia and hypernatraemia are more common with steroid use (63). Multiple trials investigating the effect of vitamin C in sepsis are ongoing.

Supportive ICU Care

Septic patients should receive standard ICU care bundles that include venous thromboembolism and stress ulcer prophylaxis, glucose control and sedation care for ventilated patients.

CONCLUSION AND FUTURE WORK

Sepsis remains a challenging condition to manage. Heterogeneity of presentation, similarity to other inflammatory states and disease time-course makes diagnosis challenging and mortality remains high despite global efforts in improving treatment. International collaboration has been extraordinary and provides a good starting point for management. Treatment recommendations are not without controversy and are probably best used as a platform to design personalized treatment regimens, rather than rigidly sticking to protocols. Ongoing work must focus on improved diagnostics, alongside novel therapeutics. The need for an sensitive and specific biomarker seems most pressing, as it would enable appropriate management for the individual patient, assist in antimicrobial stewardship to protect drugs for the population and provide the ability to more accurately recruit participants with true sepsis to interventional trials.

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

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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.

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Role of Complement and Histones in Sepsis

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The wide use of the mouse model of polymicrobial sepsis has provided important evidence for events occurring in infectious sepsis involving septic mice and septic humans. Nearly 100 clinical trials in humans with sepsis have been completed, yet there is no FDA-approved drug. Our studies of polymicrobial sepsis have highlighted the role of complement activation products (especially C5a anaphylatoxin and its receptors C5aR1 and C5aR2) in adverse effects of sepsis. During sepsis, the appearance of these complement products is followed by appearance of extracellular histones in plasma, which have powerful proinflammatory and prothrombotic activities that cause cell injury and multiorgan dysfunction in septic mice. Similar responses occur in septic humans. Histone appearance in plasma is related to complement activation and appearance of C5a and its interaction with its receptors. Development of the cardiomyopathy of sepsis also depends on C5a, C5a receptors and histones. Neutralization of C5a with antibody or absence of C5aR1 blocks appearance of extracellular histones and cell and organ failure in sepsis. Survival rates in septic mice are greatly improved after blockade of C5a with antibody. We also review the various strategies in sepsis that greatly reduce the development of life-threatening events of sepsis.

Keywords: anaphylatoxins, histones, NLRP3 inflammasome, ROS, NETs, METs, C5a, C5b-9

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BACKGROUND

Development of infectious sepsis in humans often results in a series of events that can lead to death. In spite of nearly 100 clinical trials, no drug has been approved by the FDA for use in septic patients. The mouse model of infectious sepsis is polymicrobial sepsis produced by cecal ligation and puncture (CLP). This model has been widely used for four decades (1, 2) because it appears to mimic events occurring in septic humans. In the early phases of polymicrobial sepsis (first 1–3 days), there is hyperactivation of the innate immune system, releasing a flood of cytokines, chemokines, and extracellular histones, all of which cause injury to cells and organs (especially lungs, liver, spleen, heart, kidneys, and the central nervous system) (3–5). It is also established that aged septic mice (20–24 months of age) or septic humans (>60 years of age) have more severe sepsis and much greater lethality when compared to younger mice or humans, respectively (6–10). Reasons for these responses are not established, except, perhaps, for the fact that aged mice or humans have progressive impairments of the innate immune system (7, 11, 12). Beginning around 3 days after onset of sepsis, the innate immune system becomes progressively non-responsive, and immunosuppression sets in (5, 13). If the septic mouse or human is able to contain these responses, within about a week the innate immune system recovers, immunosuppression subsides and organ dysfunction is reversed, with return to the pre-septic state (14). It has recently

been shown that, after discharge of patients from the hospital, there is evidence of residual medical problems in many individuals, especially the elderly (15, 16). For instance, nearly 50% of these individuals have a shortened life span over a 2-year period post-sepsis, resulting in a doubling of death rates when compared to a non-septic cohort. Obviously, there is a great deal to be learned about events in sepsis and how survival can be improved. Septic mice with various genetic manipulations appear to be critical resources for such studies. While the NIH/NIGMS has discouraged the use of mice for sepsis studies and is no longer engaged in supporting sepsis studies in humans, we have been able to identify mechanisms in septic mice that can be extrapolated to septic humans. Studies such as neutralization of histones or C5a have been shown to greatly improve survival in septic mice, employing interventions that could not be done in septic humans.

POLYMICROBIAL SEPSIS MODEL AND BLOCKADE STRATEGIES TARGETING C5aRs OR EXTRACELLULAR HISTONES

Most of the septic studies in mice use the polymicrobial sepsis model with CLP as the standard procedure (17, 18). The polymicrobial experimental model mimics sepsis in humans and is helpful for understanding the sepsis process in the human body (19, 20). In human sepsis, there is excessive C5a generation associated with inflammatory responses. In 2009, Xu et al. described the role of histones in mice with polymicrobial sepsis, endotoxemia or infusion of TNF (21). We have shown the harmful effects of C5a in septic mice, resulting in cardiomyopathy and cardiac dysfunction (22, 23). Blockade of C5a or its receptors (mainly C5aR1) significantly preserved heart dysfunction in septic mice. The same result developed in septic mice lacking C5a receptors (C5aR1 or C5aR2) (23). In these studies, cardiovascular performance was measured by ECHO/Doppler technology in mice before and 8 h after induction of CLP. Echocardiograms were also obtained before and after sepsis, according to the recommendations of the American Society of Echocardiography (23). Cardiac performance (especially isovolumic relaxation time) showed preserved levels of this parameter in the septic mice which lacked either C5a receptor (especially C5aR1) compared to the septic wild type mice (23).

Another strategy for reducing the harmful effects of sepsis was neutralization of extracellular histones (with clone BWA3 antibody targeting H2A/H4). Our functional studies showed remarkably reduced cardiac dysfunction in septic mice. Septic mice receiving this neutralizing antibody against histones showed preserved cardiac function as measured by Echo/Doppler studies (24).

PHASES OF POLYMICROBIAL SEPSIS

It is now well-established in polymicrobial sepsis in mice that there is an early phase of robust activation of the innate immune system (1–3 days after CLP) during which time neutrophils (PMNs) and monocytes/macrophages release powerful proinflammatory mediators (TNF, IL-6, IL-1β, IL-17, etc.) in a "cytokine burst" that causes cell damage and multiorgan dysfunction, especially affecting kidneys, heart, liver, and other organs (13, 25, 26). As polymicrobial sepsis progresses, many of the proinflammatory responses are attenuated as immunosuppression develops at 3–7 days, resulting in reduced innate immune cellular responses which may compromise the natural protective responses that combat a variety of infectious agents (bacterial, fungal, viral) (5, 27, 28). If the protective immune responses are adequate, the inflammatory responses subside and mice are returned to health within 7 days, including recovery of organs from damage and reversion to the pre-sepsis state (14, 29).

The early phases of sepsis are associated with strong activation of the three complement pathways (classical, alternative, and lectin), generating a variety of strong proinflammatory peptides, especially anaphylatoxins C3a and C5a. C3a was originally identified by its ability to react with its receptor (C3aR), resulting in increased vascular permeability and smooth muscle contraction in a variety of tissues (3, 30, 31). The other anaphylatoxin, C5a, was recognized as a very powerful activator of PMNs and macrophages. Following its rapid binding to C5a receptors (C5aR1 and C5aR2), C5a·C5aR1 interaction causes activation of both PMNs and macrophages, the result being release of proinflammatory cytokines and chemokines, chemotaxis, generation of powerful and harmful oxygen-free radicals, and release of a variety of enzymes and lipid mediators that positively and negatively modify inflammatory responses (32–34).

Sepsis-Induced Release of Histones and Mechanisms of Cell and Organ Damage Related to Histones

It is commonly found that septic mice and septic humans develop activation of all three complement pathways (classical, alternative and lectin). As emphasized in **Figure 1**, early in sepsis in both mice and humans (over the first 24 h), via C5a·C5aR1 interactions, there is extensive cell and organ damage related to the surge in plasma of proinflammatory peptides. In addition, there is also an early (1–3 days) appearance in plasma of IL-1β associated with activation of the NLRP3 inflammasome in PMNs and macrophages (35, 36). TNF, IL-6, and the IL-17 family of factors are early proinflammatory peptides appearing in plasma (13, 37, 38). The role of complement and C5a and its receptors in activation of the NLRP3 inflammasome in septic mice has been described (39), as well as NLRP3-induced activation of CD4+ T cells (40). As time progresses, like the rest of the innate system, the NLRP3 inflammasome becomes functionally defective, resulting in diminished release of IL-1β. We have recently shown that the cardiac dysfunction and proinflammatory cytokines (including IL-1β) levels are significantly diminished in septic mice lacking NLRP3 (35). We also have shown the levels of extracellular histones were significantly lower in plasma from mice lacking NLRP3 or C5a receptors (24). It has been demonstrated that sepsis causes in

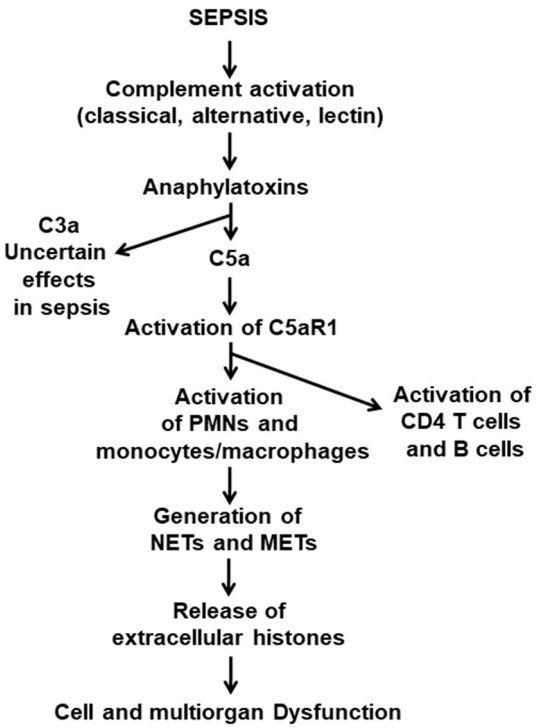


FIGURE 1 | Pathophysiological consequences of sepsis. This figure is a composite of what is currently known about the pathophysiology of polymicrobial sepsis in mice. Onset of sepsis is associated with activation of the three pathways of complement activation, which results in appearance of the anaphylatoxins, C3a and C5a. Both anaphylatoxins have strong proinflammatory effects, and both interact with their receptors, C3aR and C5aR1 and C5aR2, especially on PMNs and macrophages. The literature indicates that the C5a axis of activation, involving C5aR1 and C5aR2, causes proinflammatory responses that are harmful to cells and organs. In addition, C5a interaction with C5aR1 activates PMNs and monocytes and macrophages which form NETs and METs that are strongly proinflammatory and prothrombotic. Similar events occur in septic humans.

PMNs the appearance of neutrophil extracellular traps (NETs) and in macrophages the appearance of macrophage extracellular traps (METs) (41–44). In both cases, these traps cause adherence of bacteria to the traps resulting in bacterial killing. At the same time, these traps contain a host of products from leukocyte granules, such as proteases, proinflammatory peptides as well as extracellular histones, which are strongly proinflammatory and prothrombotic, the composite resulting in extensive injury to cells and organs (41–43, 45).

Biological Roles of Extracellular Histones in Sepsis

In the setting of sepsis, many of the proinflammatory and thrombogenic events affecting cells and organs can be attributed to extracellular histones which derive from activated PMNs and macrophages (41–44). We have shown numerous biological responses, including vascular leakage, buildup of PMNs and macrophages in organs, cell swelling and cytotoxic outcomes, LDH release, increased $[Ca^{2+}]i$ in cells, and release of cytokines and chemokines (46). We have also shown the biological activities related to purified individual histones (H1, H2A, H2B, H3, H4) (46). We have not yet determined to what extent various biochemical changes that develop in histone proteins (acetylation, methylation and ubiquination) affect the biological function of extracellular histones, but this likely happens in a manner that amplifies or reduces biological responses, induced by histones.

Role of Toll-Like Receptors (TLRs) in Biological Responses in Sepsis

It has been known for some time that TLRs function as pattern recognition receptors and are critical for numerous responses of the innate immune system. TLRs are also involved in responses to LPS, with most evidence suggesting that TLR4 is critical for cell responses to LPS (47). We have recently shown that in septic mice, as early as 8 h after onset of sepsis, cardiomyocytes develop dysfunction and that such dysfunction can be linked to the role of complement activation, C5a and its receptors, as well as extracellular histones, causing cardiomyocyte dysfunction (24). We have seen decreased levels of $[Ca^{2+}]i$ in the cardiomyocytes from knockout (KO) of either TLR2 or TLR4 after infusion of the histones, suggesting these receptors may be linked to interactions with histones (24).

Regarding other TLRs, we have also shown that the full development of cardiac dysfunction developing in septic mice requires the presence of both TLR3 and TLR9 (48). Septic mice with KO of either TLR3 or TLR9 have attenuated cardiac dysfunction during sepsis, but the precise pathways responsible for such changes are not currently known. However, in recent studies, cardiomyocytes (from normal mice) exposed to the histone mix showed release of LDH and the septic hearts showed release of proinflammatory cytokines (TNF, IL-6, and IL-1β) that are greatly diminished in hearts from TLR3 or TLR9 KO mice (48). Obviously, much more data are needed in terms of roles of TLRs in sepsis.

Protective Interventions in Septic Mice and Septic Humans

Based on the information in **Figure 1**, there are numerous interventions that protect mice from the damaging effects of infectious sepsis.

Complement Blockade

This has been done in septic mice using a variety of strategies, including KO of key components (49–51) for each of the three pathways (classical, alternative, lectin) of complement activation. The problem with such interventions is that there are extensive interactions involving activation products from each pathway reacting positively with all three pathways of complement. Accordingly, specific and limited pathway blockade of a single complement pathway does not often occur under such circumstances. The exception may be the C1 esterase inhibitor which blocks C1 of the classical pathway (52). This inhibitor has been used in human septic patients (53, 54), but the results have not been especially impressive. C1 esterase inhibitor is currently not used to treat septic humans (55).

Blockade Involving C5 or C5a Receptors

The absence (by KO) of C5 or its blockade with a mAb blocks both C5a and C5b-9 generation as obvious targets (49–51). Blockade of C5 with mAb has been shown to block inflammatory responses in rheumatoid arthritis (56, 57). The use of mAb to C5 has also been approved in patients with paroxysmal nocturnal hemoglobinuria (58) as well as patients with myasthenia gravis (59). Using KO mice, the absence of C5aR1 has been shown to block many of the harmful outcomes in septic mice (23, 51). KO of C5aR1 substantially reduces the harmful effects of sepsis in mice, reducing multiorgan injury and greatly improving survival after CLP (23, 51, 60). There are several companies that have developed small molecular weight inhibitors for C5aR1, but to date none have been approved for use in septic humans. The second C5a receptor, C5aR2 was originally described as a C5a "default receptor," functioning as a compound that binds C5a, resulting in the absence of any signal-transduction response. There is conflicting information in the literature about the biological role of C5aR2 (formerly known as C5L2) (61, 62). Until this conflict is resolved, it seems unlikely that C5aR2 will be a target for pharmaceutical companies.

Blockade of Proinflammatory Peptides or Their Receptors in Sepsis

While blockade of TNF or its receptor, or mAb to C5 has been effective in patients with paroxysmal nocturnal hemoglobinuria or myasthenia gravis, similar interventions have not been successful in the setting of sepsis in mice or humans. This is probably due to the presence of numerous other proinflammatory peptides appearing in septic humans with similar biological activities.

Blockade of Histones in the Setting of Sepsis

As indicated above, it is now clear that extracellular histones derived from activated PMNs or macrophages in septic mice (or septic humans) appear in the plasma of septic mice or septic humans in the early phases of sepsis (1–3 days) in substantial amounts (25 μg/ml) as determined by immunological assays or by mass spectrometry (24, 63, 64). In septic mice, PMNs and macrophages appear to be the chief source of the extracellular histones. As indicated above, these histones are intensely proinflammatory and prothrombotic. Our studies indicate that appearance of extracellular histones in sepsis is complement and C5aR1-dependent. This suggests an alternative strategy to block appearance of extracellular histones in the setting of sepsis. However, much more information will be needed before interventions of histones in septic humans can be considered.

AUTHOR CONTRIBUTIONS

FZ and PW were involved in the work in mice with polymicrobial sepsis. Both participated in the writing and review of this report.

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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.

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REVIEW
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Sphingolipidomics in Translational Sepsis Research–Biomedical Considerations and Perspectives

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Scientific Background: Sphingolipids are a highly diverse group of lipids with respect to physicochemical properties controlling either structure, distribution, or function, all of them regulating cellular response in health and disease. Mass spectrometry, on the other hand, is an analytical technique characterizing ionized molecules or fragments thereof by mass-to-charge ratios, which has been prosperingly developed for rapid and reliable qualitative and quantitative identification of lipid species. Parallel to best performance of in-depth chromatographical separation of lipid classes, preconditions of precise quantitation of unique molecular species by preprocessing of biological samples have to be fulfilled. As a consequence, "lipid profiles" across model systems and human individuals, esp. complex (clinical) samples, have become eminent over the last couple of years due to sensitivity, specificity, and discriminatory capability. Therefore, it is significance to consider the entire experimental strategy from sample collection and preparation, data acquisition, analysis, and interpretation.

Areas Covered: In this review, we outline considerations with clinical (i.e., human) samples with special emphasis on sample handling, specific physicochemical properties, target measurements, and resulting profiling of sphingolipids in biomedicine and translational research to maximize sensitivity and specificity as well as to provide robust and reproducible results. A brief commentary is also provided regarding new insights of "clinical sphingolipidomics" in translational sepsis research.

Expert Opinion: The role of mass spectrometry of sphingolipids and related species ("sphingolipidomics") to investigate cellular and compartment-specific response to stress, e.g., in generalized infection and sepsis, is on the rise and the ability to integrate multiple datasets from diverse classes of biomolecules by mass spectrometry measurements and metabolomics will be crucial to fostering our understanding of human health as well as response to disease and treatment.

Keywords: metabolomic analyses, sphingomyelin, ceramide, sphingosine−1−phosphate, mass spectrometry–LC-MS/MS, theranostic

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PROEM WHY SPHINGOLIPIDOMICS—WHY SHOULD WE DO ANALYSES FROM A CONVOLUTED BIOLOGICAL SYSTEM?

Why one should do analyses from a complex and convoluted biological system, from which at least a proportion is understood in detail? Because (i) we are interested in a complete picture encompassing all or at least many members of this set of biomolecules, because (ii) these molecules are often interconvertible into each other by tightly regulated and dynamic mechanisms, because (iii) individual biomolecules exert synergistic, but also antagonistic properties in issues of life or death of a cell or of an organism, because (iv) we minimize on the basis of an unbiased and comprehensive profile the probability of misinterpretation, whenever we have a look to the bigger picture of a distinct phenotype, and finally, because (v) we are only that way able to discover relationships and mechanism of potential drugs in complex diseases. This justification should be captivating to task with thousands of molecules and complex bioanalytical procedures, since it is unknown which of the throng of metabolites in the samples will be of interest.

SPHINGOLIPIDS—A MAJOR LIPID CLASS WITH BROAD STRUCTURAL AND PHYSICOCHEMICAL DIVERSITY

Backbone and Structure of Sphingolipids

One of the few common features of sphingolipids (SP) is the hydrophobic sphingoid backbone as a building block, a long carbon chain with a nitrogenous head group (**Figure 1**). The most abundant sphingoid base is SPHINGOSINE with an 18-carbon chain, which is hydroxylated at positions 1

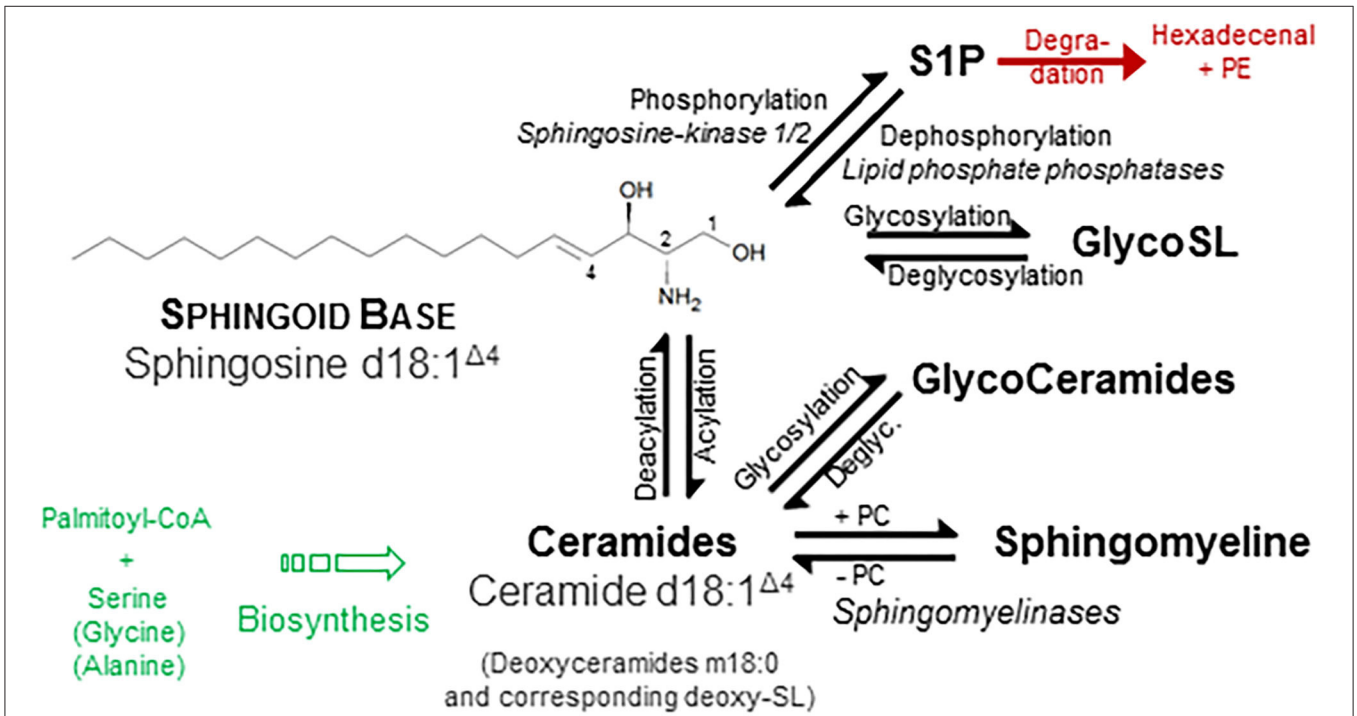


FIGURE 1 | Structural and physicochemical diversity of sphingolipids. Sphingolipids (SP) share a common hydrophobic SPHINGOID BASE structure, which most abundant species is SPHINGOSINE (d18:1 $^{\Delta 4}$) (1). Diversity of SP originates from either discrete or common substitution at hydroxyl moiety (Pos. 1) as well as at the amino moiety (Pos. 2) and—more categorical—variation of the backbone with respect to number and position of hydroxyl groups and unsaturated bonds (not shown). Discrete phosphorylation of the hydroxyl group at position 1 results in the simple sphingoid base derivatives SPHINGOSINE-1-PHOSPHATE (S1P), acting at a family of G-protein-coupled S1P-receptors. Acylation of the amino moiety at position two establish the *N*-acylated sphingoid bases, termed CERAMIDES (Cer), where the fatty acid also varies in length (16–26 carbon atoms), hydroxylation, and saturation rate in a broad range. Esterification of these ceramides with phosphorylcholine (PC) results in the phospho no series of SP, *esp.* SPHINGOMYELINES. Additionally, glycosidic bonding of the hydroxyl group with mono- up to tetra-saccharides with different compositions initiates the most complex group of GLYCOSPHINGOLIPIDS, which are considered as derivatives of ceramide. For instance, a β-glycosidical linkage of D-glucose to the 1-hydroxyl moiety of ceramide results in formation of glucosylceramide (GlcCer). Ceramides, glycosphingolipids, and SM do not display a net charge and are therefore neutral sphingolipids, whereas linkage of glyco-sphingolipids with sialic acid or sulfate is negatively charged. Other sphingoid bases differ in number of unsaturated bonds (SPHINGANINE d18:0, syn. dihydrosphingosine; SPHINGADIENE d18:2 $^{\Delta 4,14}$) as well as number and position of hydroxyl groups: PHYTOSPHINGOSINE t18:0 and DEOXYSPHINGOSINE m18:0. Use of alanine instead of serine as a building block in the first condensation step produces deoxysphingosine derivatives (m18:1). A plethora of additional variations are found in other organisms (2). Along chemical structures of individual lipids and their derivatives, categorization into distinct classes and subclasses, nomenclature, and cataloging of (sphingo-)lipids and of their properties were performed by the LIPID MAPS Consortium (3), most recently revised by leading authorities in the field (4). Similar to sphingosine, also Cer is phosphorylated to the bioactive lipid mediator Cer-1-phosphate (not shown) (5). Also, major SP classes are metabolically interconvertible by enzyme-mediated pathways (6, 7), some names of which are given in *italic* style.

and 3 and unsaturated at one double bound (position 4). Written in shorthand for sphingolipidomics, thus sphingosine is d18:1$^{\Delta 4}$, where the dihydroxylation status is termed by the "d." There are over a dozen variations in the backbone in terms of chain length, saturation (number of double bonds), branching (8), and finally the number and position of hydroxyl substituents ("m" or "t" denotes one or three hydroxyl groups, respectively) (1, 2). In mammals, the d18:1 backbone is mostly predominant (9). Concerning the chain length of the acylated fatty acid, in heart tissue and also in plasma, predominantly a chain length of 16 carbons, in skin varying between 16 and 26, and in brain from 16 to 24 has been observed (9–11). One related group of simple sphingosine derivatives is the phosphorylated derivative SPHINGOSINE-1-PHOSPHATE (S1P). This lipid mediator has been identified as ligand for a family of G-protein-coupled receptors, termed by its agonist $S1P_1$ to $S1P_5$ (12, 13). Besides unspecific breakdown by some lipid phosphate phosphatases (14), the action of just one lyase—hydrolyzing S1P into phosphoethanolamine and hexadecenal in an irreversible manner—is the only known escape strategy from the universe of SP (15). A short overview is given in **Figure 1**.

Biosynthesis

Beyond structural and molecular diversity, the function of SP is also widely spread from constitutive, inert component of cellular membranes up to highly active compounds involved in cell–cell recognition, growth, and signal transduction, to mention a few (6, 8, 16–19). Sphingolipds and glycosphingolipids also undergo constitutive degradation in the endosomal/lysosomal compartment of a cell, which is in detail also a pathogenetic correlate for a series of inherited diseases, but beyond of the scope of this report (20, 21). Instead of serine as substrate for the first, rate-limiting step of SP DE NOVO biosynthesis, also the amino acid alanine or glycine can be used, resulting in the formation of deoxysphingolipids (22). Due to the lack of the essential hydroxyl moiety for phosphorylation, these aberrant species are unable to attain the conventional pool of SP or to be degraded by canonical pathways tending to accumulation with subsequent induction of mitochondrial dysfunction (23). Resolution of the extensive compositional and structural diversity of SP in biological systems is far from being completed, novel species are continuously discovered (24).

SPHINGOLIPIDS—A MAJOR LIPID CLASS WITH BROAD (PATHO-) PHYSIOLOGICAL ACTIVITY PROFILE

Biosynthesis and Distribution

For *De novo* SYNTHESIS of all SP, biosynthesis of ceramides (Cer) in the endoplasmic reticulum is the common starting point, which underlies extensive intra- and extracellular transport and secretion mechanisms and salvage pathways [excellently reviewed in (19) and references cited therein]. Beyond accumulation of SP in membranes of cells, platelets, and erythrocytes, more than 200 species are found in plasma (10) distributed to lipoproteins and albumin (**Table 1**). Interestingly, in a recently published study a universal and nearly linear decline of all major lipid classes in the second half of human lifespan in healthy individuals was reported, which was more pronounced in females. Interestingly, the lipidome in the super-aged subgroup (95 years+) was found to be mostly similar to that among younger subjects (30).

Despite that SP are present in all eukaryotic cells and body fluids, the relative abundance of individual SP species varies significantly among tissue, cell type, and activation status undergoing pathophysiological response or transformation. Then, SP often function as a consequence of physicochemical properties as well as pacemaker and biomarker in a series of conditions such as cancer (31–33), endotoxin triggered activation of macrophages (34, 35) or in liver tissue following inflammation (36) as well as severe infection by bacteria (37) or viruses (38), to mention a few. Principal functions of SP are given in **Table 2**. In overall terms, SP composition and content have the potential to provide "specific profiles" of cell types and underlying activation

TABLE 1 | Distribution of plasma-secreted sphingolipids.

Class	Number	Rate	Distribution			Comment	References
			VLDL	LDL	HDL		
SM	101	87%	63–75%		25–35%	~ 20% of total plasma lipids	(10, 25)
Cer	41	3%	48%		52%		(10, 25)
GluCer	56	9–10%	8–14%	46–60%	28–44%		(10, 25, 26)
bases	5	< 1%	n.d.	n.d.	n.d.		(10)
S1P*	1	~ 1%	2–5%	8 20%	45–60%	Albumin 25–40%	(10, 15, 27, 28)

*The distribution pattern of most four relevant SP classes in plasma of healthy human subjects (d18:1 backbone) are given with the relative proportion of SP and distribution between lipoproteins with very low density (VLDL), low density (LDL), and high density (HDL). More than 200 species are identified in plasma (10), whereas isobaric species were not considered. Note that, for sphingomyelin (SM) and ceramides (Cer), there is a rapid transfer between VLDL and LDL. SM in plasma accounts for around 20% of total plasma lipids. A considerable amount of S1P is also bound to albumin, and—in the particular fraction of whole blood—to the membranes of platelets and erythrocytes. * Here, data for S1P with d18:1-backbone are given. Using a sophisticated fractionation technique with subsequent derivatization for improved detection characteristics of low abundant species ("deep profiling"), also S1P with divergent backbones has been described (d16:1, d18:0, d18:2, and d20:1) (29).*

TABLE 2 | Coordinative function of sphingolipids.

Coordinative function of SP	Cellular response	References
Membrane architecture	Regulating membrane's thickness, stiffness, and curvature	(39, 40)
	Biogenesis of vesicles	(41)
Phase segregation & self-association	Lateral sorting (formation of lipid rafts) Organization of both protein distribution and its bioactivity	(42, 43)
Cellular signaling	Processing of extracellular signals by GCPR	(6)
Surface recognition molecules	Cell–cell interaction (metabolism, tumor invasion)	(44)
	Host–pathogen interaction and adhesion	(45)

An overview on the principal coordinative function of different SP regulating cellular response is given. Action of SP regulates the activation status of the cell, receiving, and processing of extracellular signals by G-protein-coupled receptors (GPCR) in issues of life or death, and interaction with either host cells or the prefacing contact during host response toward pathogens.

status as a specific criterion, of which a number of implications for translational value of SP analyses increased (6).

Membrane Stabilization

As a major component of the plasma membrane of all eukaryotic cells, SP represent up to a third of the membrane lipids, of which sphingomyelin (SM) is predominant and enriched in the outer leaflet of the membrane (46). Due to the fixed cylindrical formation and the intrinsic physical phase behavior, regulation of SP content in the membrane provides a biophysical mechanism to control the membrane's rigidity and shape (46). Softening the tight package is driven either by hydrolysis of SM to Cer and/or by the incorporation of cholesterol molecules (46–49). The interaction of linear acyl chains (either from SM and Cer) and flattened sterol rings (from cholesterol) results in lateral heterogeneity and spontaneous formation of so-called lipid rafts with unique scaffold properties and implications for protein assembly affecting signaling capacity (50, 51). The formation of these submicrometric lipid domains greatly contributes to membrane curvature, vesiculation properties and function-associated shaping of cells, platelets and erythrocytes (52, 53).

Signaling Performance

Beyond this membrane-stabilizing action, a small proportion of SP is held responsible to directly affect signaling or to act as a signal mediator: especially, low molecular weight SP such as ceramides, sphingosine, and the phosphorylated derivatives Cer-1-phosphate as well as SPHINGOSINE-1-PHOSPHATE are best characterized to bear instant signaling performance (**Figure 1**) upon generation (6, 54). As a general role, the unphosphorylated form (Cer, Sph) is associated with rather antiproliferative and apoptosis-triggering effects, whereas the phosphorylated species (Cer-1-P and S1P) often show proliferative and anti-inflammatory response (6, 55). In addition to the action of all of these four lipids as a second messenger, only S1P is binding and signaling with a specific family of five G-protein-coupled receptors $S1P_1$ to $S1P_5$ (13), which are expressed by nearly all mammalian cells. Action of S1P is pleiotropic and complex, for example the egress of lymphocytes from thymus and peripheral lymphoid tissue; the progression of coronary artery disease and tamoxifen resistance in breast cancer are given (56–58).

SP as Markers and Mediators

In addition to disorders with (monogenetic) defects in lysosomal degradation, a series of SP have been shown to be altered in a variety of diseases: dysregulated SP profiles were reported for metabolic diseases (19, 59) such as obesity (60), non-alcoholic fatty liver disease (61, 62), and type 2 diabetes (63). Since SP function as important mediators in the central nervous system, it is not surprising that SP are also orchestrated as markers or mediators of autoimmune diseases such as multiple sclerosis (64, 65), in neuronal cell death (66), Alzheimer's disease (66, 67), stroke, post-stroke inflammation, and neuroprotective mechanisms (68, 69), as well as other age-related diseases (70). For example, Cer accumulate within tissues and circulation during metabolic dysfunction, dyslipidemia, and inflammation. Elevated levels of just four ceramide species (16:0, 18:0, 24:1, 24:0) in plasma are predicting major adverse cardiovascular events including death in a diverse patient population referred for coronary angiography (71).

SPHINGOLIPIDOMIC IN INFECTION AND SEPSIS

In today's understanding, sepsis is recognized as a clinical syndrome that results from the dysregulated inflammatory response to infection ultimately leading to organ dysfunction and death (72). Thereby, sepsis should not be categorized to be either a sole pro- or anti-inflammatory syndrome but rather as a dynamic and variable continuum of overlapping immune mechanisms. Despite evolving progress in better understanding the complex role of SP in infection biology (73, 74), clinical sphingolipidomics of this highly conserved stress response pathway might offer a systematic reflection of pathogenetic pathways during host response in the presence of pathogens or pathogen-associated molecular patterns. Close supervision of systemic changes of the sphingolipidome could therefore help for a better understanding of pathobiology as well as development of drugs, for early diagnosis and for therapeutical monitoring of systemic infection (75, 76).

A bacterial toxin (*Staphylococcus aureus* α-toxin) is able to activate the inflammasome and mediate the formation and release of cytokines by activation of the SM-degrading stress responsive enzyme acid sphingomyelinase with release of Cer, which was shown to be abrogated by pharmacological inhibition of the activated enzyme (77).

As another factor of pathogenicity, pathogens (e.g., *Legionella pneumophila*) are found able to directly target the host's SP metabolism by degradation of S1P by microbial lyase activity, probably received by horizontal gene transfer, ultimately resulting in inhibition of autophagy during macrophage infection (78). Also, pathogens causing respiratory infections such as *Chlamydia pneumoniae*, *Streptococcus pneumoniae*, and *Mycobacterium tuberculosis* are known to exploit SP metabolism for their opportunistic survival by decreased S1P content in both circulation and lung tissue. The dysregulation of host's SP metabolism results in inadequate maturation of the phagolysosomal compartment, decreased activation of macrophages, and subsequently, ineffective control of mycobacterial replication/growth in macrophages (79).

In preclinical studies (minipigs and mice), inhalation of sphingosine was safe and effective to increase the tissue level in the luminal membrane of bronchi and trachea of an agent held responsible to be potent for elimination of pathogens such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, or *Acinetobacter baumannii.* This observation is opening a new promising opportunity for therapeutical management of complicated lung infections (80, 81). Inhibition of the S1P-degrading enzyme (S1P-lyase) exerted tissue protective effects mediated by S1P-$S1P_3$ signaling as a potential therapeutic target increasing disease tolerance against murine sepsis (82).

There is a series of reports of deranged plasma composition of SP and/or of changes in the activity of circulating enzymes affecting SP in blood or at the outer leaflet of cellular membranes during infection (**Table 3**). In sepsis, endothelial dysfunction, especially barrier disruption, results in increased vascular permeability, edema, and insufficient tissue oxygenation; all of these pathogenetic phenomena are controlled (beyond others) by S1P. This SP is a signaling lipid that regulates important pathophysiological processes including vascular endothelial cell permeability, inflammation, and coagulation. In preclinical observational studies, reduced S1P levels in serum or plasma of sepsis patients were associated with the disease (83). Beyond other metabolites, serum concentrations of SP were altered in sepsis compared to systemic inflammatory response syndrome; thus, SM (d18:1/22:3) combined with a glycerophospholipid was recommended for sepsis diagnosis. Furthermore, changes of metabolites between sepsis and severe sepsis/septic shock also varied according to the underlying type of infection, showing that SM (d18:1/16:1) combined with other metabolites is associated with unfavorable outcome in community acquired pneumonia, intra-abdominal infections, and bloodstream infections, respectively (86).

In lung tissue pathobiology, alteration in SP metabolism is closely related to inflammatory reaction and Cer increase, which in particular favors the switch to pathological hyperinflammation (93).

In community-acquired pneumonia (CAP), the metabolite profile obtained from serum samples differentiated healthy controls from patients in a severity-specific manner, where a combination of lactate, sphingosine, and an androsterone derivative was superior to conventional clinical scoring (94).

TABLE 3 | Summary of studies identifying alterations in the sphingolipidome during infection.

Disease entity	Alteration in Plasma	Comment	References
Polymicrobial sepsis	⇓ S1P	Inverse association with disease severity	(83, 84)
	⇓ S1P	Redistribution of S1P from albumin to HDL	(85)
	⇓ SM d18:1/22:3	In combination with a lyso glycerophospholipid for early diagnosis	(86)
	⇓ SM d18:1/16:1	In association with other metabolites for prediction of outcome	
Community acquired pneumonia (CAP)	⇑ S1P	Correlation with severity/prediction of outcome	(87)
	⇑ SM d18:0/16:0 SM d18:1/16:0 LacCer d18:1/16:0	Distinguishing CAP from the non-infection and from extrapulmonary infection as well as non-CAP respiratory tract infection subgroups	(88)
	⇑ Sphingatrien d18:3	SP of bacterial origin as a diagnostic biomarker	(88)
	SM d18:1/22:1 & 22.2	Association with outcome, also in COPD patients	(89)
	⇓ SM (also PC, lysoPC)	Trend to normalization during time course	(12)
	⇑ Cer	Association with activity and transcription rate of acid sphingomyelinase	
COVID	⇓ S1P	Raise in subset of patients at hospital discharge	(90)
	⇑ SM d18:1/18:1	Increase (top 10 marker)	
	⇓ Glucosylated Cer	Decrease (top 10 marker)	
Dengue fever	⇓ SM d18:1/16:0	Major changes in early febrile stages, normalization to control levels at convalescent stage	(91)
	⇑ ceramides		
Endotoxin neutralizing therapy in septic shock	⇑ S1P	S1P increase function as predictor of therapeutical effectiveness	(92)

Change of plasma concentration is given either by an up or by a down arrow.

Furthermore, plasma levels of S1P were markedly elevated in CAP patients and were inversely correlated with disease severity and with predictive power for duration of hospital stay, ICU admission, and unfavorable outcome (87). In another study, (dihydro-) SM (d18:0 rsp. 18:1/16:0) and three glycosylated ceramide derivatives distinguished (among lyso-phosphoethanolamines) also in serum samples of CAP patients from healthy controls and allowed discrimination of CAP cases from the non-infection, extrapulmonary infection, and non-CAP respiratory tract infection subgroups (88). Levels of SM species were significantly lower in CAP patients *vs.* those with exacerbation of chronic obstructive pulmonary disease (COPD), and SM (d18:1/22:1 & 22:2) were found to be associated with lower risk for short-term adverse outcomes, but not with long-term mortality rates (89). Similar results with decrease of SM in association with increase of Cer were also found, comparing CAP *vs.* patients with COPD and healthy controls. Interestingly, a disease relevant increase of the enzymatic activity of the corresponding enzyme, acid sphingomyelinase (SMPD1), was also verified, in both plasma (protein-level) and mRNA levels obtained from circulating leukocytes (95).

Among the top 10 metabolites distinguishing healthy control subjects from patients undergoing COVID-19, S1P was found to be reduced; however, its level was raised at hospital discharge relative to admission in a small subset of patients followed longitudinally (90). In these patients, increases in lysophospholipids (lysophosphatidic acids and lysophosphatidylcholines) as well as SM (d18:1/18:1, also top ten rated in this study) and glucosylated ceramides were contrasted by a decrease of neutral lipids. Most recently, serum S1P levels (determined using an immunological technique) were found to be inversely associated with COVID-19 severity: a significant correlation with markers for tissue damage, inflammation, and coagulopathy was determined (C-reactive protein, lactate dehydrogenase, ferritin, and D-dimers). Interestingly, the S1P decline was strongly associated with the number of red blood cells, the major source of plasma S1P, and both apolipoprotein M and albumin, the major transport proteins of S1P. Furthermore, S1P was exhibiting strong predictive value for admission to ICU and patient outcome, for morbidity and severity of the clinical course. As a consequence, restoration to normal S1P-values was supposed as a therapeutic strategy in patients with COVID-19 (96).

Major changes in early febrile stages in Dengue fever were observed with respect to the SM/Cer ratio with a normalization to control levels at the convalescent stage (91). Unfortunately, in this study the activity of the converting enzyme (acid sphingomyelinase/SMPD1) was not monitored, but an association of membrane SM content to replication of flaviviruses could be confirmed (97).

Most interestingly, S1P monitoring (i.e., increase) was tested to function in a reliable manner to predict therapeutical effectiveness of an endotoxin absorption strategy using polymyxin B-immobilized hemoperfusion in patients with septic shock (92).

Broad Horizon and Mean Limitations With Appropriate Quality Control in SP-Analysis

For profiling the patterns of glycero-, phospho-, and SP-based on mass spectrometry (MS), the two most common approaches include analyses either by direct infusion (SHOTGUN-MS) or following chromatographic separation.

Analytical Affairs

The two main proceedings for analysis of (sphingo-)lipids are differing in the analysis of individual molecular species (specificity), resolution, and sensitivity. In an approach termed "SHOTGUN" from a crude complex extract, SP species are analyzed without any purification and/or chromatographical separation (98–101). For identification of highly abundant lipids, this method is quite simple and effective but fails completely with respect to resolution of isobaric compounds. Also, suppression of signal intensity by matrix constituents (see below) from the lipid extract is an intrinsic and fundamental limitation. Despite attempts to overcome this restriction either by mild hydrolysis of neutral glycerophospholipids or by selective derivatization for improved detection of low abundance SP (98, 102), this technique is far beyond reliable and wide-ranging analysis of clinical samples.

In a second, more common approach an upstream chromatographic separation is performed; thus, matrix suppression by co-eluting compounds is often overcome. On the other hand, used gradient protocols for chromatographic separation are time consuming, eluents might also affect ionization efficacy (103), and finally both amount and number of species detected in parallel are limited (104, 105). Noteworthily, the acute phase protein CRP, already abundant in circulation of septic patients, is known to effectively bind phospho- and sphingolipids, raising the question, whether an observed decrease of these lipids might just be fabricated and might be caused by an ineffective extraction procedure under these circumstances. However, this competent concern could be overcome by spiking experiments with recombinant protein prior to precipitation (95).

There, a series of methods has been developed for characterization and quantitation of SP; the detailed description and discussion is beyond the scope of this review [(103), for excellent review see (105, 106) and references cited therein]. For selection of the most appropriate method, there are some critical criteria discussed in more detail.

Reliable analyses of SP from clinical samples require specialized instrumentation (i.e., triple quadrupole mass spectrometer directly coupled to a high flow capacity ion source—ESI or APCI) and qualified users. There are some excellent publications to familiarize beginners in the field with the approach of sphingolipidomics (107). These general methods should be refined for the specific sample series to be tested since the head group, sphingoid bases, and fatty acid substituents are differing in a broad range as discussed previously, also affecting analytical performance. In ESI-positive mode, unique molecular decomposition products using precursor ion scans are the method of choice (**Table 4**). The complete fragmentation

TABLE 4 | Common fragmentation ions of SP (ESI^+-mode).

Sphingolipid species	Fragment ion [m/z]
Sphingosine Sphinganine Sphingosine-1-phosphate	264, 266
Cer	264, 266
GlcCer	264, 266
SM	184

Optimal conditions for ionization as well as for collision for each SP species should be determined by representative compounds and/ or internal standard compounds, thus preventing molecular decomposition prior to reaching Q1 and also compensating intrinsic differences in the dissociation rate. Fragment ions of m/z 264 and m/z 266 are indicating a sphingoid base building block (SPB, d18:1, d18:0, respectively), which is representative for most naturally occurring sphingoid base chains. Differentiation of those SP will be achieved by either specification of retention time during liquid chromatography and/or by monitoring the specific fragment ions corresponding to molecular species (108).

TABLE 5 | Fragmentation profile of the trihexoside globotriaosylceramide Gb3 (d18:1/23:0).

m/z	
1122.8	$[M+H]^+$
1104.8	$[M+H-H_2O]^+$
960.7	$[M+H\text{-}Hexose]^+$
942.7	$[M+H-H_2O\text{-}Hexose]^+$
796.7	$[M+H\text{-}2Hexose]^+$
780.7	$[M+H-H_2O\text{-}2Hexose]^+$
636.6	$[M+H\text{-}3Hexose]^+$ i.e., Cer
618.6	$[M+H-H_2O\text{-}3Hexose]^+$
660.6	$[M+H-2H_2O\text{-}3Hexose]^+$
378.4	Long fatty acid fragment
354.4	Short fatty acid fragment

There is a sequential loss of hexoses with an apparent molecular weight of 162 each including corresponding dehydrated derivatives. The fragment with a molecular weight of m/z 636.6 corresponds to the unglucosylated ceramide molecule >Cer d18:1/23:0< with an exact mass of 635.622 u (109).

pattern should be perceived for reliable identification of a SP class. This approach will allow the differentiation of compounds in case of isobaric precursors, e.g., SP with either Cer18:1/22:0 and its isobaric dihydroderivative Cer (d18:0/22.1) as a building block (m/z 621.606 each, but difference + 2.0 in sphingoid base fragmentation pattern, 264.3 and 266.3, respectively) (**Table 5**). For unique confirmation of candidate spectra, it might also be of great advantage, since there is a known ratio between different transition intensities from internal standard compounds, which is termed the qualifier ion ratio (110).

Determining lipid profiles in large cohorts of clinical samples and translational studies motivates to complex logistic measures to minimize technical variations within and also between laboratories (111).

Sample Integrity

Due to dissimilar physicochemical properties of used anticoagulants, extraction efficiency might be affected with respect to several compounds or to the global recovery rate (25, 112). Up to now, there is no general recommendation for use of a distinct anticoagulant for sphingolipidomic studies, but the same should be used throughout the study for prevention of unforeseen discrepancies (see below).

Preanalytical Purification Procedure (PPP)

There are a number of mono- and biphasic extraction procedures from plasma or serum described, which are all used for deprivation of proteins in the sample. Most methods—with decades of experience—are either based on a modified FOLCH extraction (113) or BLIGH & DYER procedure (114). Some recent developments resulted in the validation of extraction methods combining advantages of efficiency, high throughput, and automation. There are two major issues taken into account for best preanalytical performance: from a technical point of view, harvesting of the organic layer from biphasic systems is one of the most critical issues during workflow, since a chloroform-borne organic phase due to higher density remains at the bottom of FOLCH and BLIGHT & DYER procedures. Thus, collecting this lower phase is allowing range for variability due to contamination (non-lipids, proteins, salts) or leakage (non-quantitative extraction). Since our sphingolipidome encompasses a broad polarity range of compounds—as outlined in **Figure 1** from uncharged to amphiphilic species (115)—efficiency of the extraction methods is highly pressed to work without discrimination of a class of compounds with overall coverage of almost all compounds from the sample, technical efficiency, and total throughput.

Beside sophisticated methods, also methanol-driven precipitation of proteins might have shown the best performance, as it does not lose many analytes, consisting of only a single precipitation step. Biphasic extraction procedures, in contrast, separate the analytes into two phases, and although the polar and non-polar compounds are meant to be enriched in either the aqueous or the organic phase, the amount of each analyte might still be split into both phases to some extent. Therefore, a single aqueous or organic phase might never contain as much analyte as the methanol precipitation extract where no separation step occurred. It is also known from other comparative studies that compared to biphasic extraction procedures (FOLCH, BLIGH, AND DYER) the methanol precipitation protocol (80%, V/V) yielded regularly to higher for both exogenous and endogenous compounds (116).

There are also recently developed methods for one-step extraction of both Cer and SM from human plasma by a butanol:methanol mixture (BUME) (117). Monophasic procedures are regularly characterized by higher reproducibility and recovery rate, allowing automated high-throughput procedures (118). As a specialized method for use with low-level human samples, derivatization of long-chain base phosphate derivatives with extraordinary backbone (i.e., S1P derivatives; d18:2, t20:1, or odd carbon forms) might improve the detection rate (29). Whatever you do, careful consideration and best performance of the lipid extraction method control overall quality of the analysis.

Workflow at the Machine

Some easy, feasible, and pragmatic procedures for in-line monitoring of the analytical process for metabolomic profiling control the overall output of the study. Beyond design of the clinical study and following PPP, the design of the experimental workflow is the next quality-determining step. Due to the fact of long-lasting longitudinal studies, the samples run over a period of time and/or in an intermittent manner. For prevention of bias, proper randomization is critical for minimizing bias and variance. For these aims, "block randomization" is a commonly recommended method, which run sequentially in separated, rather homogeneous subcohorts (119, 120). Another technique-borne confounding factor is given by the tendency of the mass spectrometers for a moderate drift over a long-lasting run, compromising quality of continuously running data over time in high-throughput screenings (119). There are some easy protective mechanisms against instrument-driven bias: the standard approach is preparation and use of specific quality control samples, which are collected with aliquots from every sample to generate a representative pool prior to sub-aliquoting into a set of study-specific, uniform quality control (QC) samples (121, 122). The QC specimens are running together with the experimental samples and are integrated into the workflow at the beginning, in a regular and periodic basis throughout the run and finally at the end (121, 122). As a consequence, data quality of the complete analysis can be easily evaluated by a synoptical comparison of QC samples. As a key quality factor, variance of the signals of all the QC samples should be evaluated (121, 122). With two distinct sets of QC samples, the study director is able for efficient monitoring of both drifts caused by instrumental changes and "wet-lab" non-conformance during sample preparation (see in detail **Figure 2**). Since in large, long-lasting clinical studies preparation of the QC-samples will hamper to start an interim analysis with the first batches of samples, batch-specific QC-samples from individual subcohorts should be prepared, which might differ with respect to the absolute values of compounds, but should be similar with respect to overall variance (119). Antecedent definition and documentation of maximum tolerable values of variance in the study protocol outline a supreme performance of the analyses (121). There are also initiatives to improve reproducibility, accuracy, and precision of lipid quantitation, study design,

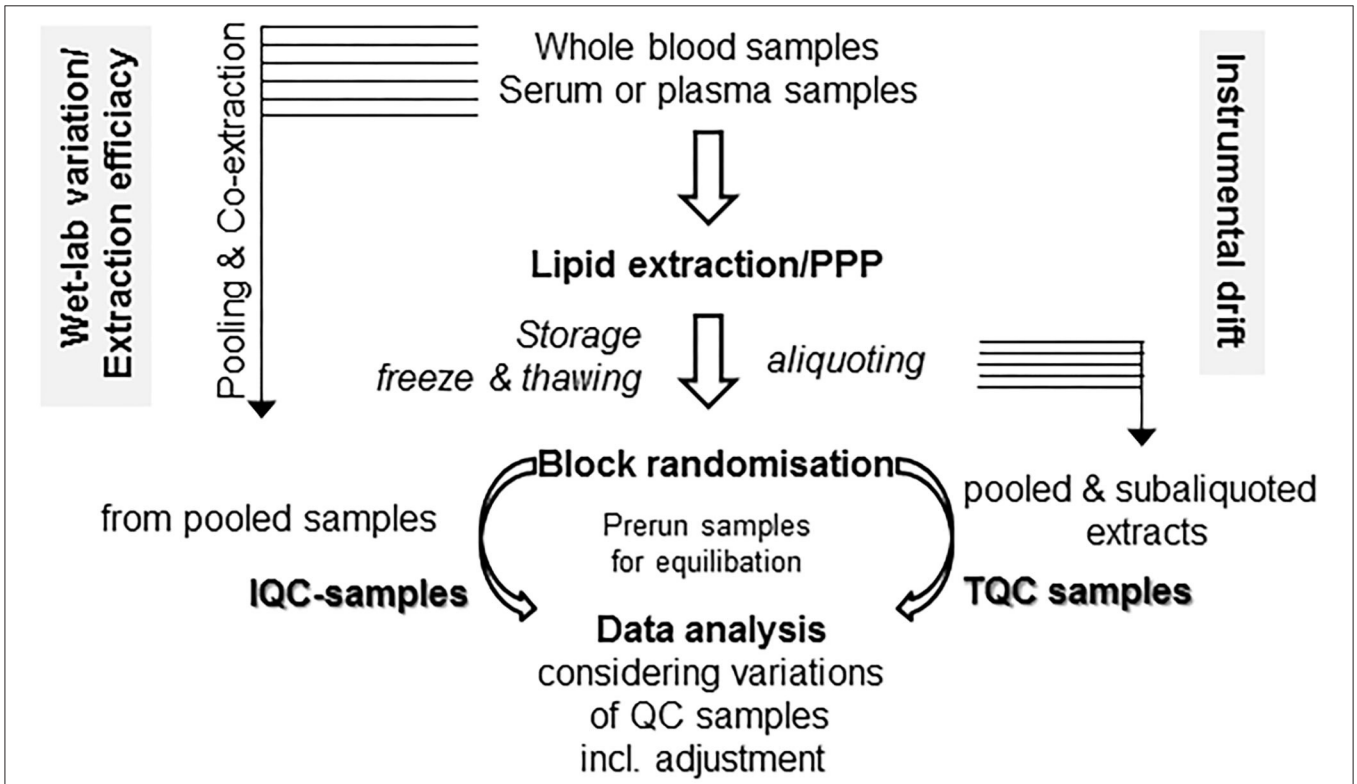


FIGURE 2 | Monitoring technical and process related variations. With two distinct sets of quality control (QC) samples, there is a clear differentiation between drifts caused due to "wet-lab" non-conformance during sample preparation (in-process QC, IQC) as well as by instrumental changes (technical QC, TQC). For these issues, two separate sets of QC samples might be prepared. For **IQC** preparation, you gather (a portion of) the raw starting material (i.e., plasma/serum) prior to extraction and co-extract these aliquots in similar conditions with the samples in your preanalytical preparation procedure (PPP) workflow. The second set is gathered from the already reconstituted solutions following PPP from either a single batch or a subcohort, then pooled and aliquoted into separate vials (**TQC**) and should display a representative pool of your analysis. Run these samples at the beginning, at the end, and in the long run in an intermittent manner. Use of biostatistical algorithms, e.g., principal component analysis, will demonstrate deviations from conformity and help to control adjustment procedures (123). For equilibration of best performance separation condition, use pre-run samples with similar matrix components, which will not be included in data analysis.

sample handling, and data/sample sharing for quantitative MS-based lipidomics of blood plasma or serum, with harmonization of data acquired on different instrumentation platforms across independent laboratories as an ultimate goal (124–127).

There are also some technical issues of LC/MS instrumentation resulting in increased variance parameters to take into account: conditioning of the separation media at the LC column seems to function as a critical factor with respect to generation of high-quality data (128, 129). Thus, in addition of equilibration of the column material in several blank runs, stabilization of the tightly balanced binding equilibrium is of great importance. For these aims, a precarriage of the column by use of samples with similar composition (especially in term of matrix composition), which are however insignificant for later data interpretation, will help to adjust retention times, mass accuracy, and signal intensity at a maximum level and excellent repeatability (119).

At the end, a sufficient number of QC samples will also help to overcome batch effects, i.e., the drift of signal intensity and retention time across different batches (130). Now, data sets might be adjusted by proper batch correction principles, when there are adequate guardians of study execution with respect to number and quality of these samples (130, 131), which ultimately also control the accuracy of the *post hoc* adjustment (123, 131).

Sample Preparation

There are different extraction methods for best performance of global profiling of SP without discrimination of low abundant species. To prevent chromatographic interference with glycero-/phospholipids (1) or to reduce a potential matrix effect (132), in some procedures an alkaline hydrolysis is reported. Despite the elimination of a plethora of co-eluting compounds by saponification of ester bounds, the slow hydrolysis of the amide bound in SP should also be taken into account as a potential biasing factor and tightly controlled by spiking of internal standard compounds.

Methods for sample preparation differ fundamentally in MODUS OPERANDI: either precipitation of proteins with more or less complete release of SP from those binding partners or methods driven by distribution of SP between phases with variable lipophilicity. It is noteworthy that the latter approach deals with a non-quantitative enrichment process, which can also result in depletion or dislocation of the original profile but also removal of matrix-driven extinction of weak signals. Finally, there are also procedures utilizing the broad affinity of SP to solid matrix materials for removal of other (plasma) constituents. Parchem and colleagues recently published an excellent synopsis on sample preparation techniques for all phospholipid classes (133).

Stationary and Mobile Phases

Silica gel particles as stationary phase combined with a linear gradient of the mobile phase with increasing polarity is able to separate cholesterol, ceramides, and derivatives thereof (GlcCer, LacCer, Gb3, and Gb4) as well as SM in an order of elution along the increasing polarity of the head groups. In this homologous sequence of SP, convincing baseline separation is a *conditio sine qua non*, which can be also achieved by smart design of the gradient for clear separation of epimeric glycosylated ceramide derivatives such as GlcCer and GalCer (132). Using a reversed-phase system (C-18 column, 150 mm × 2 mm, ∼ 2.7 μm), a segmented linear gradient has been demonstrated as an effective option for optimal and fast separation of SP (103).

Source

Coupling the outflow of an liquid chromatography apparatus online to a mass spectrometer with an atmospheric pressure chemical ionization interface (APCI) is typically used for analyzing less polar molecules from biological samples (134). Ionization by APCI is rather insensitive to signal impairing effects by the carrying solvent with an overall low tendency for adduct's formation. Due to the fact that APCI is a rather harsh ionization procedure, you often observe in-source fragmentation, where the building blocks of the SP occasionally can be verified in the full-scan spectrum of the molecule: from a synthetic and purified globotriaosylceramide with three hexosid molecules (Gb3, d18:1/23:0), all three characteristic components might be identified (109). Besides the molecular ion $[M+H]^+$ and the dehydrated ion $[M+H\text{-}H_2O]^+$, the carbohydrate moiety, the Cer-backbone, and the fatty acid residue are found, whereas there is also a stepwise loss of carbohydrate residues, each of them also with a corresponding dehydrated species (**Table 5**).

An electrospray ionization interface (ESI) is complementary to APCI, which revolutionized the ability to generate intact molecular ions from polar biomolecules, which was therefore broadly used for structural characterization as well as quantitative and qualitative analysis of SP (135–137). This technique is sensitive to adduct formation, e.g., in the presence of ammonium ions, which might increase the sensitivity of the method. In the positive mode, you regularly detect the molecular ion $[M+H]^+$ as well as a common class-specific fragment, which sometimes hampers the precise mapping of isobaric compounds of a homologous sequence.

Amount/Detection Limit

(i) In some cases (*esp.* following alkaline hydrolyses), it is strictly recommended to dispose the fractions with free fatty acids (either at the start or at the end of the run, depending on polarity of the stationary phase) to prevent contamination of the source as well as an overload of the detector with free fatty acids from glyerophospholipids, cholesterol esters, and glycerolipids. (ii) Due to the broad range of abundance of naturally occurring SP, the injection volume of the sample (i.e., the total amount of SP species on the column) must be thoroughly evaluated, since a higher amount might increase signal intensity at all, but impairs separation efficiency of the column and signal intensity of low-abundance species due to unforeseen matrix effects of co-eluting compounds. Specification of the ideal injection volume should be performed following stepwise increase and comparison of signal characteristics of low-abundance species. (iii) The lower limit of detection (LLD) normally lies around 0.05–0.1 nmol, whereas the non-substituted SP (i.e., Cer, sphingosine) have the lowest. In glycol-SP, the LLD increases with the degree of substitution. (iv) Beyond the absolute amount of a species in

the mixture for analysis, the stability of the fragment is also a critical factor to be taken into account. For example, the LLD of the cholesterol molecule is surprisingly high despite the weak stability of the detected molecular ion $[M+H-H_2O]^+$, which is but yet counterbalanced by high concentration in mostly all biological samples.

Sensitivity and Specificity

Reliable identification and quantitation can be achieved on the basis of the improved performance (*esp*. high-speed scans) of newer instrumentation in the last decades. Thus, identification is performed in tandem mass spectrometers (MS/MS) with precursor ion scans, to distinguish various SP species from crude biological mixtures by their unique decomposition products (107, 138). One significant advantage of this approach is the fact that both combination and molecular composition of building blocks can be readily determined (138). Automated suppression of the background noise of the instrument realizes much lower limits of detection. Quantitative performance is optimized by use of a specialized technique termed multiple reaction monitoring (MRM); since the time period for detection of precursor/product ion transitions is increased to a maximum, the time period for scanning regions without any interest reduced to a minimum. All in all, this approach yields in high sensitivity (with respect to LLD) and high specificity compared to precursor ion scan alone. If available, detection performance of individual lead compounds (representing a class of analytes) might also be optimized with respect to ion formation and fragmentation pattern including instrumental setting for best performance with respect to formation and detection. However, one limitation for absolute quantitation of SP is the fact that just for a minority of compound classes certified internal standard derivatives are available.

Starting Material and Its Processing Procedures

As most commonly used for the purpose of longitudinal clinical studies, we here focus on whole blood—drawn by venipuncture—and "products" thereof as starting material for sphingolipidomic analyses. There is a long list of anthropometric factors, all having an impact of SP profiles also in healthy individuals (age, gender, ethnicity, etc.) (30, 139, 140). Also clinical factors are affecting the SP profile (diet/fasting status, medication, diurnal variation, etc.), which should all be documented (141). With respect to storage, sample handling, use of quality controls, and sharing of reference material for harmonization of results between laboratories, we refer to previously published papers (121, 122, 127, 142). In general, up to two freeze/thaw cycles did not substantially affect metabolic profiles (140, 143–145), but with increasing numbers significant changes were observed, e.g., with respect to an increase of arachidonic acid content, probably a reflection of ongoing metabolism, to which also SP are sensitive (144).

Plasma or Serum?

Next, it is noteworthy, that the (anti)coagulation strategy *per se* massively affects the SP profile (25). Moreover, the liquid fractions of whole-blood preparations (plasma vs. serum) should be considered as completely different materials, which are now and to no time comparable with respect to SP profile (27, 146, 147), which might be caused at least by clotting-associated alterations and release of metabolites and enzymes further metabolizing them (148). As an example, export mechanisms of (activated) platelets are held responsible to control S1P concentration in plasma measurements (149). In a series of studies, it was shown that for metabolomic studies [reviewed in (140)] serum was detected to be superior with respect to sensitivity, but the use of plasma provided more reproducible results. Overall, variation induced by anticoagulation additives seems to be marginal; however, in a report of Hebels and colleagues, there is the strong recommendation that metabolomic studies should not mix plasma samples with different additives (112).

For generation of serum from whole blood, appropriate and uniform conditions for completion of the clotting procedure are mandatory [clotting period 30–60 min, temperature, type and concentration of additives as clotting enhancers, etc. (148)]. Use of tubes equipped with a gel barrier did not affect SP analyses on serum samples (143). Plasma preparation requires additives for prevention of coagulation such as EDTA, citrate, and fluoride, following standard procedures: centrifugal force 1.500 up to 4.000 × g, temperature range 4–15°C, centrifugation time 5–10 min [(148) and references cited therein]. In these conditions, however, those plasma specimen are not completely free from (activated) platelets, leading to differences in metabolic patterns obtained from plasma (140). Moreover, during storage these remaining platelets are lysed in an unspecific manner. Up to now, it is an open question, if and in which manner these debris from lysed platelets might cause known discrepancies in metabolomics studies with plasma as starting material (148). The only way out is preparation of platelet-free plasma by reduction of remained platelets $< 10{,}000/\mu L$ (150) by increasing either the centrifugation time or the centrifugal force in a second separation step (151). There is one report stating the use of heparin results in higher variability with respect to SP (25).

Hemolytic samples should be completely withdrawn (or at any rate analyzed with caution) due to the facts of adverse effects on metabolomic studies (152, 153) and especially of inestimable but severe alterations of SP highly abundant in red blood cells (152).

The same is true for variation of handling (i) time [post-processing $<$ 2 h. (153)], (ii) temperature profile, and (iii) centrifugal phase separation parameters (time force, shear stress) exposed to the drawn sample up to freezing (146, 154), all in all emphasizing the demand for a uniform procedure including a careful removal of the liquid phase without disturbing the particular fraction. As a consequence, consideration of the effects of pre-analytical factors is a particularly important issue for long-lasting studies, especially when samples are collected in decentralized settings with a risk of time and temperature delays prior to being completely processed and frozen for storage. Of note, preprocessing at room temperature before centrifugation might result in an increase of S1P signals (152). Prior and after PPP, the samples are stored regularly at least at −80°C, in large multicentric studies occasionally for years. Beyond quality

indicators for pre-analytical process variations such as time to centrifugation (155), levels of 56 out of 111 metabolites (also degradation of SM−14.8%) were reported in a five-year period of storage (156); the same was true for cholesterol and triglycerides (157). Thus, storage time has to be taken into account and a potential bias should be overcome by a smart batch management of samples. Also, the number of freeze-thaw cycles significantly affects a number of lipid mediators such as phosphatidylcholines (PC) and SM (25). However, other studies demonstrated minor changes but also oxidative modification of unsaturated fatty acids (100). As a consequence, the number of freeze/thaw cycles should be kept constant throughout the study, and sub-aliquoting helps to minimize possible artifacts (119).

From plasma samples, a purified platelet fraction can also be used for SP profiling; in a recent study in patients with coronary artery disease, a total of 39 SM and 23 Cer species were detected for detection of additional insights of mechanisms responsible for symptomatic thrombus formation during an acute myocardial infarction (158).

Platelets, Microvesicles, and Exosomes

Platelets have a short life span around 8 to 10 days in circulation and are activated during inflammation and infection; therefore, they function as an excellent reporting system during a variety of diseases. Moreover, activated platelets release platelet-derived microvesicles (PMV), often termed "platelet dust," playing both a pivotal role in atherosclerosis, thrombosis, and immune defense (159). The same is true in stored platelets, where the release of PMV is induced by senescence as a storage lesion (160). During storage, the content of ceramides significantly increased (+53%) and S1P decreased (−53%), all in all shifting SP metabolism toward Cer (161). The heterogeneous extracellular vesicles generated from platelets differ with respect to size, composition, and function (159). The same is true for plasma-borne exosomes, which are small vesicles released from cells and platelets after fusing of multivesicular bodies with the plasma membrane upon activation. Other types of extracellular vesicles are formed by direct budding of the membrane and are larger in size (100–1,000 nm, also termed microvesicles). Apoptotic bodies (<2,000 nm) are also formed by blebbing of membranes from cells undergoing apoptosis (162). Reported lipid compositions that are found to be enriched in these particles vary due to principles of cellular source and biogenesis, which has implications to functions as well as clinical applications as biomarkers and possible use for drug delivery (162). Especially, the majority of the blood-borne extracellular vesicles are thought to originate from either platelets or directly from the platelet precursor cell platelets, participating in a plethora of physiological functions, including hemostasis and immunity as well as in thrombogenesis (159, 163).

From plasma, the most commonly used method for isolation of exosomes is ultracentrifugation, but this procedure results in co-isolation of exosomes, lipoproteins, and lipid droplets (164). More sophisticated methods for isolation are filtration, size exclusion, or immune-affinity chromatography, all of them with advantages and disadvantages with respect to homogeneity and presence of related subpopulations of extracellular vesicles (165–168). Thus, these factors should be taken into careful consideration interpreting results drawn from preparation of extracellular vesicles. As an example, the presence of cholesterol esters or triacylglycerol derivatives in your analyses, which are regularly not present in cellular membranes (46), supports the concept that lipoproteins or lipid droplets have been co-isolated with exosomes (162). On the other hand, immunological characterization of platelet-derived exosomes allowed improvement of the early detection of the infective agent in fungal sepsis (169). A short overview for preparation of subfractions of whole blood and the properties thereof is given in **Figure 3**. In contrast to oncological studies using these extracellular vesicles for improved diagnosis and therapeutical monitoring (170, 171), in translational sepsis research they are just often only recognized with respect to their pro-coagulant and pro-inflammatory function (172).

Red Blood Cells

Similar to platelets also red blood cells (RBC) during their short life span exhibit the closest contact toward every tissue and cells in the organism and might therefore act as a carrier of information upon exchange of SP following isolation. Since inhibition of SP biosynthesis in solid tissues affects their concentration levels in plasma as well in RBC (173), analyses of SP composition of RBC opens the field for studying changes in metabolic mechanisms in deep compartments without any access in routine diagnostics. Induced by a variety of causes (osmotic pressure, oxidative stress, energy depletion, etc.) and comparable to apoptosis of nucleated cells, also RBCs may undergo suicidal death characterized by cell shrinkage and phospholipid scrambling and redistribution of the cell membrane, termed eryptosis (174, 175). Whatever the rapid clearance of eryptotic RBC, canonical mechanisms are held responsible to trigger eryptosis including formation of Cer, which might be observed by techniques of sphingolipidomics (176). Enhanced erythrocytic Cer formation was observed in fever, sepsis, hepatic failure, and metabolic diseases (177).

Recently, a specific transporter termed Mfsd2b with high S1P export capacity from platelets and RBC was identified, which is sensitive to stress (178). That susceptibility toward biomedical/pathophysiological factors but also toward handling of samples underlines the role of anucleated cells in S1P distribution, function, and metabolism.

White Blood Cells

Sphingolipids are also involved in leukocyte activation and reprogramming during sepsis (179, 180). The mixed cell population is isolated from whole blood samples using a simple and rapid centrifugation procedure based on a density gradient procedure developed by Bøyum (181, 182). For mass spectrometric analyses, contaminating platelets in the raw leukocyte fraction should be effectively removed by a second centrifugation step (183). Following cell counting, cellular proteins from a predefined number of pelleted cells are precipitated by addition of methanol and lysis of cells at low temperatures.

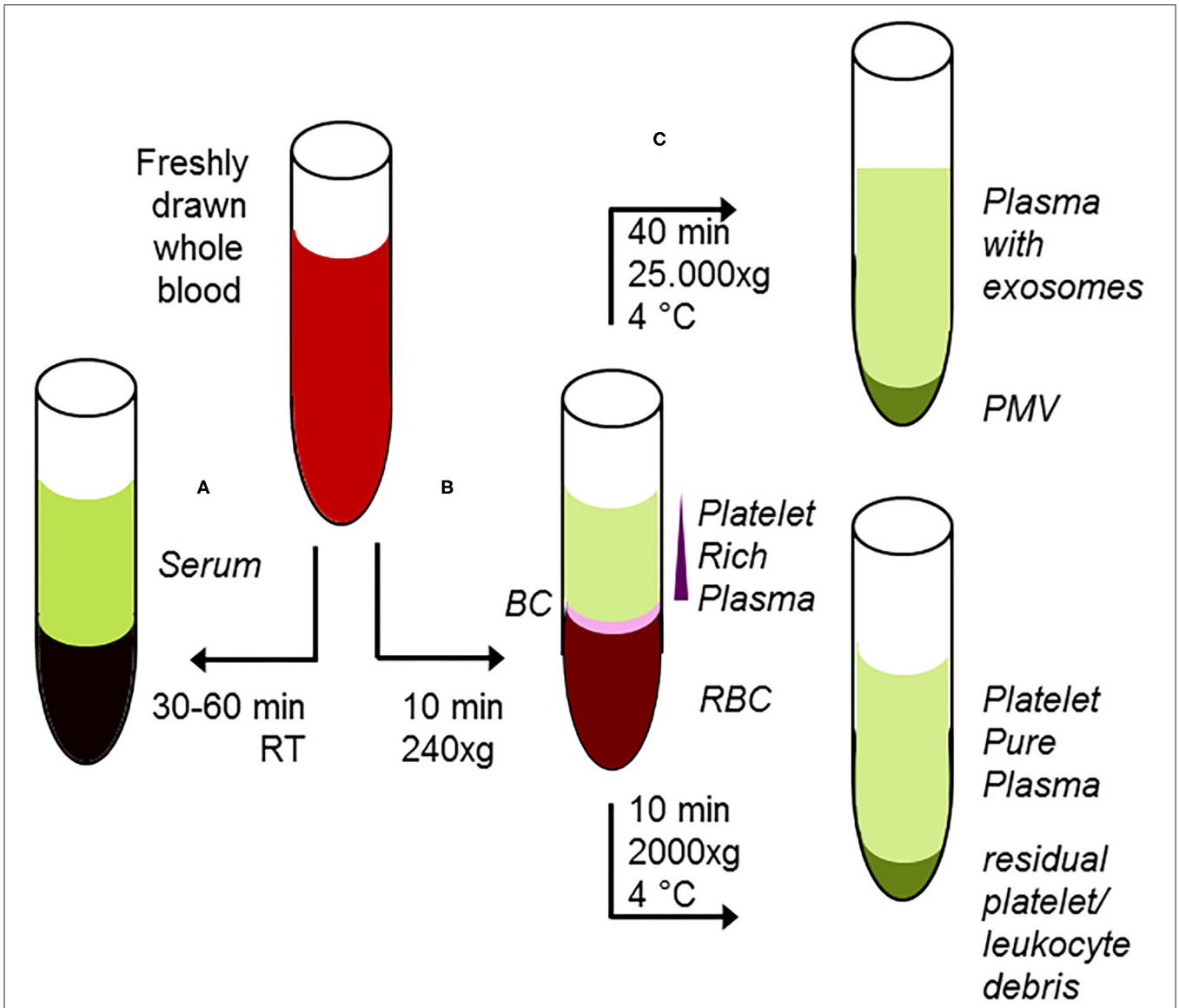


FIGURE 3 | Classical protocol for preanalytical procedures of starting material. **(A)** After adding of clotting enhancers to freshly drawn blood and completion of clotting procedure 30–60 min, room temperature), serum samples are harvested following centrifugation. **(B)** Freshly drawn, anti-coagulated whole blood is briefly centrifuged at low centrifugal forces (softspin, 240 × g, 10 min), resulting in separation of three layers: red blood cells (RBC) at the bottom, a layer termed "buffy coat" (BC) of typically whitish color containing the major proportion of leucocytes, and some platelets in an intermediate layer and acellular, crude platelet-rich plasma fraction (PRP) with varying platelet contents. For further separation, the crude PRP layer is transferred to a fresh tube. After hard-spin centrifugation, most of the supernatant is harvested as the platelet-pure plasma fraction. The final PRP concentrate at the bottom consists of an undetermined fraction of BC (containing a large number of platelets) suspended in some fibrin-rich plasma. The transfer step is often performed with a syringe or pipette, with only visual inspection. Because the manual PPP process is not clearly defined, this protocol might randomly lead to impure plasma fractions. **(C)** From the PRP fraction, platelet-derived microvesicles (PMV) are isolated by centrifugation (25.000 × g/40 min), and finally, plasma-borne exosomes can be obtained from the remaining supernatant. Using the buffy coat fraction for isolation of leukocytes, nucleated cells should be further purified by a second centrifugation step.

A series of studies—mainly carried out with cultured cells or with animal experiments—describe the formation of Cer-enriched lipid rafts as highly dynamic molecular devices for receptor protein reorganization and subsequent signal transduction (50, 51). Ceramides and S1P are also known as reciprocal modulators of cell survival and proliferation (6, 55). The increase of Cer following bacterial infection might be caused by SM breakdown (74). The activation of the enzyme responsible for conversion of SM to Cer (acid sphingomyelinase, SMPD1) is associated with maturation of the phagolysosome and intracellular degradation of pathogens (184) as well as severity of the underlying disease (185) and sensitive to anti-inflammatory therapy (186). Next, conversion of SM to Cer is involved in formation of neutrophil extracellular traps (187) and release of

reactive oxygen species (188, 189). Subsequent degradation of ceramides results in the formation of sphingosine, an SP with remarkable antibacterial activity (81). Phosphorylation thereof is leading to S1P, a molecule with complex functions during infection ranging from cell activation to trafficking and tissue protection to mention a few [excellently reviewed in (83)]. More studies from this compartment are urgently needed for rigorous association and a better understanding of SP-triggered functions of leukocytes in human sepsis.

Data Interpretation and Visualization

Data sets obtained in metabolomic analyses are large and complex; thus, numerous algorithms and a strategy for data quality improvement are required. On the basis of the broad variation in rates of dissociation not only between SP subclasses but also in a particular class, absolute quantitation of SP using only precursor ion scan is not recommended. Here, relative comparison using MRM results and a corresponding internal standard compound for normalization is an appropriate approach (108). After picking the true peak of the lipid of interest by confirmation of the presence of co-eluting qualifier or—much more specific by a precursor ion scan experiment—and determination of the areas of quantifier transitions by automated peak integration, data were normalized with corresponding, class specific internal standard compounds (190). Of note, in-source loss of hexoses of glycosylated ceramide derivatives results in an overlay with unglycosylated derivatives. It might be expected that glycosylated ceramide derivatives point out a shorter retention time with lower signal intensity.

Next, the set of raw data is subjected to filtering with the aims of i/ confirmation of minimal variations in retention time with stepwise increase in parallel with respect to increasing number of carbon atoms in the acyl chain, ii/ exclusion of potential artifacts, and iii/ definition of signal-to-noise (S/N) ratio especially to characterize low abundance SP with poor signals (quality control). Criteria for filtering are correlating with the specific aims and management of the study and should be fixed in advance, e.g., to exclude signals (= lipid species) with a S/N ratio < 10. Outliers should be defined and removed, and missing values can be carefully imputed (191). Quality samples (see above) will help to identify and to correct either drifts of the instrument (retention time, resolution of peaks, sensitivity) and/or batch effects. At the end, at least the implementation of a principal component analysis visualizes similarities or discrepancies of your sample groups in heatmaps including appropriate biostatistical univariate or multivariate approaches.

CONCLUSIONS AND TAKE-HOME MESSAGES

In translational sepsis research, generation and interpretation of data from human blood samples, either from the liquid or particular phase, using the approach of mass spectrometric analyses, opens the field of a better understanding of this complex and convoluted biological system. Whatever all interrelationships are understood in detail, these data will contribute further insights into the dark site of disease-associated signaling. We will be able to oversee a more complete picture of all interconversations of these molecules and biomarkers, even driven by host or pathogen proteins in a tightly regulated and highly dynamic process. Next, the approach will result in more elaborated pathways of the synergistic but also antagonistic properties of these compounds in the issue of life or death of a cell or of an organism. Association of the sphingolipidomic profile to a distinct phenotype will support this concept. Finally, this procedure is without any alternative to discover the action, relationships, and mechanism of potential drugs in a complex disease, especially with respect to a more personalized treatment approach in the future.

- Structural diversity of sphingolipids is the key feature of underlying pleiotropic effects.
- Sphingolipids are ubiquitously distributed biomolecules participating in membrane organization, barrier function, metabolism, and signaling to stress in every known eukaryotic cell type.
- The integration of a targeted metabolomic approach as an information-rich analytical platform can provide important insight into health as well as mechanisms and diagnosis of disease and therapy (theranostic).
- Analyzing the generation and the dynamic of sphingolipids during severe infection and sepsis in different compartments will result in a better understanding of pathogenesis and risk assessment.
- For these aims, targeted and sophisticated experimental settings are critical, starting from sample preparation and storage, analysis, processing up to data interpretation.
- Future efforts are needed for more advanced analysis tools of such datasets.

AUTHOR CONTRIBUTIONS

RC drafted the manuscript. Both authors revised the final version of the manuscript.

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DEDICATION

The manuscript is dedicated to the memory of Ms. Susann Hoffmann (1985 – 2020), a member of our group analyzing sphingolipid composition of plasma proteins in health and disease.

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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.

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Keep Your Friends Close, but Your Enemies Closer: Role of Acid Sphingomyelinase During Infection and Host Response

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Breakdown of the inert and constitutive membrane building block sphingomyelin to the highly active lipid mediator ceramide by extracellularly active acid sphingomyelinase is tightly regulated during stress response and opens the gate for invading pathogens, triggering the immune response, development of remote organ failure, and tissue repair following severe infection. How do one enzyme and one mediator manage all of these affairs? Under physiological conditions, the enzyme is located in the lysosomes and takes part in the noiseless metabolism of sphingolipids, but following stress the protein is secreted into circulation. When secreted, acid sphingomyelinase (ASM) is able to hydrolyze sphingomyelin present at the outer leaflet of membranes to ceramide. Its generation troubles the biophysical context of cellular membranes resulting in functional assembly and reorganization of proteins and receptors, also embedded in highly conserved response mechanisms. As a consequence of cellular signaling, not only induction of cell death but also proliferation, differentiation, and fibrogenesis are affected. Here, we discuss the current state of the art on both the impact and function of the enzyme during host response and damage control. Also, the potential role of lysosomotropic agents as functional inhibitors of this upstream alarming cascade is highlighted.

Keywords: sphingomyelinase (SMase), ceramide (CER), sepsis, organ failure (OF), inhibitor, FIASMA

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INTRODUCTION—WHY IS CONSIDERATION OF ASM IMPORTANT IN THE CONTEXT OF INFECTION AND HOST RESPONSE?

During severe infection and sepsis, a stress-responsive enzyme becomes present in circulation, which is known to be essentially involved in membrane repair, internalization of pathogens, maturation of phagolysosomes, and mitochondrial dysfunction. The function of the circulating enzyme is held responsible for rapid and transient formation of the highly bioactive lipid mediator ceramide from the inert membrane constituent sphingomyelin (SM), which is localized at the outer leaflet of cellular membranes. Under physiological conditions, there is a very consequent segregation of the enzyme from its substrate. But why it is important for a better understanding of the pathophysiology of organ dysfunction development, that there is a resolution of the spatial

separation? Is there an action of the enzyme for a remote signaling to regulate tissue damage apart from the infectious focus? In this review, we summarize the functions of acid sphingomyelinase in order to contribute to the question whether acid sphingomyelinase (ASM) is our friend or foe in the course of sepsis and severe infection, and further discuss the significance of both, an intended and an unattended inhibition for interpretation of results from preclinical and clinical studies with septic patients.

The first clear description of an enzymatic activity cleaving SM into ceramide with an acidic pH optimum was given by Gatt (1). Twenty-five years later, the protein was isolated and purified from human urine and biochemically characterized (2). Surprisingly, the urine obtained from patients with peritonitis was identified as a rich source for purifying human acid sphingomyelinase (3), which was at that time used as a reagent for *in vitro* experimentation of cellular membranes. Tissue, i.e., brain tissue, was also identified as an appropriate starting material useful for purification procedures (4).

ASM IS OUR BUDDY

ASM Profile

The plasma membrane with an asymmetric distribution of phospho- and sphingolipids as well as lateral segregation of SM and cholesterol has—beyond separating cellular compartments—an important function with respect to signal transduction and a plethora of other essential cellular processes (5, 6). The outer leaflet of plasma membranes is enriched in SM, phosphatidylcholines, and glycosphingolipids, whereas in the inner leaflet phosphatidylinositoles, phosphatidylserine, phosphatidylethanolamines, and phosphatidic acid are abundant (5, 7). Metabolism of SM is the entry point in a unique and highly interconnected universe of sphingolipids with a plethora of compounds differing in physicochemical properties and diverse functions regarding cellular signaling and membrane organization (8). The first step—hydrolysis of SM to ceramides and phosphatidylcholine—is catalyzed by the pacemaking enzyme sphingomyelinase, of which so far five different isoforms are known (8, 9). These sphingomyelinases can be distinguished according to primary structure, triggering of activity, cation dependence, and subcellular localization (10, 11). In this review, we focus on acid sphingomyelinase, which is primarily localized in lysosomes (12). Other sources of ceramide formation (*de novo* synthesis, synthesis from sphingosine and fatty acid, as well as hydrolysis of glucosylated or phosphorylated specimen) are playing a minor role during stress response and severe infection (13). A short overview on *in vitro* determination of ASM activity is given in **Box 1**.

Almost 30 years ago (1991), the full sequence of ASM was firstly described (14, 15). The genetic locus was identified on the short arm of chromosome 11 (15), interestingly in close proximity to the locus of other lysosomal proteins such as cathepsin D and acid phosphatase (13). The sequence of ASM is highly conserved among mammals (16) and the ASM locus [systematically sphingomyelin phosphodiesterase 1 (SMPD1)] is undergoing epigenetic regulation by paternal imprinting (17). During protein synthesis, ASM undergoes extensive posttranslational modification. In this process, glycosylation at all of the six potential sites is of major importance to ensure correct folding, sorting, and/or proper stability within the proteolytic milieu of the lysosome (18, 19). By mannose-6-phosphate-receptor shuttling, the protein is transported via the endosomal system to its final destination, the lysosome (20).

Along the primary subcellular localization, the purified protein favors an optimum pH around 5.0, but sphingolytic activity is retained also at neutral pH (21): regarding parameter of enzymatic activity, despite the pH shift up to 7.45, the maximum turnover velocity (v_{max}) remains constant (22, 23). The decrease in affinity to the substrate (K_m) is of no relevance due to the enormous excess of SM at the outer leaflet of cell membranes (24, 25). The persistence of enzymatic activity at neutral pH values is of great importance as discussed later.

In vitro experiments showed us that enzyme activity is also dependent on lipid environment (26) and addition of a detergent in order to overcome dependence from activation by specific proteins (26). Sensitivity to reducing agents such as dithiothreitol is underlining the relevance of disulfide bridges within the mature protein structure for hydrolyzing activity (27, 28).

Resolving the crystal structure of mammalian ASM (with 88% identity to the human protein) confirmed that ASM-mediated hydrolysis of SM is functioning in a canonical mechanism, where phosphoesterases are utilizing a nucleophilic attack of a zinc (Zn^{++})-activated water molecule and protonation of the leaving group for release of phosphocholine and ceramide (29). Two saposin domains are relevant for determination and stabilization of either a closed or open conformation of the enzyme. In the latter one, the enzyme is able to bind and dock to membranes, and extract SM therefrom for subsequent hydrolysis (29). Direct inhibitors of the enzyme (i.e., bisphosphonic acid derivatives) are competing with Zn^{++} binding within the active center (29). A short overview on milestones in translational research regarding ASM activity is given in **Table 1**.

Under physiological conditions, ASM is fulfilling essential house-keeping functions in the lysosomes, whereas genetic deficiency leads to extensive accumulation and deposition of SM resulting in organ abnormalities as described in Niemann-Pick disease (NBD), types A and B (30). The moment of glory has come to ASM as soon as response to external or internal signals of stress is much needed. Since ceramide generation is the common final pathway of most stressors, the panel of ASM stimulators in a large variety of cell types goes far beyond those previously described for any other molecular switch of the SM pathway and are summarized in **Table 2**. Activation of the ASM-sensitive pathway leads to a rapid and transient translocation of the enzyme toward the cell membrane ranging from seconds to hours (60, 61).

In order to highlight the importance of ceramide generation in the course of a severe systemic disease, it is noteworthy that ASM-deficient fibroblasts or mice are resistant to radiation-induced cell death (57). In addition, specific ceramide-binding antibodies rescued mice from lethal radiation gastrointestinal syndrome by preventing signaling platform formation (see below) and inhibition of endothelial cell death (62).

BOX 1 | Determination of ASM activity.

For activity determination, there is a broad range of assays, using either naturally occurring or labeled substrates for *in vitro* measurements up to quenched SM probes: highest structural similarity is given by radiolabelled sphingomyelin (either in the backbone or in acylated fatty acid), when the decrease of SM of formation of ceramide is determined *in vivo* or *in vitro*. This procedure is also suitable for *in-situ* assays without any need for detergent or any other artificial condition [5]. Next, substrates with fluorescently labeled fatty acids in SM were hydrolyzed by ASM, the generated corresponding ceramide derivative is separated using thin-layer chromatography TLC and determined using a CCD camera in a high-throughput format [6]. In addition, SM substrates with radioactively labeled phosphocholine are used following extraction of the water-soluble reaction product [7]. An artificial SM substrate with short chain fatty acids are used with highly specific mass spectrometric analysis of corresponding ceramides resulting in improved sensitivity [8]. From naturally occurring sphingomyelin, another opportunity for determination of the second reaction product (phosphocholine) is oxidation of a pro-fluorescence dye following hydrolysis and oxidation to betaine, where formation of a resorufin analog by released hydrogen peroxide is used as a reporter system. The strength of this assay format is the transfer to a multi-well-format without need of any separation step [9]. A most recent review on strengths and pitfalls of ASM assays is given by Nikolova-Karakashian [10].

Quenched fluorescent SM probes based on FAM/BODIPY dyes are allowing real-time analysis to monitor relative sphingomyelinase activities and ceramide formation of intact, living cells by techniques of flow cytometry. At the end, these probes are acting as a biosensor in a non-invasive manner and in native cellular environment with high spatial and temporal resolution [11, 12]. The specific profile of generated ceramides in affected cells is quantified by methods of mass spectrometry following lipid extraction and chromatographical separation [13].

TABLE 1 | Milestones for ASM in translational sepsis research.

Finding	Year	References
First observation of sphingomyelin hydrolysis at acid conditions	1963	(1)
ASM deficiency as molecular basis of Niemann-Pick disease, types A and B (NPD-A and NPD-B, respectively)	1966	(30)
Purification of ASM from urine (obtained from septic patients)	1987, 1989	(2, 3)
Description of a Zn^{++}-dependent secreted isoform of ASM	1989, 1996	(31, 32)
Sequencing, cloning, and locus mapping of full-length ASM	1991	(14, 15)
Generation of ASM-deficient mice as NPD model	1995	(33)
Association with severity in septic patients	2005	(34)
Phosphorylation of S508 for activation and translocation	2007	(35)
Cationic amphiphilic drugs as functional inhibitors of ASM (FIASMA)	2010	(36)
Crystal structure of mammalian ASM	2016	(29)
Triple combination of FIASMA blocking Ebola virus infection	2017	(37)
Re-evaluation of the puzzle	2019	(38)

Description of milestones, which are mostly relevant for a better understanding of the role of ASM during host response, development of organ failure, and proposed treatment thereof.

TABLE 2 | Stressors triggering ASM activity and translocation (selection).

Stressors and agonists		References
Pathogens	Viral (rhinovirus, Ebola, SinbisV)	(39–42)
	Bacterial (*Neisseria gonorrhea*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*)	(43–49)
	Parasitic (*Cryptosporidum parvum*)	(50)
Endogenous danger signals	Cytokines (IL1-β, TNF-α)	(51, 52)
	Ligation of death receptors (TNF-α, CD95, TRAIL)	(13)
Cytotoxic agents/drugs	PMA, *cis*-platin, paclitaxel, retinoic acid, doxorubicin	(35, 37, 53–55)
(Chemotherapeutics)	Rituximab	(56)
Radiation	UV-C, ionizing radiation	(57)
Oxidative stress	Ischemia/reperfusion injury	(58)
	Generation of reactive oxygen species	(44, 45, 59)

Outline of stressors and harmful events resulting in triggering extracellular ASM activity by translocation and exocytosis of the lysosome with decompartimentalization and amplification of biological effects of ASM at the outer leaflet of cellular membranes (selection).

Maturation of Phagolysosome, Translocation, and Role in Raft Modeling

ASM is essential for proper fusion of late phagosomes with lysosomes, which is crucial for efficient transfer of lysosomal antibacterial hydrolases into phagosomes (63, 64). A significant role of ASM in the phagolysosomal compartment for the defense against infection with intracellular pathogens was shown by a dramatically increased susceptibility to *Listeria monocytogenes*

in ASM-deficient mice (ASMKO). Although ASM-deficient immune cells showed intact production of reactive nitrogen intermediates and oxidative burst, they are completely incapable of restricting and controlling the intracellular growth of *L. monocytogenes in vitro* (65). Similar findings were obtained from mouse peritoneal macrophages infected with the obligate intracellular protozoan, *Leishmania donovani*. Increase of intracellular ceramide was not only a consequence of ASM-triggered activity but also from *de novo* synthesis, which resulted in upregulation of Ca^{++}-independent atypical protein kinase C (PKC)-ζ. Surprisingly, suppression of formation of reactive nitrogen species (i.e., nitric oxide) facilitated the survival of leishmanial parasites in the intramacrophageal milieu (66).

Accumulation of reactive oxygen species (ROS), as a consequence of altered redox status followed by ASM activation, ceramide generation, and subsequent clustering of CD95 in ceramide-enriched lipid rafts is a common and early event in neutrophil apoptosis, which are abundant, and short-lived leukocytes. Their death by apoptosis is central to hemostasis and the resolution of inflammation (67).

Extralysosomal Activity—Circulation in Plasma?

In 1996, Tabas et al. described an additional, plasma-secreted, circulating product of the sphingomyelin phosphodiesterase 1 (*SMPD1*) gene with a similar glycosylation pattern, but increased dependency and susceptibility to Zn^{++} ions (31, 68). Differential protein trafficking was held responsible for the regularly observed activity increase in men, mice, and cell culture experiments upon stimulation, but the underlying mechanisms remain unknown. There is an ongoing debate on the origin of the ASM isoform circulating in plasma, since the mechanisms for a differentiated intracellular trafficking upon stimulation are hard to explain. Phosphorylation of serine residue 508 (S508) by PKC-δ upon stimulation with phorbol ester or UV light was considered to be essential for activation and translocation (35). But to the best of our knowledge, there is no evidence of phosphorylated ASM in disease models or clinical samples.

However, recent studies with the protozoan parasite *Trypanosoma cruzi* revealed that conventional lysosomes are regularly fusing with the plasma membrane in response to increased intracellular Ca^{++} concentration with subsequent triggering of exocytosis (69) and release of the intralysosomal content to the extracellular space, eventually also into circulation. As a result, exocytosed ASM is capable to act at the outer leaflet of the membrane. This mechanism is thought to define the role of extracellular ASM as well as to represent the major source of extracellular form of the protein (38). The findings, that a plethora of harmful, stress/injury-triggering events induced lysosomal exocytosis including interaction with pathogens (59, 70, 71) offers a plausible perspective of the possible origin of extracellular and circulating ASM activity without any need for postulating differential trafficking (38). An overview on ASM release as a feature of stress response is given in **Figure 1**.

Exposition of immune cells to ROS resulted also in a rapid and transient, Ca^{++}-dependent translocation of ASM to the outer leaflet of cellular membranes—mediated by exocytosis of lysosomes—and formation of ceramide-enriched platforms (59). On the other hand, these microdomains are required for *Pseudomonas aeruginosa*-induced activation of NADPH oxidase and production of ROS, demonstrating a positive feedback mechanism for amplification of ASM-mediated redox signaling (43).

On a molecular level, the generation of ceramide is an integral part of intrinsic repair mechanisms following perforation of the plasmalemma by pathogenic pore-forming toxins, whereby lysosomes fuse with the plasma membrane. Hereby, lysosomes externalize their contents including acid sphingomyelinase and facilitate exposure to the abundant SM at the outer membrane bilayer (70, 72). Subsequent formation of ceramide-enriched, topically restricted domains in the outer leaflet of the lipid bilayer is an essential step contributing to tighter packing of the membrane, forwarding a negative curvature and inward vesiculation of the damaged area. At the end, the resulting internal degradation contributes to cellular integrity and survival (72, 73), e.g., after exposition of cells toward listeriolysin O or pneumolysin (74).

Microdomain Formation, Protein Organization, and Ceramide Signaling

In cultured immune cells, exposure to endotoxin led to activation of ASM, generation of ceramide, phosphorylation of PKC-ζ, assembly of Toll-like receptor 4 (TLR4) within lipid rafts, activation of the stress-responsive kinases, and release of tumor necrosis factor-α (TNF-α). These pathogenetic mechanisms could be abrogated by CD14 blockade or inhibition of ASM but reversed by treatment with the central effector molecule ceramide (51). In addition to other proteins associated with the lipid raft, ASM is contributing to TLR4 signaling triggered by endotoxin and non-microbial endogenous ligands (75) (**Figure 1**).

Moreover, ASM is involved in most effective host membrane remodeling during enteropathogenic infection with *Shigella* spp. resulting in decreased binding of pathogens to epithelial cells and therefore, impeding abovementioned or other pathogens from reinfection, which was proposed as a novel stress-responsive cell-autonomous defense mechanism (76).

In concert with the endogenous danger signal adenosine triphosphate (ATP), ASM is involved in secretion of membrane TNF-α within microvesicles bypassing conventional pathways. These cytokine-carrying microvesicles are biologically more potent than soluble TNF-α *in vivo*, evolving significant lung inflammation in mice, which might have crucial implications for the biological activity of this prototypically proinflammatory cytokine (77).

Ceramide as Second Messenger

Beyond the membrane reorganizing capacities (78), ceramides also act as second messengers transducing cellular signals (79). These functions widely differ among the diverse cell types, ranging from induction of senescence to apoptosis. As an example, the role of ceramide generation and the function of generated ceramides in mitochondria were extensively studied (80): organelle-specific accumulation of ceramide is

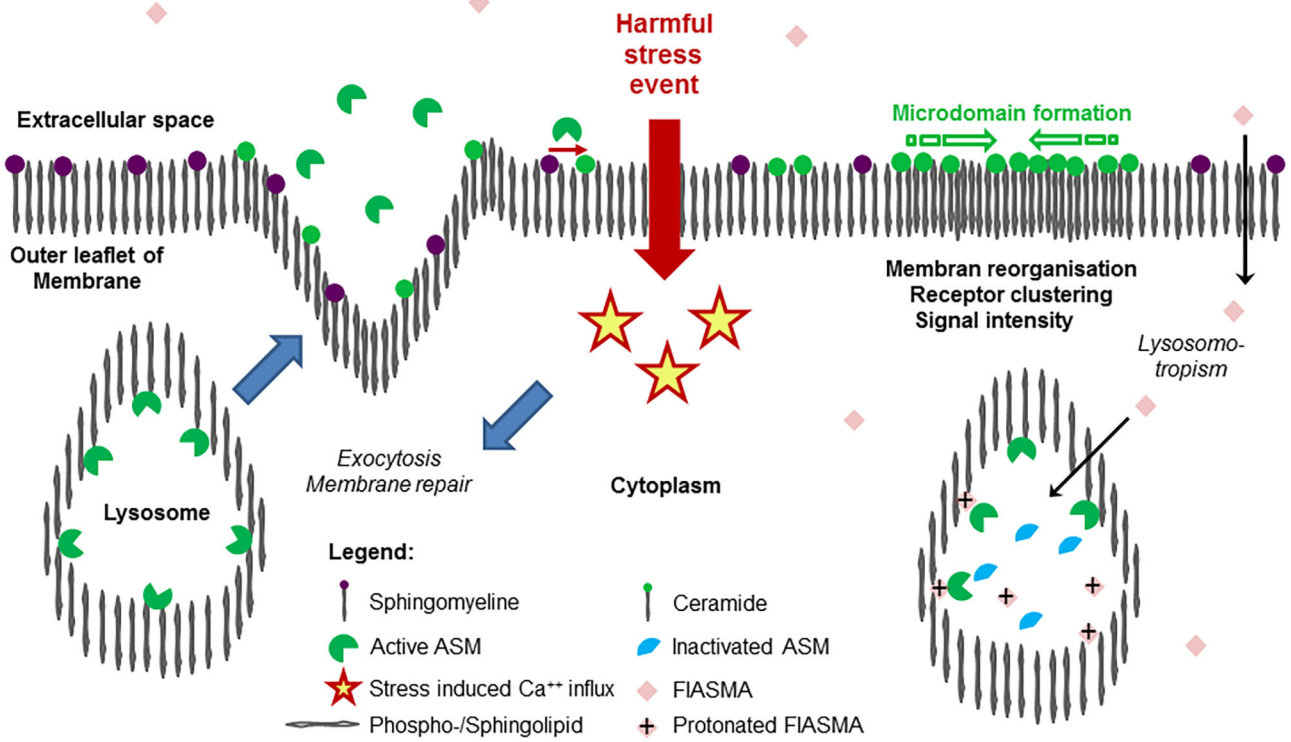


FIGURE 1 | Release of acid sphingomyelinase (ASM) in the course of stress response and mode of action of its inhibition. Exposition of a cell membrane to external harmful stress events such as endogenous or exogenous stimuli (bacterial endotoxin, pro-inflammatory cytokines, pore-forming toxins, **Table 2**) is followed by intracellular Ca^{++} influx, triggering the exocytosis of lysosomes, where ASM was bound to the inner lysosomal membrane by SAP domain. In the extracellular space, ASM mediates hydrolysis of SM (abundantly embedded into the outer leaflet of the membrane), generates ceramide, facilitating remodeling, and repair of the membrane (restoring integrity), as well as pathogen entry. As a hallmark of ceramide-induced signal transduction, due to the trend for self-aggregation and formation of ceramide-enriched microdomains, subunits of receptor proteins are reorganized to functionally active receptor complexes such as TLR4, TNFR, etc. As a result, an increase in signaling quality and intensity is observed, controlling an adequate cellular response to external harmful stimuli. On the right panel, the mode of action of cationic amphiphilic substances is illustrated: in an uncharged form at physiological pH value, the compounds diffuse across the cellular membrane through the cytoplasma into the lysosome, where the weak basic nitrogen atom of the compound is protonated because of the acid pH value of the lysosome. The protonated compound interacts with the sapsonin domain of ASM, detaching them from the membrane, then undergoing proteolytic inactivation. Following stimulation and release, the inactivated ASM is unable to contribute to ceramide generation at the outer leaflet of the membrane. Due to similar physicochemical properties, a long list of cationic amphiphilic substances with a broad range of clinical indications, but most of them used in daily care as antidepressive drugs, are found to function as functional inhibitors of acid sphingomyelinase (FIASMA) and to effectively control stress-induced ceramide generation. Most of the available FIASMAs are licensed for medical use in humans, are minimally toxic, and may therefore be applied for disease states associated with increased activity of ASM.

critically involved in progression of mitochondrial apoptosis and mitophagy, defining ceramides as a *bona fide* transducer of mitochondrial (dys-)function [excellently reviewed in (80) and references cited therein].

In the next paragraphs, consequences of ASM depletion in the course of local or systemic infections with the presentation of interesting phenotypes will be discussed. In 1995, the generation of ASM-deficient mice was of great value for studying the pathogenesis and treatment not only of type A/B-Niemann-Pick disease (see below) but also for investigations into the role of ASM in ceramide signaling, induction of apoptosis, and ceramide-induced tissue damage (33).

Hyperresponsiveness

Mice, genetically lacking ASM (33), showed an exaggerated response to polymicrobial sepsis with an increased bacterial burden, an enhanced phagocytotic activity, a more pronounced cytokine storm and decreased survival rate (81). Moreover, on a functional level, leukocyte-endothelial interaction was found diminished in ASMKO animals corresponding to a distinct leukocytes' phenotype with respect to rolling and sticking as well as expression of cellular surface proteins (81). A similar phenotype was found in cultivated lung epithelial cells with controlled ASM activity, where increased neutrophil recruitment, elevated levels of cytokine mRNA, and a pro-oxidative shift could be observed (82).

Moreover, the availability of ASMKO mice provides the opportunity of a better understanding of ceramide generation for immune response on a molecular level. In a fundamental experimental setting, injection of endotoxin and its putative effector TNF-α, into mice induced disseminated apoptosis in endothelium of intestine, lung, fat tissue, and thymus accompanied with cytokine release and ceramide increase. This endothelial cell death was ASM dependent, since ASMKO

mice were protected against endothelial apoptosis and animal death (83).

In mice and men, complete loss of function of ASM resulted in a diminished CD1d-restricted antigen presentation of invariant natural killer cells (iNKT), decreased levels of this cell population and resistance to iNKT cell-mediated inflammatory conditions, supporting the concept of a tight link between cellular sphingolipid metabolism and immunity (84).

Pneumonia in ASM-Deficient Patients

Niemann-Pick disease (types A and B) is an autosomal recessive lysosomal storage disorder caused by biallelic mutations in the *SMPD1* gene resulting in a severe human disease state characterized by deficient ASM activity (85). A recent study reported around 185 mutations with ASM-deficient NPD worldwide (86), while the detection of new mutations is not yet completed (87). The majority of mutations are predicted to affect proper folding, stability, as well as membrane binding of the enzyme (29). Type B patients are characterized by hepatosplenomegaly and progressive alterations of the respiratory system, but the central nervous system is usually less affected, which is more profound in type A, resulting in early death (∼2 years of age) (85). Lung involvement is the most important prognostic factor in NPD-B, with recurrent respiratory infections starting in infancy being the major cause of morbidity and mortality (82, 88, 89). In lung epithelial cells, decreasing ASM activity by 50% leads to an increased neutrophil recruitment via elevated levels of cytokine expression, both at baseline and in response to bacterial stimulation. Instead of preventing the host defense responses, decreased ASM activity results in an inflammatory response even in the absence of infection supporting the hypothesis of a chronic inflammatory state impairing host defense mechanisms (82). Despite the low incidence of progressive pulmonary disease in a cohort of more than 100 patients diagnosed with NPD-B, pneumonia was the leading cause of death of juvenile patients (<21 years) (90, 91). On the one hand, pathophysiology of the pulmonary disease is presumably related to the accumulation of SM in alveolar macrophages, on the other hand, inflammation, abnormal surfactant catabolism, as well as composition contributing to lung abnormalities was shown in ASMKO mice (92, 93). In parallel, endogenous lipid pneumonia, interstitial fibrosis, and accumulation of foamy macrophages were found in human lung biopsies (94) supporting the hypothesis that the lung is a primarily affected organ of NPD-B contributing to morbidity and mortality (90, 95).

In a brief summary of this section, ASM is a highly conserved stress-responsive enzyme, activity of which is triggered by a variety of harmful events including infection and inflammation. As a key consequence of activation, there is a disorganization of the previously strict segregation of the enzyme (located in the lysosome) and the corresponding substrate (SM in the outer leaflet of cellular membranes). Besides switching the current pH status by two orders of magnitudes, the enzyme is capable of rapid and transient ceramide formation upon activation. The resulting self-aggregation of ceramide molecules is reorganizing cellular membranes. Now it is time to put this critical event of signal amplification as a general mechanism into the scene of stress response, membrane repair, oxidative stress, proper maturation of phagolysosomes, and regulation of host defense against invading pathogens. As our buddy—keeping us well and fine by its integration in imperative actions of life—the enzyme and its regulation of translocation and activity received also value for further elucidation with respect to pathophysiology and pathogenesis of a series of diseases.

ASM IS A DUBIOUS FRIEND

Invasion of Pathogens

Non-human sphingomyelinases are essential factors for virulence of extracellular, facultative, or obligate intracellular pathogens. These enzymes contribute to phagosomal escape or phagosomal maturation avoidance and even immune response evasion (96). Whether the activity profile of these prokaryotic proteins contribute to the analyzed hydrolysis rate of SM to ceramide in conventional assays in samples obtained from patients with blood stream infection (**Box 1**) is still discussed controversially. Some prominent and representative mechanistic approaches are shown here in an exemplary manner.

Infections by *Staphylococcus aureus* are a major clinical problem ranging from mild infections (skin and soft-tissue) to severe and even lethal infections (e.g., pneumonia, endocarditis, sepsis, osteomyelitis, etc.). *S. aureus* stimulates ASM via CD44-triggered release of ROS, resulting in ceramide release, clustering of CD44 in ceramide-enriched membrane platforms, co-activation of GTPases, and translocation of linker proteins with subsequent rapid rearrangement of the cytoskeleton. In the absence of either CD44 or ASM, reduced internalization of macrophages is counteracted by a reduced killing capacity (44).

A major toxin of *S. aureus* (α-toxin) caused in bone marrow-derived macrophages ASM-dependent ceramide formation, release of cathepsin B and D from lysosomes, inflammasome activation, and induction of pro-inflammatory cytokines, which could be abrogated by pharmacological ASM-inhibition (45). In *in vitro* experiments with endothelial cells, ASM activation by α-toxin was linked to degradation of tight junctions, which could be blocked by pharmacological inhibition. However, most importantly, in *in vivo* experiments, genetic deficiency prevented severe degradation of tight junctions in the lung and edema formation (46). Combination of antibiotic treatment and ASM inhibition are proposed to exhibit synergistic and super-additive effects (47).

Inflammasomes are important for host defense against invading pathogens; ASM activation is critically involved in the activation of endothelial inflammasomes including recruitment of adapter proteins and caspases (97–99) and subsequent oxidative signaling by lipid raft-associated redox platforms, release of cytokines, activation of stress kinases, and altering tight junctions in epithelial cells (45).

For exposition of proteins, non-enveloped viruses undergo partial uncoating in order to get access to the cytoplasm by

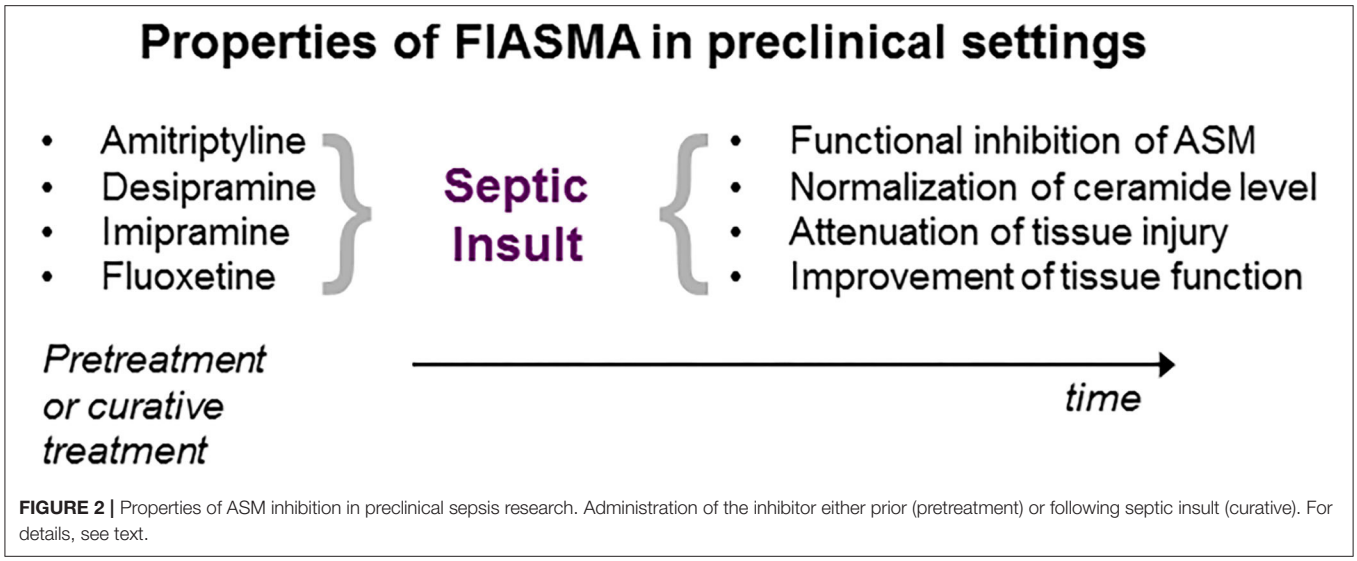


FIGURE 2 | Properties of ASM inhibition in preclinical sepsis research. Administration of the inhibitor either prior (pretreatment) or following septic insult (curative). For details, see text.

membrane lysis. Cellular ASM is a critical player in this process, since ASM is induced and hijacked by mimicking wound removal processes facilitating the adenovirus further membrane disruption and infection. In addition, stimulation of Ca^{++} influx and lysosomal exocytosis are key steps for efficient membrane penetration of the virus (71). Most recently, influenza A virus infection was found to be associated with suppression of ASM activity in cultured alveolar epithelial cells, whereby depletion of SM content either in the epithelial membrane or in the virus envelope impaired virus infection and reduced virus entry as well as reduced virus infectivity and impaired its attachment and internalization, respectively. Inhibition of ASM by desipramine did not affect influenza virus infection (100), which is in line with the observation that ceramide generated by *de novo* synthesis might play an antiviral role (101).

For meningococcal internalization into brain endothelial cells, transient ASM activation and ceramide release are also determinative factors for invasiveness among a defined set of pathogenic isolates of *Neisseria meningitidis* (102).

In the pathogenesis of cystic fibrosis, abrogation of ceramide accumulation by inhaled functional inhibitor of ASM (FIASMA, see below) restored normal ceramide concentrations in murine bronchial epithelial cells, reduced age-dependent pulmonary inflammation and deposits of DNA in bronchi, as well as prevented infection with *P. aeruginosa* (103, 104). ASM activation (by endotoxin) and subsequent ceramide formation play a pivotal role in *Escherichia coli*-induced apoptosis of immature dendritic cells, a phenomenon contributing to sepsis-induced immunosuppression (105). In liver specimen, inhibition of activity in endotoxin challenged mice correlated with a reduced rate of hepatocellular apoptosis (34).

It is also known that ceramide-containing microparticles in packed red blood cells contribute to adverse effects following transfusion, which was abrogated by treatment with a FIASMA during storage time (106).

ASM activation is a critical factor for redirection of TNF-α trafficking, thus the cytokine is embedded in microvesicles bypassing conventional pathways in a highly potent manner. Rerouting has crucial implications for the activity profile of the pacemaker cytokine of inflammation, inducing a significant TNF-dependent inflammation status and allowing long-range TNF signaling to target cells more effectively than soluble TNF, which is of particular interest therapeutically targeting TNF in acute inflammatory diseases (77).

Marker and Mediator in Sepsis and Pneumonia

Endotoxin challenge in mice resulted in a mild, 2-fold increase of ASM plasma activity, which was accompanied by release of cytokines (107). These data were confirmed by a similar observation of an increase of ceramide content in lipoproteins paralleled by an increased activity of circulating ASM (108).

Intratracheal administration of an ASM inhibitor in a model of acute lung injury improved lung function and decreased pulmonary inflammation (109). In critically ill preterm infants with acute lung injury, beyond other markers and mediators, ASM activity was found to be increased in tracheal aspirates (110). Inhibition of NF-κB function attenuated pulmonary inflammation of acute respiratory distress syndrome in a neonatal piglet model with decreased leukocyte concentrations in bronchoalveolar lavage, reduced ASM activity, and subsequently decreased ceramide levels (111). This emphasizes the pivotal role of sphingolipid signaling controlling pulmonary edema formation and lung function.

In patients with community-acquired pneumonia (CAP), a plasma decrease of SM was found to be closely associated with an increase in ceramides (16:0, 18:0, 24:1), but normalized toward clinical remission (112). Furthermore, the sphingolytic activity of plasma-secreted ASM was nearly 3-fold increased

with same tendency for normalization. A similar course was also observed with respect to gene expression rate of SMPD1 in circulating leukocytes, supporting the concept of a disease-relevant regulation of ASM expression in CAP at both, protein and mRNA level. These observations might qualify ASM as a potential target for host-directed treatments to reduce end-organ damage in pneumonia (112).

Endothelial Integrity

Cultivated endothelial cells are affected by serum obtained from patients with sepsis, resulting in clustering of receptors relevant for signal transduction and suppression of a stress-sensitive transcript marker of these cells (i.e., ADAMTS13), which is abrogated by direct and indirect inhibition of ASM activity (113). Both, plasma activity and amount of ASM were found to be increased in septic patients dependent on clinical severity (34, 113). Thus, ASM is involved in the dysregulation of ceramide metabolism in endothelial cells leading to macrodomain formation, cytotoxicity, and downregulation of ADAMTS13 expression (113), which is held to function as an adverse effect to endothelial dysfunction and microthrombus formation in sepsis (114). These results obtained in *in vitro* experimentation were recently confirmed in a monocentric clinical study, where a plasma decrease of the endothelium-stabilizing mediator sphingosine-1-phosphate (S1P) was strongly negatively associated with an increase in ceramide levels (115). Moreover, the association with severity of clinical course could be outlined by a highly powerful value of an integrative analysis of the S1P/Cer ratio for prediction of unfavorable outcome, superior to established severity scores (SOFA) and HLA-DR expression on circulating monocytes (115).

Role of Alternative Splicing and SNP in Sepsis and Depression

Beside predominant localization in lysosomes especially of monocytic, hepatic, and endothelial cells, and delocalization of the protein to the outer leaflet of cellular membranes in response to multiple stimuli including pathogens (116), encoding $mRNA_{SMPD1}$ is undergoing alternative splicing as an independent matter of stress response. A series of *SMPD1* mRNA splice isoforms are described to date, but only ASM-1 as the full-length transcript, Ref_mRNA NM_000543 has been shown to be translated into a catalytically active protein (14, 117, 118). It might be speculated, that the extensive variability is a marker of negative regulation with highest variability in healthy controls but decreasing in a stress condition such as severe infection by skipping of exon 3 and/or elongation of exon 2 (119). Thus, an association of specific pattern of alternatively spliced *SMPD1* transcripts with disease severity (healthy and non-infectious patients undergoing intensive care as controls vs. sepsis) was observed (119). At the end, alternative splicing of *SMPD1* might act in a dominant negative manner upon overexpression making alternative splicing a promising target in regulation of ASM activity (118, 119). Interestingly, a similar phenomenon was observed in patients with major depression, a disease, where despite clear associations of ASM activity with the severity of the disease (120) the regulatory mechanisms and cause-effect links are not well-understood (121). Strikingly, alternative splicing of SMPD1 was also found to be reduced in depressed patients, and most interestingly, normalized upon subchronical treatment with FIASMA in both, patients and healthy volunteers (118, 122).

There are a small number of reports on polymorphisms of the *SMPD1* gene locus: V36A, A487V, and G508R as well as hexanucleotide repeat in the signaling peptide (123–127). In contrast to the variety of missense mutations, there is only a minor effect of these sequence variations on overall activity and function of the enzyme, but they might increase the susceptibility for common diseases such as allergy (125). The G508A transition—exchange of an uncharged with a charged amino acid—is discussed to be significant for recognition of a potential phosphorylation site, which is relevant for the control of protein activation and secretion (35, 52). In line with this hypothesis, G508A is associated with plasma-secreted activity in a gene-dosage-dependent manner (125), since subjects homozygous for the minor A allele displayed half of the plasma activity compared with the major G allele. Whether this observation on a molecular level might contribute to control the susceptibility to infections is still a matter of debate, but as discussed below, a decreased ASM activity is associated with lower respiratory tract infections (90, 91).

In brief, ASM is also held responsible for internalization of pathogens (bacteria and viruses). In acute and chronic infection, inhibition of ASM improved outcome in (pre-)clinical studies in patients with cystic fibrosis and CAP. ASM inhibition also diminished inflammation-responsive fibrogenesis following sepsis in a preclinical study. A multilevel regulation with association to favorable outcome was observed in patients undergoing CAP. Due to the facts that (i) the impact of single nucleotide polymorphisms and alternative splicing of ASM with respect to susceptibility and progression of infection is not fully understood and (ii) pharmacological inhibition of the enzyme resulted in improved outcome from chronic infection, the role of ASM should not be evaluated as exclusively favorable without any restriction. Our friend is becoming dubious and needs more critical consideration.

BUT ASM IS ALSO WORRYING

In a series of clinical and preclinical studies, ASM activity was positively associated with the severity of the disease. In a small, mixed population from an intensive care unit (ICU), ASM activity was found to be increased and to remain elevated in the presence of a low level of procalcitonin-discriminating non-surviving patients after systemic inflammation. Also, a posttrauma effect with a significant increase in ASM after surgery parallel to a postoperative increase of procalcitonin and C-reactive protein was observed (128). Strikingly, patients with severe sepsis exhibit an enhanced, 2-fold sphingolytic activity in comparison with controls. A further increase was associated with greater risk by the severity of illness and with fatal outcome (34). Supporting the hypothesis that ASM activity is highly correlated to inflammation, we also found ASM activity mildly increased in patients with rheumatoid arthritis, which

was associated with oxidant activity, markers of inflammation, and endothelial activation (129). Interestingly, treatment of these patients by biologicals with TNF-α-binding capacity suppressed the increase completely to levels as found in healthy controls along with improvement of the clinical condition (130). These data support the concept that activation of circulating ASM may play a critical role in the development of apoptosis and organ failure in inflammation-associated disease, especially sepsis. An inhibition of ASM should be explored further as a potential target in the complicated puzzle of sepsis. At sepsis diagnosis, leukocyte-associated ceramide content as a result of ASM activity was significantly elevated and correlated to TNF-α concentration as both a marker of cytokine release as well as mediator of ASM activation (131). Ceramide concentration was highly predictive for risk of development of organ failure (131).

In hemophagocytic lymphohistiocytosis (HLH), a rare systemic inflammatory syndrome resulting from unrestrained immune cell activation, levels of circulating ASM were found elevated. Also, there was a shift in ceramide and sphingosine ratio (increase), while levels of S1P were decreased (132). Interestingly, an elevated ratio between ceramide and sphingosine was predictive for unfavorable outcome (132). Data were confirmed in a small cohort with a 10–20-fold increase of ASM activity with a trend to normalization during recovery (133).

As an indirect measure for ASM-induced unfavorable outcome, fluoxetine and desipramine reduced in an lipopolysaccharide (LPS)-induced model of septic shock the levels of circulating TNF-α similar to prednisolone, accompanied with an improved outcome to untreated controls (134). In murine models of peritonitis and endotoxemia following LPS challenge, amitriptyline-treated mice were protected from overwhelming cytokine release (KC, MCP-1) and from pulmonary edema as well as exhibiting improved survival. Anti-inflammatory effect of amitriptyline treatment is reflected by increased IL-10 levels and decreased accumulation of immune cells at the site of infection (135).

Eryptosis

In patients with hyperbilirubinemia, as often observed in the acute phase of severe infection due to sepsis-induced cholestasis and also a long-term consequence of survivors (136, 137), high levels of conjugated bilirubin may also lead to progressive sclerosing cholangitis (138) and are able to stimulate suicidal death of human erythrocytes. As an underlying mechanism, bilirubin triggered rapid Ca^{++} influx resulting in the release of ASM, formation of ceramide, and subsequent translocation of phosphatidylserine to the erythrocyte surface (139), a phenomenon, which is also compatible with the anemia status of these patients exerting an increased mortality rate (140).

Platelets

Ceramide accumulation over time, generated by ASM in stored and aged platelets, caused lung injury in endotoxin-challenged mice, examined by neutrophil accumulation, endothelial barrier dysfunction, and histological evidence of tissue injury (141). Interestingly, this adverse effect of pulmonary complications following transfusion could be overcome by ASM inhibition (141).

Mechanisms and Effects of ASM Inhibition

A broad panel of cationic amphiphilic compounds is known to inhibit the activity of ASM by lysosomotropism due to their unique physicochemical properties, which was firstly observed by Albouz et al. (142). Interaction with the membrane-embedding *N*-terminal saposin domain of ASM caused detachment from the inner lysosomal membrane and a consecutive proteolysis of the enzyme (143, 144). As a result, there is a significant decrease in sphingolytic activity, therefore these compounds are termed as FIASMA (36). The compounds differ markedly in molecular structure. A prediction of inhibitory capacity is available by a structure-property-activity relation (SPAR) model in order to specify the structural and physicochemical characteristics including variables referring to pK_a, logP, as well as a factor depicting the steric hindrance of the most basic nitrogen atom of the compound modulating the free presentation of a protonated nitrogen atom at the inner lysosomal surface (145). It is noteworthy that these compounds are licensed for medical use in humans, are minimally toxic, and in use for a broad range of clinical indications, including the treatment of intensive care patients. There is a long list of FIASMA of ASM including amitriptyline, imipramine, desipramine, doxepine, fluoxetine, maprotiline, nortriptyline, paroxetine, sertraline, suloctidil, terfenadine, and famotidine, to mention a few (145) (**Figure 1**).

Furthermore, evaluating lysosomotropic and ASM-inhibiting activities in appropriate cell culture models are an applicable approach of newly designed substances to identify novel compounds with anti-apoptotic and anti-inflammatory capacity, which results ultimately in a decreased response of prototypic inflammatory mediators. At the end, in new candidate drugs, based on established SPAR models, physicochemical and biological properties will be selected and identified to enrich the pool of compounds, which might be beneficial for the treatment of the adverse effects of ASM upon activation (146).

In the next paragraph, there is a short overview on the proposed beneficial effects of ASM inhibition in a series of conditions associated with development of organ failure and long-term effects of sepsis sequelae, classified by the affected tissue.

Muscles, Diaphragm—Heart, and Cardiovascular System

Cardiac dysfunction, in particular of the left ventricle, is a common and early event in sepsis and is strongly associated with an increase in patients' mortality. Surrogates of cardiomyopathy cardiac function, ceramide formation, markers of oxidative stress, as well as troponin I levels were found to be improved in FIASMA-treated animals in a semi-lethal peritonitis model (147). Interestingly, in this study, an activation of *de novo* synthesis of ceramides could be identified to be responsible for cardiac ceramide increase (147). In an *in vitro* and *ex vivo* experimental setting, Ferreira et al. showed that mouse myotubes and diaphragm muscle fiber bundles are sensitive to ASM

treatment mediated by release of mitochondrial ROS, resulting in significant depression of diaphragm force and accelerated fatigue in a time and concentration manner (148, 149). Also, the p47(phox) subunit of NADPH oxidase is held responsible to play an important role on oxidant-mediated diaphragm weakness triggered by ASM (150). Similar results could be obtained in a chronic malfunction of skeletal muscles and dysregulation of sphingolipid turnover in insulin-responsive tissues at old age. In parallel with a progressive increase of ceramide content and Cer/SM ratio during aging of rats, there is a decrease of insulin responsiveness, which can be overcome by ASM inhibition (151). Interestingly, in muscle tissue, the counteracting activity of sphingosine-1-phosphate is once more evident, promoting cell survival, Ca^{++} mobilization, fiber growth and repair, as well as fatigue resistance [excellently reviewed in (152)].

ASMKO mice are protected from TNF-α-induced hypotension and tachycardia (153), which might be regarded as clinical symptoms of systemic inflammation (154). One hypothesis for this surprising observation might be a ceramide-triggered activation of NOS, resulting in vasodilation and hypovolemic shock (153), which was in a similar manner also observed for ceramide generation by the neutral isoform of sphingomyelinase (155). Therefore, pharmacological inhibition of ASM-triggered ceramide generation and presumed prevention of shock should be considered to contribute to the overall improved survival (153) in the acute phase as observed in a series of peritonitis models in mice (147, 156).

Lung

Pulmonary outcome following endotoxin challenge in mice was improved by ASM inhibition, resulting in attenuating alveolar collapse (157). Inhibition of ASM was identified as a possible target in acute lung injury and pulmonary edema, induced by administration of platelet-activating factor and endotoxin (158). Fluoxetine and desipramine reduced in ovalbumin-sensitized rats the number of migrated immune cells into bronchalveolar lavage fluid but did not exert anti-inflammatory activity by attenuation of bronchial hyperreactivity (134).

Liver Tissue—Long-Term Effects on Tissue Function and Fibrosis Following Sepsis

Long-term sepsis survivors might develop hepatocellular/hepatobiliary injury and fibrosis (159–162). ASM, also an important regulator of hepatocyte apoptosis and hepatic stellate cell (HSC) activation (163), is linked to the promotion of liver dysfunction in the acute phase as well as to fibrogenesis in the long term. In both, the acute and the postacute phase, pharmacological inhibition of ASM displayed a beneficial effect on oxidative stress levels, hepatobiliary function, macrophage infiltration, hepatic stellate activation, and overall survival (156). ASM inhibition exhibited a protective effect on liver function in the acute phase, and the reduction of HSC activation diminished development of sepsis-associated liver fibrosis in the postacute phase of sepsis (156). In this context, dysregulation of hepatic biotransformation capacity, especially of the cytochrome P450 (CYP) system, represents an important distress factor during host response (164–166). Thus, these enzymes are estimated to be responsible for metabolizing >75% of drugs which are in daily clinical use. Pharmacological inhibition of ASM has an important impact on expression and activity of different hepatic CYP enzymes using an animal model of polymicrobial sepsis in the acute as well as in the postsepsis phase (167).

In murine models of peritonitis and endotoxemia following LPS challenge, amitriptyline-treated mice were protected from overwhelming cytokine release (KC, MCP-1) and from pulmonary edema as well as exhibited improved survival. Anti-inflammatory effect of amitriptyline treatment is reflected by increased IL-10 levels and decreased accumulation of immune cells at the site of infection (135).

In all studies enrolling patients with severe infection, sepsis and multiple organ failure as well as in all reports modeling the disease continuum *ex vivo* or *in vivo*, we found a clear association of ASM activity with morbidity and unfavorable outcome. These observations are independent from the underlying disease such as peritonitis, pneumonia, and HHL. On the other hand, inhibition of ASM activity is capable to prevent destructive events of overwhelming immune activation in all affected tissues and circulating cells, be they nucleated or not. Here, we come to the point for further consideration of the presumed beneficial effects of ASM inhibition in daily clinical care, because a broad panel of drugs is exhibiting ASM inhibition.

CONCLUSIONS AND FURTHER PERSPECTIVES

The expenditure for locally restricted or disseminated ceramide generation by ASM for effective host response is remarkable. The multifaceted roles of ceramide in this context, specific function in individual tissues and organs, the flux and interconnections of lipid mediators with agonistic functions requires further investigation and redefining. Nonetheless, there is conclusive preclinical evidence that the conserved stress enzyme ASM plays an essential role in the pathogenesis of sepsis/host response and that its inhibition might improve the outcome. However, the gap between preclinical and clinical trials has not been convincingly closed so far. Therefore, we suggest as a first step that a retrospective analysis of sepsis patients in the intensive care unit, who are coincidentally treated with FIASMAs, should be initiated to overcome the missing link from bench to bedside. Our knowledge gained from preclinical experiments indicates that FIASMAs might be promising candidates for future pharmacological studies targeting ASM in sepsis and host response keeping in mind that these drugs are already FDA approved and just need to be repurposed.

FIASMA—From Unattended ASM Inhibition at ICU to an Intended Use

FIASMA are a class of drugs, which are widely used for (often chronic) treatment of a number of symptoms such as major depression, neuropathic pain, fibromyalgia, etc., to mention a few (168). From that list, major depression was recently ranked as the third leading burden of non-fatal diseases worldwide

with a prevalence of >10% in all analyzed regions and with an increasing incidence to be first during the next decade (169, 170). In the *Global Burden of Disease* survey for 2017, more than 264 million cases were reported worldwide (169).

In Germany, the 12-month prevalence of major depression is 12% in adults, of whom the majority is treated with antidepressant drugs (171).

In cultured PBMC from healthy volunteers treated either with imipramine or amitriptyline in a therapeutical dosage, activity of ASM is rapidly reduced to levels around 30% of original activity over a period of 3 weeks. Interestingly, removal of the drugs resulted in a gradual and prolonged, but complete normalization of activity over a period of 5–6 days (172). Considering this scenario combined with the facts that (i) daily use medication is often or regularly spared on ICU admission and (ii) that drugs initially used at the newly admitted ICU patient might also exert an inhibitory capacity on ASM, it seems quite possible that the septic patients at least in the early days following admission might be subdivided in separate groups: one with decreased ASM activity due to antecedent treatment with FIASMA due to other indication and gradual normalization of ASM activity, one with inhibition of ASM activity only just at ICU submission, and one without any interference with ASM activity. In addition, it might be further speculated that the presented ASM activity in circulation is also superimposed by the conserved mechanisms of stress response as discussed in detail previously.

This scenario in mind, there are some principal opportunities, risks, as well as consequences examining host response and its unintended, but presumptive treatment by FIASMA.

1. For a more precise interpretation of results from clinical sepsis studies, the definition of the as-yet unknown biasing factor of ASM inhibition by consideration of both ASM activity and inhibition by FIASMA due to other indication prior to hospitalization is needed (anamnesis, documentation of prehospital medication, identification of FIASMA therein). In sepsis trials, depression at least (and treatment thereof) should be defined as a significant comorbidity.
2. The incidence of sepsis is determined including clinical parameters of organ dysfunction in a cohort undergoing prehospital treatment with FIASMA compared with those without. Answering this question will also result in the assessment, whether FIASMA-treated patients are at higher risk for a severe course due to putative impairment of phagolysosomal elimination of invading pathogens or—on the contrary—at lower risk for development of tissue damage and organ failure due to potential beneficial effects of FIASMA. From an epidemiological point of view, two studies came up with interesting results: in a general population (∼60,000 individuals) with self-reported anxiety and depression symptoms, severe depression was found to be associated with an increased risk for blood stream infection, moderate were not. However, an increased mortality risk was found for the later subcohort only; unfortunately, no data on antidepressant treatment were given in this study (173). Similar results were obtained from a Danish cohort study, where the underlying depressive disorder was assessed either by psychiatric diagnosis or by at least two antidepressant prescription redemptions within a 6-month period prior to hospitalization due to sepsis (174).
3. On ICU, ASM activity is also determined in septic patients treated with FIASMA due to other indications, comparison with an untreated group, and association with clinical parameters.
4. Ultimately, there is also a need for placebo-controlled trial with a carefully selected FIASMA (on the basis of data from observational trials) for a prospective investigation of the proposed beneficial effect of resulting ASM inhibition with respect to severity (development, duration, resolution of organ failure) and overall outcome.

Drug Repurposing

Repurposing of established drugs to treat both, common, or rare diseases is becoming an increasingly attractive and fast-track approach because it involves the use of compounds with known pharmacokinetic and safety profiles, with potentially lower overall development costs and shorter development timelines due to existing approval by the regulatory authorities (175). In the case of ASM, it is of remarkable importance since there is a long list of potential candidates available (36), and both, the structure-activity relationship (145) as well as the underlying mechanism of effective inhibition are known (144). Also, there are promising results from preclinical and clinical studies as discussed in this review, and there are some more outcomes using an explorative design for identification of beneficial effect of ASM inhibition.

Out of around 800 unique three-drug combinations, two sets were identified to effectively inhibit Ebola virus entry into human cells and were further validated for inhibition of live Ebola virus infection—at least two drugs of the triple exhibiting effective ASM inhibitory capacity (176).

There is also an excellent review [Beckmann et al., (177) and references cited therein], critically outlining the effectiveness of amitriptyline in a series of serious conditions such as cancer, infection, and metabolic and neurological diseases, all of them assessed as ASM-related diseases. For this drug, potential new applications for therapeutical treatment are demonstrated which might also be considered a general opportunity, since amitriptyline is proposed to function PARS PRO TOTO for this group of drugs exhibiting inhibitory capacity for ASM. Beside other drugs, these studies revealed that amitriptyline is a promising candidate for further consideration for the treatment of infectious diseases and overwhelming host response. However, adverse effects of a missing residual activity as shown by incompetence for phagocytosis and increased bacterial burden were also observed (65, 81). On the other hand, there is a clear association of ASM activity with the severity of sepsis and unfavorable outcome (34, 113, 128). It has to be mentioned that in heterogeneous mice by FIASMA treatment, a temporary status similar to complete loss of function was shown, underlining the effectiveness of functional ASM inhibition (167). A residual activity of 15% is insufficient to prevent clinical features of Niemann-Pick diseases over time (17).

BOX 2 | Take Home messages.

- ASM activity in circulation is a marker and mediator of harmful events, increased in patients with sepsis and associated with the clinical course regarding development, duration, and resolution of organ failure and outcome;
- In septic patients, different phenotypes with respect to ASM activity might be expected due to antecedent treatment with drugs for other indications (i.e., major depression etc.), which are also inhibiting ASM by lysosomotropic mechanisms;
- Further investigation in upcoming clinical studies is necessary to examine the potential consequences of an unattended ASM inhibition;
- Intended inhibition of ASM, also by repurposed drugs, is a promising approach to control the adverse effects of an overwhelming ASM activity associated with unfavorable outcome in these patients.

It is of major interest that fluoxetine, a widely used antidepressant drug, efficiently inhibited the entry and propagation of SARS-CoV-2 in the cell culture model without cytotoxic effects and also exerted potent antiviral activity against two currently circulating influenza A virus subtypes, an effect which was also observed upon treatment with the FIASMAs amiodarone and imipramine (178).

The recently proposed concept of a stress/injury-induced lysosomal exocytosis (59) as the major source of extracellularly circulating ASM (38) is in line (i) with a restoration of dependency for Zn^{++} ions, since due to rapid displacement of these ions after reaching the extracellular space, an additional regulating factor participates in the resolution of ASM activity beyond the outfall of the lysosomal content, and (ii) the effectiveness of activity decreases in circulation upon treatment with FIASMA, resulting in intralysosomal proteolysis of the mature ASM protein and subsequent release of inactive protein fragments without sphingolytic activity, and (iii) missing evidence of phosphorylated ASM in circulation of a patient undergoing stress response. However, the validation of this concept in septic patients still needs further examination, e.g., by comparison of the fragmentation and phosphorylation pattern of ASM isolated from both compartments, lysosomes and plasma, obtained from patients undergoing treatment with FIASMA. Another important point with respect to the dosage of FIASMA for treatment of host response is the fact of similarity to that of the original indication (often major depression) due to a very similar mode of action regarding ceramide generation.

Résumé and Take Home Messages

During an episode of sepsis, a broad panel of cell, tissue, and organ response is controlled by stress-induced ceramide generation; there is a broad understanding of potentially harmful effects of ceramide generation during sepsis and there is a broad panel of well-established and approved drugs with effectiveness for ASM inhibition (**Box 2**), at the end encouraging systematic studies for detailed examination of an unattended or an intended inhibition of ASM during sepsis to improve patients outcome.

Methods

The objective of this narrative review is to evaluate the changes of sphingomyelinase activity during infection and host response and the discussion of translation into the field of translational sepsis research including development of new strategies for diagnosis and treatment. A search in the main biomedical databases (PubMed, Medline, Scopus, and Web of science) was conducted for a 20-year period ending in July 2020, focused on primary research articles in the field of interest. Keyword search for abstracts and titles included ("sphingomyelinase" OR "SMPD1") AND ("sepsis" OR "inflammation" OR "infection"). The search identified 723 references, which were selected by particular importance for this review. The prescreened references were completed by other publications related to the issue. We apologize if a valuable work of any appreciated colleagues could not be included due to space limitations and the narrow scope of this review.

AUTHOR CONTRIBUTIONS

RC and H-YC drafted the manuscript. Both authors revised the final version of the manuscript.

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REVIEW
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Antimicrobial Resistance in *Enterobacterales* and Its Contribution to Sepsis in Sub-saharan Africa

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Antibiotic resistant *Enterobacterales* (formerly *Enterobactereaceae*) are a growing threat to Sub-Saharan Africa. Genes causing antibiotic resistance are easily spread between the environment and humans and infections due to drug resistant organisms contribute to sepsis mortality via delayed time to appropriate antimicrobial therapy. Additionally, second or third-line antibiotics are often not available or are prohibitively expensive in resource-constrained settings leading to limited treatment options. Lack of access to water and sanitation facilities, unregulated use of antibiotics, and malnutrition are contributors to high rates of antibiotic resistance in the region. Improvements in the monitoring of drug resistant infections and antibiotic stewardship are needed to preserve the efficacy of antibiotics for the future.

Keywords: resistance, *Klebsiella pneumoniae*, *E. coli*, sepsis, Sub-Sahara Africa

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INTRODUCTION

Globally, sepsis is estimated to cause an excess of 5 million deaths annually with low and middle income countries disproportionately affected (1). Sepsis is a significant problem in Sub-Saharan Africa (SSA) where mortality rates can be as high as 43% (2–4). While this high mortality is in part due to a lack of critical care resources being available in much of SSA (5), drug resistant pathogens play an important role in the increased mortality due to sepsis in this region.

Antimicrobial resistance (AMR) is a significant threat to human health that hampers our ability to treat a wide range of bacterial, parasitic, fungal, and viral infections (6). Antimicrobial resistance is recognized by the World Health Organization (WHO) as an increasingly serious threat to global public health that requires action across all government sectors and society (7). Although much of the research into AMR to date has been conducted in high-income countries (8), low and middle income countries (LMICs) bear a growing burden of AMR (9). Countries in SSA, in particular, are understudied in terms of rates and excess mortality due to AMR infections (10), yet experience a significant burden of disease (11, 12) and are expected to bear a disproportionate mortality burden (13). Additionally, parts of SSA have comparatively high rates of people living with HIV, which has been shown to increase the risk of invasive infection and subsequent sepsis (14, 15).

It is well-established that there are worse outcomes with drug-resistant infections in high-income countries (16–18). These poor outcomes can be exacerbated in low income regions where drug susceptibility testing can be delayed or absent and second or third-line antibiotics

are either unavailable or prohibitively expensive (19). Additionally, AMR has been forecast to increase the number of people living in extreme poverty globally (20), exacerbated by global inequities and poor access to healthcare.

Enterobacterales (formerly *Enterobacteriaceae*) are of particular concern in SSA given their ability to rapidly colonize and spread (21–23) and the limited treatment options available for drug-resistant *Enterobacterales* in resource-constrained settings (24–26). Both the WHO and the CDC recognize drug-resistant *Enterobacterales* as extremely concerning pathogens (27, 28). This review will focus on the rates and causes of AMR in *Enterobacterales* in SSA and the subsequent contribution to sepsis and mortality in the region. A limitation of this review is that more data are needed from future studies to more precisely estimate the rates and mechanisms of resistance in specific bacterial species, and the patient outcomes associated with infections caused by specific multidrug-resistant organisms.

GENETIC DIVERSITY OF AMR IN SSA

Antimicrobial resistance can occur through a variety of mechanisms. For *Enterobacterales*, resistance to the beta-lactam class of antibiotics is particularly concerning as beta-lactams are the cornerstone of treatment for these infections (29–31). Resistance to beta-lactam antibiotics can occur via efflux pumps, altered penicillin binding sites, and beta-lactamases which cleave the beta-lactam ring and inactivate the target antibiotic (32), with beta-lactamases being the primary means of resistance for cephalosporins and carbapenems. Development of resistance occurs through new mutations or upregulation and expression of existing genes, *AmpC* being the most common occurrence of the later (33). Spread can then occur through clonal dissemination, horizontal gene transfer via plasmids, or translocation of resistance genes between mobile genetic elements (32, 34).

As AMR genes have spread worldwide they are now found throughout Africa in varying rates. The beta-lactamase genes bla_{CTX-M} are responsible for much of the spread for ESBL-Enterobacterales (ESBL-E) worldwide (35–37). bla_{CTX-M} are most commonly transferred via plasmids (38), which allow for the spread of multiple resistance genes at one time (39). In Africa, rates of ESBL-E are increasing, largely as a result of CTX-M genes. A review of AMR isolates in East Africa found high rates of a variety of AMR genes, with the bla_{CTX-M} genes being the most common etiology of ESBL-E infection, found in 45.7% of isolates (40). A study in Ethiopia showed high rates of ESBL infections among gram negative isolates with 95% of those carrying CTX-M genes (41). High rates of CTX-M have similarly been found in studies from Nigeria (42), Tanzania (43, 44), Malawi (45, 46), and Ghana (47). Other ESBL-encoding genes including bla_{TEM}, bla_{OXA}, and bla_{SHV} are also found through the region (40, 46).

In addition to ESBL-producing organisms, carbapenemase-producing organisms are a growing threat to the region. The carbapenemase gene KPC is the most prevalent carbapenemase in the United States (48), but is less frequently found in Africa (49). In contrast, the carbapenemase genes bla_{IMP}, bla_{VIM}, and bla_{OXA} appear to be more common in Africa (40, 50). For carbapenemase genes, it remains unclear if clonal expansion or horizontal transfer via plasmids are the primary means of dissemination (51), however they have been shown to spread easily and rapidly in hospitals and other healthcare settings (52). This is of particular concern in SSA where rapid diagnosis is not always possible and strict hygiene and infection control measures can be difficult to achieve (53, 54).

BURDEN OF INTESTINAL AMR CARRIAGE IN SSA

There is substantially less data characterizing the rates of AMR carriage and infection in SSA when compared to North America and Europe. Studies that have been done are primarily from urban areas, despite 60% of the population of SSA living in rural regions (55). Studies can be broadly categorized as those that determine rates of AMR carriage in community-dwelling (asymptomatic) individuals and those that are hospital-based and determine rates in patients with active infection.

Although infection rates are an obvious and important source of information to assess the contribution of AMR to sepsis in SSA, carriage rates are equally important, as intestinal colonization with AMR pathogens precedes and predicts subsequent infection. The intestinal microbiome is a well-described and important reservoir for AMR bacteria that may cause subsequent infections (56–58) and the likelihood of invasive AMR infections is dramatically increased in people who are colonized (59, 60).

Rates of intestinal AMR carriage can be studied in a variety of ways, which can make comparison between studies difficult, however rate of ESBL-E carriage is a common method. In SSA, rates of ESBL-E carriage vary widely by the region and specific population being studied, from 5 to 59%. Carriage rates from a number of studies and their populations are summarized in **Table 1**. Notably, many of these studies include individuals with little to no healthcare exposure or antibiotic use, suggesting there is significant community spread.

RATES OF AMR INFECTIONS IN HOSPITALIZED PATIENTS

Asymptomatic carriage can be a precursor to disease, and, while there are fewer studies from SSA than from other regions, hospital infections with AMR pathogens is an important contributor to disease in this region. This is particularly relevant for bloodstream infections (BSI), as BSI remain a leading cause of death of both adults and children in the region (74). Ceftriaxone is often used empirically for sepsis in the region due to the relative ease of dosing and low cost. Thus, ceftriaxone resistance is particularly worrisome. The MERINO study showed that carbapenems are the preferred treatment option for invasive infections caused by ceftriaxone-resistant enteric bacteria (75). Unfortunately, carbapenems are not available to many patients with sepsis in SSA.

A recent meta-analysis of bloodstream infections in SSA found an overall prevalence rate of third generation cephalosporin resistance of 18.4% for *Escherichia coli* isolates and

TABLE 1 | Studies evaluating rates of ESBL-E carriage in SSA.

References	Country	Population	Percent with ESBL intestinal carriage (%)
Sanneh et al. (61)	The Gambia	Food handlers	5
Farra et al. (62)	Central African Republic	Children (age 0–59 months)	59
Mshana et al. (63)	Tanzania	Any age	16.5
Chereau et al. (64)	Madagascar	Pregnant women at time of delivery	18.5
Ribeiro et al. (65)	Angola	Healthy community dwellers	22.2
Albrechtova et al. (66)	Kenya	Nomadic pastoralists	17
Tellevik et al. (67)	Tanzania	Healthy children <2 y/o	11.6
Moremi et al. (68)	Tanzania	Street children	31.8
Chirindze et al. (69)	Mozambique	University students	20
Fortini et al. (70)	Nigeria	Healthy pregnant women on day of admission	31.7
Ruppé et al. (71)	Senegal	Children in remote village	10
Lonchel et al. (72)	Cameroon	Student volunteers	6.7
Ouchar Mahamat et al. (73)	Chad	Healthy community volunteers	38

54.5% for *Klebsiella* isolates (19). Some studies suggest the rates of drug resistance are increasing dramatically in the region. For example, a study of BSI from a large referral hospital in Malawi showed an increase in ESBL resistance from 0.7 to 30.3% of *E. coli* isolates and from 11.8 to 90.5% in *Klebsiella* spp. isolates between 1998 and 2016 (76). Similarly, they found methicillin resistance of *Staph aureus* isolates increased from 7.7 to 18.4% over the same period. More data are needed from future studies to more precisely estimate the rates and mechanisms of resistance in specific bacterial species, and the patient outcomes associated with infections caused by specific multidrug-resistant organisms.

OUTCOMES OF AMR INFECTION IN HOSPITALIZED PATIENTS

The dramatic rise in AMR detection is associated with worse outcomes. A study in pediatric patients at a hospital in Senegal showed that AMR carriage is associated with delayed time to appropriate antibiotics and a substantial increase in mortality from 15.4 to 54.8% in patients with ESBL-producing *Enterobacterales* BSI compared to susceptible strains (77). This was confirmed by another study from Tanzania which found AMR to be an independent risk factor for mortality in both children and adults with BSI, with mortality rates increasing from 13.7 to 48.4% when organisms were multidrug resistant compared to those that were susceptible (78). Similarly, a study of adult patients in Ethiopia showed significant increase in mortality with BSI if the isolate was resistant to third generation cephalosporins, with all patients with resistant infection in that study having died (79).

Aside from BSI, resistance presents a challenge to other infections as well. Clinical isolates from multiple sources including wound, urine, blood, and sputum at a large Rwandan hospital showed 75.9% of isolates were resistant to ceftriaxone, a third generation cephalosporin (80). A study of clinical isolates growing *Klebsiella pneumoniae* from Cote d'Ivoire likewise showed high prevalence of ESBL resistance, at 84% of all isolates and 94% of pediatric isolates (81). These findings have implications for the treatment of urinary tract infections, wound infections, and surgical infections, among others.

Although many patients present from the community with AMR infections, hospital acquisition is a common means of becoming infected or colonized with AMR pathogens and can occur soon after admission. A study from Rwanda looking at ESBL-E acquisition during hospitalization found that overall carriage rates in patients increased from 49.7% on admission to 64.6% on discharge, however this number was as high as 93% on discharge from the pediatric unit (82). Another study of neonates in Kenya showed that 55% acquired ESBL carriage during hospitalization (83).

DRIVERS OF AMR IN SSA

AMR is driven by multiple factors, however overconsumption with unnecessary or inappropriate use of antibiotics is a significant contributor (84). Consumption of antibiotics varies widely between regions, however, as many as half of all antibiotics in developing countries being used inappropriately (85). A recent WHO report from a limited number of countries in Africa shows rates of antibiotic consumption that range from 4.4 to 27.3 daily doses per day per 1,000 inhabitants (86). Notably, this only includes antibiotics dispensed through regulated agencies, not unregulated or non-prescription sales. Dispending antibiotics without a prescription is a common occurrence in much of Africa. In one region of Tanzania, over 88% of antibiotic prescription were found to be irrational with 76% occurring without a prescription (87) and in Cameroon 47% of antibiotics dispensed at pharmacies were without a prescription (88). This lack of regulation and consumption of inappropriate and non-prescription antibiotics is a contributor to the spread of drug resistance in the region (89–92).

Human consumption of antibiotics is only one driver of AMR. Livestock and veterinary overuse of antibiotics for commercial farming are a large contributor of AMR worldwide (93, 94) and this holds true in SSA as well (95). The idea that humans, animals, and the environment are connected and all contribute to the health of society is termed a "One Health" approach (96). Antibiotics used in farming practices are discharged into the environmental through animal waste where they contribute to the "resistome" of the environment and can lead to human colonization or infection with AMR pathogens, raising the overall

AMR burden in communities (97). Antibiotic use in commercial animal farming has largely focused on Europe, North America, and certain middle income countries including China, Brazil, and India where use rates are high (98), however high rates of antibiotic use and subsequent AMR have been found in SSA ranging from 74 to 100% of farms (99), with little to no surveillance systems in place on national levels to ensure accurate tracking of use (100). Additionally, antibiotic use for livestock worldwide is expected to rise significantly in the coming years, including in SSA, with an estimated 67% increase to 105,000 tons by 2030 (93).

The multidisciplinary nature of AMR was highlighted by a recent study showing that the spread of AMR genes (as opposed to selective bacterial pressure) may be the dominant factor contributing to high rates of AMR in the region (101). To this end, improving access to safe drinking water and sanitation facilities (102) are urgently needed to help limit AMR (103).

In addition to the above, malnutrition may also contribute to AMR in SSA, particularly in the pediatric population. It is well-established that malnutrition places individuals at increased risk for infection (104) and thus is may facilitate the spread of AMR genes (105). Additionally, malnutrition may place individuals at greater risk specifically for drug-resistant infections. In Senegal, malnutrition was associated with twice the risk for contracting an ESBL-BSI compared to a susceptible BSI (77). Similarly, another study found high rates of multidrug resistant urinary tract infections in malnourished children in Tanzania (106).

Africa has the highest number of people living with HIV (PLWH) in the world. There is growing evidence that HIV may increase the carriage rates of AMR bacteria in the GI tract (107), a precursor to invasive infection. Two studies of pediatric patients hospitalized in South Africa with BSI found that HIV infection was associated with increased likelihood of having a resistant isolate and a corresponding increase in sepsis-related mortality (108, 109). Similarly, a study of pediatric bacteremia in Tanzania found children living with HIV were more likely to receive inappropriate initial antimicrobial therapy due to drug resistance and subsequently had a higher risk of mortality compared with their HIV negative counterparts (110).

GLOBAL IMPORTANCE OF AMR

Although this review focuses on AMR and its contribution to sepsis in SSA, drug resistance is a global problem that extends beyond national borders. As travel and immigration lead to increasing globalization, AMR genes can quickly spread worldwide (23). A recent meta-analysis found that a quarter of migrants to Europe were colonized with multi-drug resistant organisms, with the rate being higher in asylum seekers (21). Similarly, a study of Eritrean migrants in Europe showed high rates of gram negative and polymicrobial carriage with AMR genes detected (111). International travel is also a means of dissemination, with 24% of returning Swedish travelers carrying CTX-M-15 (112) and a meta-analysis showing overall acquisition of ESBL organisms among travelers to Africa to be over 15%, with wide variation between regions and higher rates in those who experienced travelers diarrhea or used fluoroquinolone antibiotics while abroad (113). In addition, acquisition is not confined to the traveler, as another study showed the probability of transmitting AMR genes to another household member after return was 12% (114), indicating the potential for dissemination to the surrounding community.

CONCLUSIONS

Antimicrobial resistance is growing as a worldwide threat and contributes to sepsis-associated mortality, with alarming rates in SSA. Moving forward, a global coordinated response is needed to halt the spread of AMR and improve outcomes for patients with sepsis due to these organisms in SSA. To this end, the World Health Organization developed the Global Action Plan Against Antimicrobial Resistance in 2015 to create a framework for member states to focus research and resources on AMR while attempting to reduce the need for antibiotics through improved infection prevention and sanitation (115). That same year, the WHO launched the Global Antimicrobial Resistance Surveillance System (GLASS) to uniformly track rates of AMR worldwide and estimate the attributable mortality due to AMR (116). As AMR continues to pose a threat to human health across the world it is clear that a multi-pronged approach is needed to halt the spread. This will require cooperation between countries and across disciplines and is imperative to ensure the continued efficacy of antimicrobials.

AUTHOR CONTRIBUTIONS

KT, JJ, and DD searched and reviewed the literature, summarized the data, and reviewed and edited the final content. KT wrote the first draft of the paper. All authors contributed to the article and approved the submitted version.

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Early Detection of Sepsis With Machine Learning Techniques: A Brief Clinical Perspective

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Sepsis is a major cause of death worldwide. Over the past years, prediction of clinically relevant events through machine learning models has gained particular attention. In the present perspective, we provide a brief, clinician-oriented vision on the following relevant aspects concerning the use of machine learning predictive models for the early detection of sepsis in the daily practice: (i) the controversy of sepsis definition and its influence on the development of prediction models; (ii) the choice and availability of input features; (iii) the measure of the model performance, the output, and their usefulness in the clinical practice. The increasing involvement of artificial intelligence and machine learning in health care cannot be disregarded, despite important pitfalls that should be always carefully taken into consideration. In the long run, a rigorous multidisciplinary approach to enrich our understanding in the application of machine learning techniques for the early recognition of sepsis may show potential to augment medical decision-making when facing this heterogeneous and complex syndrome.

Keywords: sepsis, machine learning, artificial intelligence, early diagnosis, supervised learning, unsupervised learning

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INTRODUCTION

Sepsis, defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection, is a major cause of death worldwide (1–5).

Sepsis is a complex and evolving concept: (i) complex, because it involves two different actors (the infection and the host response) and their relative contribution to the organ damage may vary across patients and over time in the same patient (6, 7); (ii) evolving, because various definitions have been developed and adopted over the last years, reflecting the complexity of the elusive concept to be defined. Although the evolution of the concept of sepsis could reflect a positive trajectory aimed to provide more precise definitions for both clinical and research purposes, the long-lasting process of defining sepsis has led to the use of different terminology and definitions across research studies (8, 9). As a consequence, this has reduced the comparability of study results, and also hindered a smooth development of predictive models of sepsis. Nonetheless, the development of good predictive models of sepsis remains a timely topic, since it would help clinicians to

readily identify patients at higher risk of (or likely to already have) sepsis, thereby allowing close monitoring and/or early treatment (thereby potentially reducing mortality) (10).

Prediction of sepsis through the use of machine learning models has gained particular attention over the past few years (11–17). In the present perspective, we provide a brief clinician-oriented vision on some relevant aspects concerning the use of machine learning predictive models for the prediction of sepsis in the daily practice. In particular, although predictive models may be developed for either prediction *sensu stricto* or early detection (i.e., as early diagnostic tools), we will mainly focus on the ability of these tools to early detect patients with sepsis.

METHODS

On 25 July 2020, we performed a PubMed search using the following key words: "sepsis" AND "machine learning." Reference lists and abstracts of the pre-screened 189 studies were manually checked, using an iterative approach, and selected for potential eligibility. Selected full texts and pertinent references were further reviewed, with the ultimate decision on inclusion being based upon authors' judgment. Of note, additional searches through PubMed and Google Scholar performed on 23 December 2020 led to the evaluation of 18 additional full texts for potential inclusion. The final text was structured in the following sections: (i) brief introduction on prediction of clinical events through machine learning models and the need for a multidisciplinary approach; (ii) the controversy of sepsis definition and its influence on the development of prediction models; (iii) the choice and availability of input features; (iv) the measure of the model performance, the output, and their usefulness for clinicians; (v) limitations and conclusions.

Brief Introduction on Prediction of Clinical Events Through Machine Learning Models and the Need for a Multidisciplinary Approach

Through machine learning algorithms, computers are conferred the ability to learn from data (18, 19). Conceptually, machine learning, which is a branch of artificial intelligence, is different from standard computer expert systems for helping clinicians in daily decision making (20). Indeed, the latter are explicitly programmed to perform a given task, whereas machine learning algorithms are more generally programmed to find out associations. For example, in supervised learning, an outcome (e.g., sepsis), is predicted through calculations starting from input features (e.g., patient demographic and clinical characteristics). Notably, the outcome and the input of machine learning algorithms may also represent the dependent and independent variables of classical statistical predictive models. Not surprisingly, there is an important conceptual overlap between classical statistics and machine learning techniques (21). A difference in their continuum may rely on the fact that some machine learning algorithms could be able to find out composite features not easy or impossible to be defined by humans. In turn, this may improve the accuracy of prediction (22). However, this also fuels the issue of the interpretability of the model, with computations that may become less transparent and not completely explainable once results are produced (23). In turn, this may hamper recognition of biases, thereby leading to additional ethical and legal implications connected to the use of machine learning techniques in healthcare (24–26).

In addition, it should be also taken into consideration that the field of machine learning is closely connected with the concept of "big data," since, at least in general, the possibility of including complex features in prediction models requires far larger samples than those usually employed in classical prediction studies (22, 27). Therefore, the possible future availability of large datasets of medical data from electronic medical records (EMR), laboratory databases, and vital signs monitors will inevitably require a multidisciplinary approach. This would guarantee standardized extraction of data, data security, interpretability or sufficient explanation of employed machine learning models, extrapolation of useful outputs from a clinical perspective, and full compliance to all the most updated ethical and legal laws and regulations. This will also apply to the prediction of sepsis, both for research studies and for real-time prediction at the bedside in the daily clinical practice.

The Controversy of Sepsis Definition and Its Influence on the Development of Prediction Models

According to the recent Sepsis-3 criteria, sepsis is formally defined as an acute increase of $\geq$2 points in the Sequential Organ Failure Assessment (SOFA) score, consequent to a suspected or proven infection (1). Increases in the SOFA score, detected through monitoring of laboratory and clinical parameters, reflect possible impairments in cardiovascular, respiratory, renal, hepatic, coagulation, and neurological systems (28). This is in line with the novel definition of sepsis as a life-threatening and progressive organ dysfunction. Previously, sepsis was defined as the presence of a systemic inflammatory response syndrome (SIRS) caused by a proven or suspected infection and based on laboratory and clinical parameters (white blood cell count, respiratory rate, heart rate, and body temperature) that could be altered before manifestation of sepsis-related organ dysfunction (29). However, this previous, more general definition, lacked specificity, since SIRS may be present in several non-infectious conditions. On the other hand, it has very high sensitivity because of the large denominator of patients with SIRS (although some patients with organ dysfunction due to infection may not present with SIRS, and a few cases may still be missed) (30). Overall, the novel definition offers a better performance than the previous one for identifying septic patients at higher risk of mortality in intensive care units (ICU) (1). However, a better prediction of mortality does not inherently reflect the best timing for intervention. For example, it still remains unclear the optimal timing for starting antimicrobial therapy. More specifically, while it is intuitive to initiate antimicrobials if sepsis is first recognized at the time of organ dysfunction (according to the novel sepsis definition), what if the patient is already suspected to have sepsis before developing any organ dysfunction (according to

previous sepsis definitions)? Can we wait, or should we initiate antimicrobials immediately? Notably, there is not a high-level evidence-based response to this question yet, and the choice is usually made upon clinical judgment at the patient's bedside, on a case-by-case basis.

However, this is hardly reproducible for defining what should be predicted by both classical statistics and machine learning models to support clinicians in the administration of antimicrobials at the best time to reduce mortality of sepsis, and, at the same time, without administering antimicrobials indiscriminately to all patients with SIRS but no sepsis, in line with antimicrobial stewardship principles. Prediction of sepsis through machine learning techniques is not exempt from this unresolved controversy, as reflected by the various definitions of sepsis employed in the different models available in the literature (see **Table 1**) (85). Against this backdrop, it is worth noting that the possible solution of using expert physicians' judgement for labeling sepsis cases as gold standard for model training may not resolve the issue because of suboptimal agreement across physicians (86). A reasonable alternative strategy explored by some authors may be the use of unsupervised machine learning techniques (i.e., by recognizing patterns in the data without a labeled outcome as in supervised learning) for the identification of novel phenotypes of sepsis based on clinical and laboratory values (77, 87, 88). This could help in the identification of specific subgroups of patients to be included in dedicated studies (preferably randomized clinical trials) to assess the impact of early antimicrobial therapy (77, 89, 90). Furthermore, this would allow to use the trial outcome as a measure of *a posteriori* accuracy of sepsis classification based on the phenotypes identified with machine learning techniques.

The Choice and Availability of Input Features

The increasing use of EMR implies an immediate availability of an electronic form of relevant clinical and laboratory data, that support the development and real-time use of right-aligned predictive models for the early detection of sepsis (91–93). Indeed, these models may continuously update their prediction of sepsis by utilizing the unceasing stream of electronic data from the EMR and/or vital signs monitors (57, 74, 85, 92, 94). A good predictive model may thus allow one to correctly classify in real time a true case of sepsis in the controversial gray area in between the previous and novel sepsis definitions. Amongst others, two important questions need to be addressed: (i) which and what minimum number of input features should be used for developing a good predictive model for the early detection of sepsis? (ii) once a good predictive model is developed, would an early antimicrobial treatment improve the prognosis of these patients?

Intuitively, there can be no answer to the latter question without first developing a good predictive model. Therefore, the appropriate selection of input features remains paramount. In this regard, it is still unclear which between a parsimonious (even of a few vital and/or laboratory parameters) and an expanded (for example, considering also information from unstructured physicians' free text in EMR notes) selection of input features could be preferable, and where precisely is any possible desirable middle in between these two extremes. In the literature, there are encouraging experiences with the use of a few vital and/or laboratory parameters for the prediction of severe sepsis according to previous definitions, with also a positive impact on survival having been registered in a small but randomized single center clinical trial and in some observational studies (44, 95–97). However, the possibility of employing input features relying on patients' clinical data and medical histories remains attractive if only for attempting a concomitant prediction of etiology (e.g., of multidrug-resistant causative agent based on a combination of previous microbiological isolates and risk factors for multidrug-resistant infections, such as previous antibiotic use), that may impact appropriateness of empirical antimicrobial therapy while waiting for blood cultures results (21). Notably, this paves the way to some important issues regarding the automated extraction of unstructured data from EMR. Examples are the need for standardization of extraction, sufficient accuracy of automated extraction, internal/external validation, and continuous re-calibration of automated extraction over time. Furthermore, besides these technical aspects, the presence of missing data [which is inherent in EMR, since they are not an instrument designed for research purposes (50)] and their potential impact on the uncertainty of prediction should be properly handled.

It should also be considered that a different amount of information is expected between ICU patients (more closely monitored clinically and through laboratory tests) and patients in other wards. Therefore, besides inherent differences in their characteristics, also the different amount of available data may influence both the selection of the best set of features and the predictive performance of models developed for ICU patients and models developed for other populations (e.g., to be used in the emergency department), that may be not interchangeable. Finally, the choice of the input features could also be dictated by the local availability of the necessary infrastructures. For example, lack of validation or ability to properly extract clinical data from the local EMR may only allow the use of laboratory values for building predictive models (manual extraction of additional clinical data from EMR may be unfeasible or extremely time-consuming if large samples are concerned).

The Measure of the Model Performance, the Output, and Their Usefulness for Clinicians

As shown in **Table 1**, the model performance is frequently measured in term of area under the receiver operating characteristics curve (AUROC), which provide an aggregate measure of performance. Strictly speaking, the AUROC identifies how well predictions are classified considering both the number of true positive and true negative, i.e., how many true cases of sepsis or true cases of not sepsis have been correctly classified. However, this measure may not inherently reflect the clinical usefulness of the algorithm. Indeed, the global value of AUROC is independent of the chosen cut-off, whereas, in the setting of sepsis, the major interest for comparing models may be in

TABLE 1 | Characteristics of selected observational studies on the use of machine learning for the prediction/early diagnosis of sepsis*.

References	Population	Sample size**	Input features (or data on which input features are computed)	Type of employed model/platform***	Endpoint	Output metrics****
Thiel et al. (31)	Mixed-ward patients	27,722	Vital signs, laboratory data	RPART(6)	Septic shock defined according to ICD-9 codes for acute infection matched to codes for acute organ dysfunction and the need for vasopressors within 24 h of ICU	Proportion of correctly classified patients, PPV, NPV, MCR
Mani et al. (32)	Neonates in NICU	299	Laboratory, clinical and microbiology data available in EMR	SVM(3), NB(1), TAN(6,1), AODE(1), KNN(2), CART(6), LR(10), RF(4), LBR(1)	Sepsis labeled based on antibiotic use, microbiological data, and laboratory data	AUROC, sensitivity, specificity, PPV, NPV
Henry et al. (33)	ICU patients in the MIMIC-II dataset (34)	16,025	Vital signs, laboratory data	Supervised learning based on Cox proportional hazards model(18) (TREWScore)	Sepsis according to SIRS criteria (35) plus relevant ICD-9 coding or clinical note mentioning sepsis	AUROC, sensitivity, specificity
Calvert et al. (36)	ICU patients in the MIMIC-II dataset (34)	1,394	Vital signs, laboratory data, age	*InSight*(19)	Sepsis according to SIRS criteria (35) plus relevant ICD-9 coding	Accuracy, AUROC, sensitivity, specificity
Desautels et al. (37)	ICU patients in the MIMIC-III dataset (38)	22,853	Vital signs, laboratory data, age	*InSight*(19)	Sepsis according to Sepsis-3 definitions (1)	AUROC, APR
Brown et al. (39)	Patients admitted to the ED	226,481	Vital signs, laboratory data, age	NB(1)	Severe sepsis according to ICD-9 coding	AUROC, sensitivity, specificity, PPV, NPV
Horng et al. (40)	Patients admitted to the ED	230,936	Vital signs, demographics, and free text notes	SVM(3)	Angus Sepsis ICD-9-CM abstraction criteria (41)	AUROC, sensitivity, specificity, PPV
Kam et al. (42)	ICU patients in the MIMIC-II dataset (34)	6,362	Vital signs, a few laboratory data, age	*InSight*(19), DNN(12)	Sepsis according to SIRS criteria (35) plus relevant ICD-9 coding	Accuracy, AUROC, sensitivity, specificity
Liu et al. (43)	Patients between 16 and 80 years admitted to ICU or medical ICU	185	General information, medical history, progress notes, laboratory data	FANN(11), SVM(3), RF(4)	Bacterial sepsis labeled by the treating physician found in the medical records	AUC, accuracy, sensitivity
McCoy et al. (44)	Mixed-ward patients	1,328	Vital signs, laboratory data	*InSight*(19)	Severe sepsis according to 2001 definitions (29) and sepsis according to Sepsis-3 definitions (1)	AUROC, sensitivity, specificity
Shashikumar et al. (45)	ICU patients	242	Vital signs, clinical data	Elastic-net LR(9,10)	Sepsis according to Sepsis-3 definitions (1)	Accuracy, AUROC, specificity
Taneja et al. (46)	Mixed-ward patients	444	Vital signs, laboratory data	LR(10), NB(1), SVM(3), AdaBoost(5), RF(4)	Eleven categories from never septic to septic shock	AUROC
Nemati et al. (47)	ICU patients	27,527	Vital signs, laboratory data, demographic and clinical data from EMR	AISE algorithm (based on a modified Weibull-Cox proportional hazards model)(18)	Sepsis according to Sepsis-3 definitions (1)	Accuracy, AUROC, sensitivity, specificity
Lamping et al. (48)	Pediatric patients admitted to the PICU	807	Laboratory data, clinical variables	RF(4)	Sepsis as defined Goldstein et al. (49)	AUC, sensitivity

(Continued)

TABLE 1 | Continued

References	Population	Sample size**	Input features (or data on which input features are computed)	Type of employed model/platform***	Endpoint	Output metrics****
Mao et al. (50)	Mixed-ward patients	90,353	Vital signs	*InSight*[19]	Sepsis according to 2001 definitions (29)	AUROC, sensitivity, specificity
Kamaleswaran et al. (51)	Critically ill children	493	Continuous high frequency stream of physiologic data	LR[10], RF[4], DCNN[13]	Severe sepsis according to criteria modified from Goldstein et al. (49) and Sepanski et al. (52)	Accuracy, sensitivity, specificity, PPV, NPV, LR+, LR–
Saqib et al. (53)	ICU patients in the MIMIC-III dataset (38)	38,270	Vital signs and laboratory data	LR[10], RF[4], LSTM neural networks[12]	Sepsis according to ICD coding system [Angus criteria (54)]	F1 score, Mathew's Correlation Coefficient, AUROC, sensitivity, PPV
Faisal et al. (55)	Patients admitted to the ED	57,243	Vital signs, laboratory data	LR[10]	Sepsis according to ICD-10 coding system (56)	AUROC, sensitivity, specificity, PPV
van Wik et al., (57)	ICU patients	1,161	Continuous high frequency stream of physiologic data	RF[4], SVM[3], LR[10], MLP[10], RNN[12]	Sepsis defined as SIRS plus presence of blood cultures and administration of antibiotics, along with relevant ICD-10 coding	F1 score, accuracy, AUROC, PPV, sensitivity, specificity
Delahanty et al. (58)	Patients admitted to the ED	2,759,529	Vital signs, laboratory data, demographics, medications, nursing notes, key words	Gradient boosting[5]	Sepsis according to Rhee clinical surveillance criteria (59)	Alert rate, AUROC, sensitivity, specificity, precision
Van Wik et al. (60)	ICU patients	586	Continuous high frequency stream of physiologic data	RF[4]	Sepsis defined as SIRS plus administration of antibiotics, along with relevant ICD-10 coding	F2 score, accuracy sensitivity, specificity, PPV
Kaji et al. (61)	ICU patients in the MIMIC-III dataset (38)	36,176	Laboratory data, demographics, prescribed medications	LSTM neural networks with attention mechanism[12]	Sepsis according to SIRS criteria (35) plus relevant ICD-9 coding	AUROC, sensitivity, PPV
Calvert et al. (62)	Multiple datasets	122,672	Vital signs	Gradient-boosted decision treesclassifier[5]	Severe sepsis according to SIRS criteria (35) plus relevant ICD-9 coding	Accuracy, AUROC, sensitivity, specificity, PPV, NPV, DOR
Masino et al. (63)	Neonates in NICU	1,188	Routine EMR data	LR[10], NB[1], SVM[3], KNN[2], Gaussian process[16], RF[4], AdaBoost[5], gradient boosting[5]	Sepsis defined as positive blood culture or negative blood culture plus administration of antibiotics	AUROC, sensitivity, specificity, PPV, NPV
Barton et al. (64)	Multiple datasets	112,952	Vital signs, age	Gradient boosting[5]	Sepsis according to Sepsis-3 definitions (1)	AUROC, sensitivity, specificity. DOR, LR+, LR–
Giannini et al. (65)	Mixed-ward patients	172,700	Vital signs, laboratory data, demographics	RF[4]	Severe sepsis according to ICD-9 coding plus positive blood culture plus increased serum lactate or reduced blood pressure	AUROC, sensitivity, specificity, LR+, LR–

(Continued)

TABLE 1 | Continued

References	Population	Sample size**	Input features (or data on which input features are computed)	Type of employed model/platform***	Endpoint	Output metrics****
Scherpf et al. (66)	ICU patients in the MIMIC-III dataset (38)	Various (see original publication)	Vital signs, laboratory data, age	RNN[12], *InSight*[19]	Severe sepsis according to SIRS criteria (35) plus relevant ICD-9 coding	AUROC, sensitivity, specificity
Schamoni et al. (67)	Surgical ICU patients	620	Clinical data, laboratory data, demographics	Ordinal regression[15]	Sepsis defined according to attending physicians' daily judgements of patients' sepsis status	AUROC, features weights
Fagerström et al. (68)	ICU patients in the MIMIC-III dataset (38)	~59,000	Vital signs, laboratory data, medical procedures, medications, journal notes, diagnoses, patient demographics	LSTM neural networks[12]	Septic shock according to SIRS criteria (35) plus relevant ICD-9 coding or clinical note mentioning septic shock	AUROC
Le et al. (69)	Mixed-ward pediatric patients	9,486	Vital signs, laboratory data, GCS	Boosted ensembles of decision trees[5,6]	Severe sepsis defined according to Goldstein et al. (49)	AUROC, sensitivity, specificity, DOR
Yee et al. (70)	ICU patients in the MIMIC-III dataset (38)	9,165	Laboratory data, diagnosis and procedures codes according to ICD-9, demographics	BN[17]	Septic shock defined according to Kadri et al. (71)	AUROC, sensitivity, specificity, PPV, NPV
Scott et al. (72)	Pediatric patients admitted to the ED and urgent care sites	2,464	Vital signs, demographics, clinical data	Elastic net regularization[9]	Septic shock defined according to Goldstein et al. (49)	AUROC, sensitivity, specificity
Dhungana et al. (73)	ICU patients	200	Laboratory data, clinical data, demographics	Computable phenotypes[6]	Sepsis labeled by manual data abstraction from EMR	Sensitivity, specificity
Bloch et al. (74)	ICU patients	600	Vital signs	LR[10], SVM[3], neural networks[11]	Sepsis according to 1992 definition (35)	AUROC, APR, sensitivity, specificity, PPV, NPV
Choi et al. (75)	Mixed-ward patients	7,743	Laboratory data	LR[10]	Sepsis according to ICD-10 coding system	Accuracy, AUROC, sensitivity, specificity, PPV, NPV
Kim et al. (76)	Patients admitted to the ED	49,560	Vital signs, laboratory data, demographics	SVM[3], gradient boosting[5], RF[4], MARS[14], LASSO[8], ridge regression[7]	Septic shock according to Sepsis-3 definitions (1)	AUROC, APR, sensitivity, specificity, PPV, NPV
Ibrahim et al. (77)	ICU patients in the MIMIC-III dataset (38)	13,728	Vital signs, laboratory data	RF[4], gradient boosting[5], SVM[3]	Manually validated extracted sepsis mention in EMR, overall and according to phenotypes	AUROC, sensitivity, specificity, LR+, LR–
Yuan et al. (78)	ICU patients	1,588	Minute-by-minute collection of vital signs, laboratory data, examination reports, text data, and images	Gradient boosting[5]	Sepsis labeled by the in-charge intensivist every day	F1 score, accuracy, sensitivity, specificity, PPV

(Continued)

TABLE 1 | Continued

References	Population	Sample size**	Input features (or data on which input features are computed)	Type of employed model/platform***	Endpoint	Output metrics****
Lauritsen et al. (79)	Mixed-ward patients	3,126	Vital signs, laboratory data, clinical data, medications, images	Combination of CNN[13] and LSTM neural networks[12]	Sepsis labeled by the treating physician based on the 2001 definitions (29)	AUROC, APR, mean average precision, net benefit
Tran et al. (80)	Adult patients with ≥20% TBSA burns	121	Vital signs, laboratory data, and a few clinical variables and clinical scores	Different ML algorithms optimized by MILO	Sepsis status based on 2007 ABA consensus guidelines (81)	Accuracy, AUROC, sensitivity, specificity
Burdick et al. (82)	Adult patients from inpatient wards and emergency department admission	270,438	Vital signs and a few clinical variables	Optimized distributed gradient boosting[5]	Severe sepsis according to ICD-9 coding system	AUROC, specificity, accuracy, DOR, LR+, LR–
Bedoya et al. (83)	Patients admitted to the ED	42,979	Patient demographics, vital signs, comorbidities, medications and laboratory data	RNN[12], CR[8,18], LR[10],RF[4]	Sepsis defined as presence of 2 or more SIRS criteria (35), a blood culture order, and at least one element of end-organ failure	AUROC
Abromavičius et al. (84)	ICU patients	40,336	Vital signs, laboratory data, demographics	DT[6], NB[1], SVM[3], Ensemble learners[5]	Sepsis labeled according to Sepsis-3 criteria (1)	AUC, APR, accuracy, F-measure, MCC

ABA, American Burn Association; AISE; artificial intelligence sepsis expert; AODE, averaged one dependence estimators; APR, area under precision-recall curves; AUROC, area under the receiver operating characteristics curve; BN, Bayesian networks; CART, classification and regression trees; CR, Lasso-penalized Cox regression; DC, decision tree; DCNN, deep convolutional neural networks; DNN, deep neural networks; DOR, diagnostic odds ratio; ED, emergency department; EMR, electronic medical record; FANN, Fast artificial neural network; GCS, Glasgow coma scale; ICD-9-CM, International classification of diseases, ninth Revision, clinical modification; ICU, intensive care unit; IPSCC, International Pediatric Sepsis Consensus Conference; KNN, K-nearest neighbors; LASSO, least absolute shrinkage and selection operator; LBR, lazy Bayesian rules; LR, logistic regression; LR+, positive likelihood ratio; LR–, negative likelihood ratio; LSTM, long short-term memory; MARS, multivariate adaptive regression splines; MCR, misclassification rate; MILO, Machine intelligence learning optimizer; MCC, Matthews correlation coefficient; MLP, multi-layer perceptron; NB, naïve Bayes; NICU, neonatal intensive care unit; NPV, negative predictive value; PICU, Pediatric Intensive Care Unit; PPV, positive predictive value; RF, random forests; RNN, recurrent neural networks; RPART, recursive partitioning and regression tree; SVM, support vector machine, TAN, tree augmented naïve Bayes; TBSA, total body surface area.

**Selected studies based on the literature search performed for the purpose of this narrative review. Not meant to be systematic and exhaustive.*

***Variously divided in training, validation, and test sets.*

****Numbers in parentheses refer to specific boxes of **Supplementary Figure 1**, which provides a brief summary of the characteristics of the different ML model.*

*****Sensitivity reported at some fixed specificity and vice versa; the same applies to other metrics dependent on cut-offs.*

the portion of the curve that maximizes sensitivity (in order not to miss true cases and not to delay a potentially life-saving antimicrobial treatment). This would allow, once a low risk of missing true cases is determined, to compare models in terms of specificity (aiming at reducing the number of false positive cases treated with antimicrobials, thereby reducing useless toxicity and resistance selection). In any case, the definitive proof of usefulness of any classification model should be provided by randomized clinical trials comparing sepsis management based on its use vs. standard identification of sepsis with respect to a clinically relevant primary endpoint (e.g., short-term mortality). Finally, it should be kept in mind that the present perspective was aimed to highlight some issues that may hamper the comparability and extrapolation of results of available models, and not to primarily assess their performance, which is not reported in detail. Nonetheless, we think it may be of interest to highlight the wide heterogeneity of model results. For example, the best AUROC for the prediction of sepsis ranged from 0.68 to 0.99 in a recent systematic review, whereas sensitivity and specificity were inconsistently reported to allow proper comparison (92).

Another important aspect is the output of the model. While sepsis is the outcome (or dependent variable), the output of the model may be different according to the type of model (for a brief summary of the technical characteristics of different ML models employed in available studies of sepsis prediction, see **Supplementary Figure 1**) (98). For example, the output of logistic regression (but also, for example, of a sigmoid function-based output layer a of neural network), intended for a single patient, is his/her probability of experiencing the outcome (in this case having sepsis or not), based on his/her input features and the calculations of the trained predictive algorithm. Let's say, for example, that the algorithm calculates for a novel given patient, based on their clinical and laboratory data, a 40% probability of having sepsis. In terms of usefulness, this probability provided to doctors may be a clinically understandable value that can be easily weighted in the balance when deciding whether or not to administer antimicrobials, possibly also improving acceptance of the implementation of machine learning-based sepsis alerts in daily practice (99). However, clinicians should also be aware of the limitations of the model, in order to further improve an appropriate understanding and use of the output. In this regard, there are no standardized directives on the optimal way to present model pitfalls to clinicians together with the model output, although "model facts" labels have started to be proposed (100).

LIMITATIONS AND CONCLUSIONS

Given the narrative (and not systematic) nature of this brief narrative perspective, some original works may have not been included, a fact that, together with the lack of an in-depth description of computational aspects of the different algorithms, may represent major limitations of the present paper. Nonetheless, our intention was to provide a brief perspective for clinicians by addressing some topics of clinical interest concerning the application of machine learning techniques for the early detection of sepsis in the daily clinical practice. For this reason, we feel an extended focus on technical aspects would have been beyond the scope of the present manuscript.

In conclusion, the use of predictive tools based on machine learning may support medical decision-making by providing novel elements to improve the correct and early identification of patients with sepsis. Although at the present time it cannot be said yet whether or not this will ultimately improve patient survival and relevant antimicrobial stewardship outcomes, the increasing involvement of artificial intelligence and machine learning in health care cannot be ignored. In the long run, a rigorous multidisciplinary approach to enrich our understanding of the application of machine learning techniques to the early recognition of sepsis may be worth the trip and truly augment medical decision-making when facing this heterogeneous and complex syndrome.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

DG made substantial contributions to the study concept and design, first drafting of the manuscript, and critical revision of the manuscript for important intellectual content. AS, MG, PP, SM, LC, CR, LB, and MB made substantial contributions to the study concept and design and critical revision of the manuscript for important intellectual content. FD, AV, and FB made substantial contributions to the literature review and critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.617486/full#supplementary-material

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REVIEW
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Immunological Endotyping of Chronic Critical Illness After Severe Sepsis

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Improved management of severe sepsis has been one of the major health care accomplishments of the last two decades. Due to enhanced recognition and improved management of severe sepsis, in-hospital mortality has been reduced by up to 40%. With that good news, a new syndrome has unfortunately replaced in-hospital multi-organ failure and death. This syndrome of chronic critical illness (CCI) includes sepsis patients who survive the early "cytokine or genomic storm," but fail to fully recover, and progress into a persistent state of manageable organ injury requiring prolonged intensive care. These patients are commonly discharged to long-term care facilities where sepsis recidivism is high. As many as 33% of sepsis survivors develop CCI. CCI is the result, at least in part, of a maladaptive host response to chronic pattern-recognition receptor (PRR)-mediated processes. This maladaptive response results in dysregulated myelopoiesis, chronic inflammation, T-cell atrophy, T-cell exhaustion, and the expansion of suppressor cell functions. We have defined this panoply of host responses as a persistent inflammatory, immune suppressive and protein catabolic syndrome (PICS). Why is this important? We propose that PICS in survivors of critical illness is its own common, unique immunological endotype driven by the constant release of organ injury-associated, endogenous alarmins, and microbial products from secondary infections. While this syndrome can develop as a result of a diverse set of pathologies, it represents a shared outcome with a unique underlying pathobiological mechanism. Despite being a common outcome, there are no therapeutic interventions other than supportive therapies for this common disorder. Only through an improved understanding of the immunological endotype of PICS can rational therapeutic interventions be designed.

Keywords: inflammation, immunosuppression, aging, acute kidney injury, PICS

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SEPSIS IS A NATIONAL CRISIS

Sepsis afflicts over 1.7 million Americans annually, and accounts for over 250,000 deaths in the United States alone (1). More patients die from sepsis annually than from lung cancer, the number one cause of cancer-related deaths. Sepsis remains the most common cause of death in the intensive care unit (ICU), accounting for 1 in 3 hospital deaths (2). Sepsis is also the most expensive

in-hospital diagnosis in the U.S. today (3). Even with these staggering numbers, the impact of sepsis on patients, their families, and the community is grossly underestimated as survivors experience multiple ongoing morbidities (4). Sepsis has an annual patient care cost approaching $23 billion (more than $55 million per day) (5), which again is likely an underestimate, as the chronic effects of sepsis months to years post-intensive care unit (ICU) discharge are unknown (6).

Sepsis induces a profound state of both acute and chronic immune dysregulation, which contributes to both the mortality and long-term morbidities (7). These long-lasting morbidities include frequent re-hospitalization within the year following sepsis diagnosis, with the most common admission diagnoses being pneumonia or urinary tract infection (8). Interestingly, there are no FDA-approved therapeutics for the immunologic treatment of sepsis despite over 150 clinical trials and successful pre-clinical testing (9). In-hospital management remains primarily supportive in nature. Post-hospitalization, Prescott and Angus in their 2018 *JAMA* review of enhancing recovery from sepsis had only three recommendations: (1) identify new problems and treat appropriately, (2) review and adjust long-term medications, and (3) evaluate for treatable conditions that result in rehospitalization (10). None of these approaches target the unique immunologic and physiologic consequences of critical illness, as our understanding of the acute sepsis survivor remains incomplete!

SEPSIS IS AT ITS MOST BASIC CORE AN IMMUNOLOGICAL DISEASE

Sepsis is associated with an early/immediate "systemic inflammatory response" (11). Authors have used the terms "cytokine storm" and "genomic storm" for this early response (12, 13), but these are gross over-simplifications of an integrated innate and adaptive immune response. At its most fundamental, the host response we define as "sepsis" is due to the recognition and response of host protective immune systems to microbial pathogens and their products (termed pathogen-associated molecular patterns; PAMPS) or the consequential release of endogenous alarmins (danger-associated molecular patterns; DAMPs) (14, 15). Acutely, the response appears aimed at isolating microbial growth and limiting replication, although it is well-known that when the response is either exaggerated or becomes systemic, can be associated with microcirculatory defects, organ injury, and death (16, 17).

The term "storm" is prescient, since the response, especially when the exposure is systemic, can produce devastating widespread host responses, including microcirculatory failure, profound vascular and organ injury, shock and death (18, 19). In the 1980's and early 1990's when many of the cytokines/mediators involved in this "storm" were originally identified and cloned, the complexity and breadth of the immediate host response to microbial products was not fully understood. Initial efforts to modify the host response to sepsis targeted this early/immediate response by interfering with individual microbial products and early cytokine appearance (20). Unfortunately, antibodies or immunoadhesins to endotoxin, TNFα, IL-1, IFNγ to name a very few, have all failed to improve outcomes to sepsis (21).

Although the reasons for the failure of these attempts to prevent this early "storm" are clearly multifactorial, including timing, redundancy of action, and heterogeneity of the patient population, there was also an over-assumption that treating sepsis would only require identifying the "silver bullet" responsible for the immediate organ damage (22). If there is anything that the failures of the past three decades have taught, it is that successful treatment of sepsis is, and will continue to be, an iterative process dependent upon both increasing our basic understanding of sepsis pathophysiology and translating this knowledge into improved clinical management.

What was learned was that one key to improving in-hospital survival was earlier sepsis recognition and initiation of treatment (23). Rapid diagnosis and initiation of sepsis treatment bundles have been a major hospital-systems' accomplishment (24). A second important key was the implementation of standardized best practices in the management of the sepsis patient (25). Almost two decades after implementation of the Surviving Sepsis Campaign, a 25% improvement in compliance with best-practice has resulted in a 9% absolute reduction in 28 day all-cause mortality (26). In the largest study to date, with over 1 million subjects, the state of New York-mandated early interventions significantly improved sepsis survival compared to states that did not mandate early intervention (27).

CHRONIC POOR OUTCOMES ARE REPLACING IN-HOSPITAL MORTALITY

Earlier recognition and more consistent best-practice management have resulted in fewer patients dying early from the consequences of the "storm" (28, 29). This has resulted in more in-hospital survivors, and the appearance of late immunological complications of trauma and sepsis are now becoming the norm (30). As many as 33% of all sepsis survivors do not rapidly or fully recover but, instead, develop a new syndrome of "chronic critical illness" (CCI) (31–33). Chronic critical illness is represented by a persistent low grade inflammatory and chronic immunosuppressive phase associated with functional declines that can last from months to years following the acute event (**Figure 1**) (32).

These mechanisms underlying post-sepsis immunosuppression and inflammation are poorly understood, limiting our ability to prevent secondary infections and improve long-term outcomes in sepsis survivors. Chronic critical illness does not have a single consensus definition, like sepsis, but all define it as ongoing/persistent manageable organ dysfunction requiring hospitalization and increased resource utilization (e.g., ICU management) (31, 34). Based upon length-of-stay based mortality data from ICU patients at Shands UF Health, we set the duration of hospitalization at 2 weeks, and this definition has gained acceptance by others (35).

Our own studies have suggested that the severity of the initial acute phase, the age of the patient, the number of pre-existing comorbidities, and the influence of kidney injury, all impact

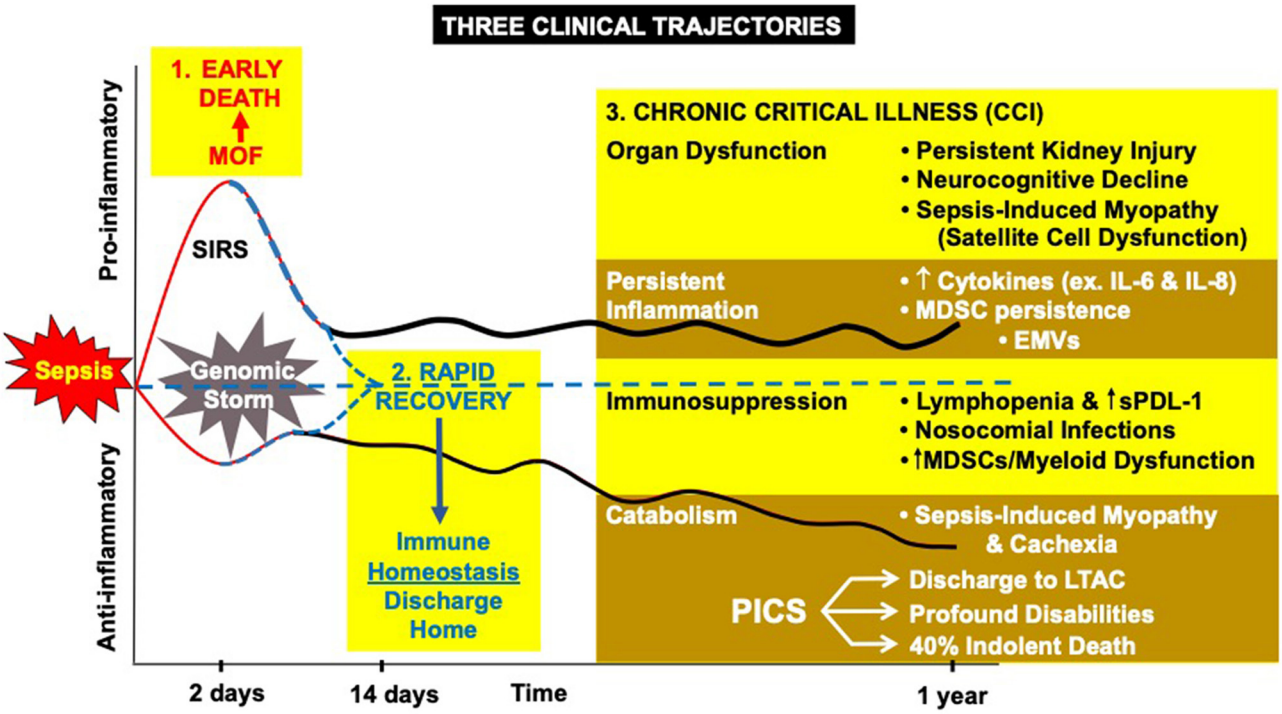


FIGURE 1 | The host response to severe sepsis can have three different clinical trajectories: (1) early MOF leading to death, (2) rapid recovery, or (3) the new appearance of chronic critical illness (CCI) characterized by organ dysfunction, inflammation, immune suppression, and protein catabolism. MDSC, myeloid derived suppressor cells; EMVs, endothelial microvesicles; sPDL-1, soluble protein death ligand 1; LTAC, long-term acute care hospital.

the development of CCI (36). Sepsis survivors who develop CCI experience a higher frequency of secondary infections, have longer hospital stays, and poor disposition (37). Dramatically, 60% of CCI patients are readmitted in the first 6 months (38), usually for recurrent infections, and 40% of these patients are dead at 6 months (39). Not unexpectedly, 70% of deaths are preceded by a withdrawal of care (40, 41).

CHRONIC POOR OUTCOMES AFTER SEPSIS ARE THE RESULT OF A MALADAPTIVE IMMUNOLOGICAL DYSCRASIA

Chronic critical illness represents the clinical manifestation/endpoint of a complex immunological "dyscrasia" that results in increased susceptibility to secondary infections; poor functional, physical and cognitive outcomes (34, 38, 39). We have argued that the metabolic and immunologic underpinnings of this response is the immunological endotype we have termed the Persistent Inflammatory, immunosuppressive and protein Catabolic Syndrome (PICS) (32, 42, 43). As summarized in **Table 1**, sepsis survivors with CCI experience the very common PICS endotype reflected by persistently elevated inflammatory cytokines and DAMPs, immune suppression, and an increased number of opportunistic infections.

A "phenotype" is defined as a set of observable characteristics of an individual resulting from the interaction of its genotype with the environment. CCI can be classified as a phenotype because of its reproducible characteristics: ongoing manageable organ injury requiring at least 14 days of ICU care. In contrast, an endotype is defined as a subtype of a condition represented by a **distinct functional or pathobiological mechanism** (44). Importantly, endotypes differ from phenotypes because the former requires a common underlying mechanism. Endotypes, unlike phenotypes, can be associated with clusters of disease. PICS would classify as an endotype, since it may well be a common underlying mechanism that can explain not only sepsis and trauma CCI, but could also explain in part, cancer cachexia, and the chronic inflammation and lean tissue wasting associated with chronic obstructive pulmonary disease, cardiac cachexia and chronic renal disease.

The immunological dyscrasia that defines PICS is multifactorial. Primary mechanisms leading to sepsis-induced impairment of adaptive immune system include: (i) apoptosis-induced T-cell depletion, (ii) T-cell exhaustion due to upregulation of inhibitory receptors or downregulation of essential co-stimulatory receptors, (iii) decreased bone marrow lymphopoiesis, and (iv) myeloid-based T-cell suppression, and myeloid cell dysregulation (45–47).

"Emergency myelopoiesis" is defined as inflammation-induced hematopoiesis, which is critical for the immediate management of tissue injury and controlling infection (48–50). In contrast to adaptive immune cells, such as T cells and

TABLE 1 | CCI phenotype in severe trauma and sepsis survivors.

Phenotype		Sepsis CCI
Persistent inflammation	Acute Phase Reactants—↑CRP, ↓Albumin	↑↑↑
	Cytokines	↑↑
	DAMPS, mtDNA, ncDNA, S100A8/A9, HMGB1	↑↑↑
Immune suppression	Absolute lymphocyte count	↓↓↓
	Secondary infections, sepsis readmission	↑↑↑
	MDSCs	↑↑↑
	$CD14^+$ HLA-DR expression	↓↓↓
	ELISpot T-cell IFNγ	↓↓↓
Protein catabolism	Body weight loss	↑↑
	Loss of lean tissue	↑↑
	Loss of physical function	↑↑

CRP, c-reactive protein; DAMPs, damage associated molecular patterns; mtDNA, mitochondrial DNA; s100A8/A9, calcium binding proteins that form calprotectin; HMGB1, high-mobility group box 1 protein; HLA-DR, Human Leukocyte Antigen DR isotype; IFNγ, Interferon gamma. ↑ increased, ↑↑ moderately increased, ↑↑↑ markedly increased; ↓ decreased, ↓↓ moderately decreased, ↓↓↓ markedly decreased.

B cells, that proliferate in response to their specific antigens, innate myeloid populations are continuously replenished from hematopoietic stem cells (HSCs) and progenitors in bone marrow (BM) and extramedullary (42, 51). However, the molecular mechanism of emergency myelopoiesis during infection remains incompletely understood. HSCs and hematopoietic progenitors can directly sense the presence of pathogens or endogenous alarmins *via* pattern recognition receptors (PRRs) such as Toll-like or NOD-like receptors (TLRs, NLRs), and they can also respond to pro-inflammatory cytokines such as interferon (IFN)-α, IFN-γ, interleukin (IL)-1, tumor necrosis factor (TNF)-α, and granulocyte colony-stimulating factor (G-CSF) (42). IFN-α and IFN-γ have pleiotropic effects on many cell types, including HSCs and hematopoietic progenitors (52). Importantly, these cytokines, along with IL-27, have been demonstrated to induce an expansion of HSCs and myeloid progenitors, leading to the production of differentiated PMNs, macrophages and dendritic cells at the cost of both lymphopoiesis and erythropoiesis (49, 50).

Dmitry Gabrilovich has argued that the long-term host response to cancer, chronic infection, and sepsis (**Figure 2B**) results in what he has termed "pathological activation of neutrophils and monocytes" (53). Weak activation signals that occur in sepsis survivors during CCI, such as endogenous alarmins released from low grade organ injury, secondary colonization and infection from invasive ICU procedures, immobility, and delirium, all result in the mild but consistent elevated production of inflammatory cytokines and signals that drive persistent "emergency myelopoiesis." What Gabrilovich has argued (54), and we have experimentally demonstrated in both trauma and sepsis (55), is that long-duration, low-grade inflammation drives pathologic myeloid cell activation and T-cell exhaustion, leading to both persistent inflammation and immune suppression (PICS).

During unresolved inflammation, such as following microbial infection, ongoing tissue injury, and other chronic conditions; the nature of signals affecting T-cells and myeloid cells differs from that seen during the early or immediate "genomic or cytokine storm" (56). Reduced bone marrow and thymic generation of new T-cells and increased expression of immunosuppressive receptors favors exhaustion and apoptosis of T-cell populations resulting in lymphopoiesis. The expansion of myeloid-derived suppressor cells (MDSCs) in sepsis is a complex and gradual phenomenon governed by multiple factors. Gabrilovich has argued that accumulation of MDSC requires two groups of signals: the first leading to expansion of immature myeloid cells and the second, pathological activation as MDSCs. The first group of signals is driven as a direct host response to the microbial pathogen and includes: GM-CSF, G-CSF, M-CSF, SCF, VEGF, and polyunsaturated fatty acids (PUFAs) (53, 57). Transcriptional factors/regulators including STAT3, STAT5, IRF8, C/EBPβ, and NOTCH play a major role in this process (58). Other factors involved in this process include adenosine receptors A2b, NLRP3, and alarmins S100A8 and A9 (59). Importantly, the second group of signals, resulting in the pathological activation of MDSCs, does not require an infectious process and can be provided by inflammatory cytokines and endogenous alarmins alone, which include interferon (IFN)-γ, IL-1β, IL-4, IL-6, IL-13, IL-27, TNF-α, and the TLR ligand, HMGB1 (59).

First and foremost, these stimuli drive the expansion of bone marrow and extramedullary myelopoiesis. Neutrophils and monocytes generated under these conditions display a variant phenotype and morphology. They are characterized by relatively weak phagocytic activity, increased levels of reactive oxygen species (ROS) and nitric oxide (NO) production, and high expression of arginase 1, PGE_2, and a number of anti-inflammatory cytokines (60, 61). Most of these features are absent in classically activated neutrophils and monocytes, which is why Gabrilovich has characterized this activation as "pathologic" (54). This state of activation leads not to the elimination of the threat or activation of host protective immunity, but to the inhibition of adaptive and innate immunity. Cells in this pathologic state of activation can be identified functionally, biochemically, and, to some extent, phenotypically, and are now termed MDSCs. The longer the myeloid compartment is exposed to the effects of factors described above, the more potent the pathologic activation of these MDSCs. Therefore, at any given moment, there is a heterogeneous population of cells in tissues represented by classically activated neutrophils, monocytes, and pathologically activated MDSCs (**Figure 2**). In the early stages of sepsis, bona-fide immune suppressive MDSCs are rarely detected (62, 63). However, there are cells with some biochemical and genomic characteristics of MDSCs, which probably represent an intrinsic part of MDSC development (53, 62).

The evidence that expansion of immunosuppressive MDSCs is a constant response to prolonged sepsis is incontrovertible. As early as 2007, we demonstrated that by 7 days post-sepsis, up to 95% of bone marrow cells are of myeloid lineage, mostly immature and functional MDSCs (64). These cells also overwhelm secondary lymph tissues, such as the spleen, lymph

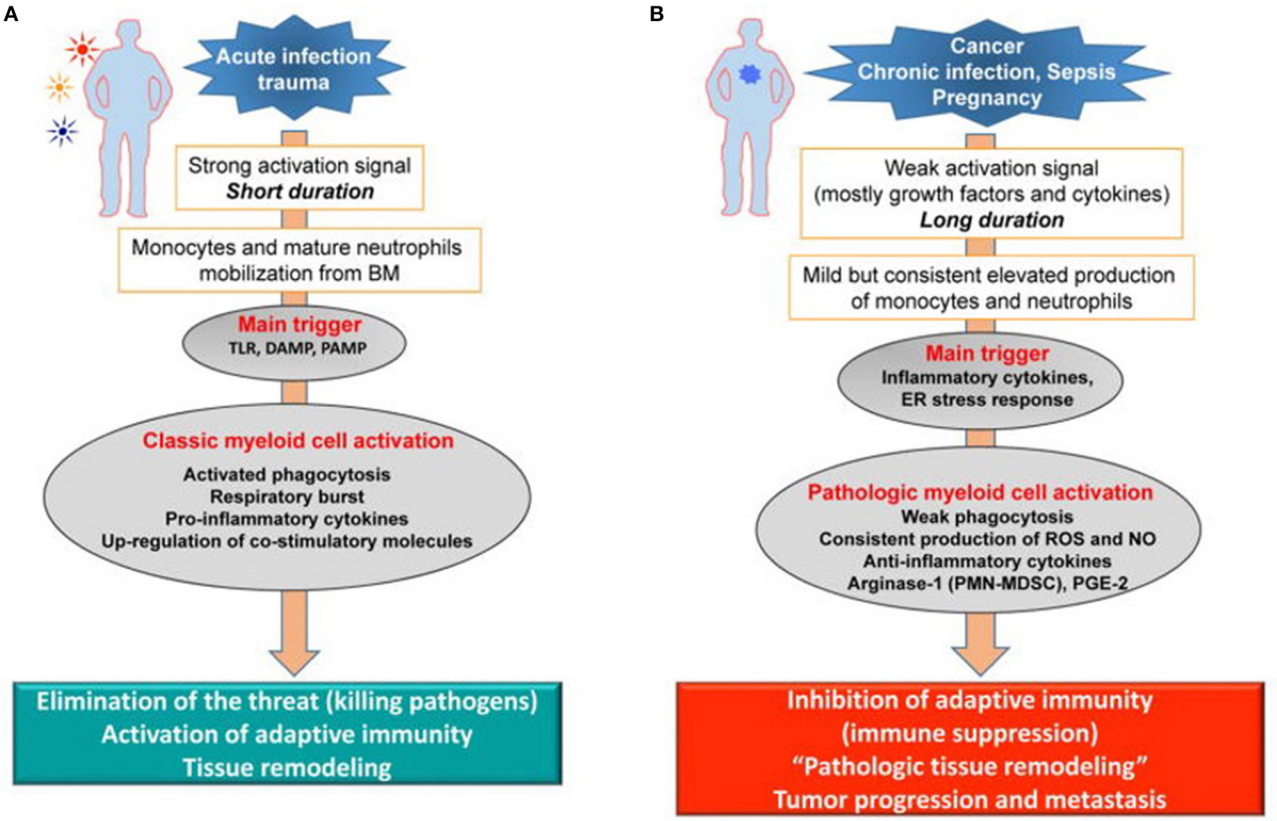


FIGURE 2 | Pathologic activation of neutrophils and monocytes. **(A)** In the presence of strong activation signals coming from pathogens in the form of toll-like receptors ligands (TLRL), damage associated molecular pattern (DAMP), pathogen-associated molecular patterns (PAMP) molecules monocytes and neutrophils are mobilized from the BM. This response results in classic myeloid cell activation. **(B)** In the presence of weak activation signal mediated mostly by growth factors and cytokines, myeloid cells undergo modest but continuous expansion. Pro-inflammatory cytokines and ER stress responses contribute to pathologic myeloid cells activation that manifests in weak phagocytic activity, increased production of reactive oxygen species (ROS), nitric oxide (NO), arginase 1 (not expressed in human monocytes and M-MDSC) and prostaglandin-E2 (PGE2). This results in immune suppression. Endoplasmic reticulum = ER. Modified from Veglia et al. (53).

nodes, and reticuloendothelial tissues (such as the lung and liver) (65). Importantly, we have demonstrated that rapid, sustained presence of MDSCs, and their quantitative levels are strong predictors of nosocomial infections and poor discharge outcomes in sepsis patients (7). These findings were confirmed by Uhel, who established that sepsis survivors with expanded MDSC populations had a higher rate of reinfection and hospital readmission (66).

Additionally, McCall et al. demonstrated that these cells evolve functionally over time, becoming more immunosuppressive (32). With regard to MDSC suppressor activity, Hollen et al. have observed considerable time-dependent differences in MDSC suppression of T-cell proliferation. Much to our surprise, but consistent with Gabrilovich's overarching hypothesis (**Figure 2**), pathological activation of MDSCs in humans did not occur immediately after sepsis, but required 7–14 days to develop fully. Regardless of whether MDSCs came from sepsis survivors who developed CCI or rapidly recovered, PBMC-derived $CD11b^{+}CD33^{+}HLA\text{-}DR^{dim}$ MDSCs obtained prior to day 7 were not immunosuppressive, while MDSCs obtained at or after day 14 (all CCI patients) suppressed both autologous $CD4^{+}$ and $CD8^{+}$ T-cell proliferation to antiCD3/CD28 (**Figure 3**). Also, septic MDSCs from day 14 (late), but not from day 4 (early), potently suppressed stimulated T-cell production of IL-2 and, to a lesser extent, IFNγ.

More interestingly, most of the MDSCs in septic CCI patients were granulocytic with a gene expression profile reflective of a highly inflammatory and immunosuppressive transcriptome (67). Analysis of individual gene transcripts from bulk cell-sorted human $CD11b^{+}CD33^{+}HLA\text{-}DR^{dim}$ MDSCs was consistent with suppressed HLA gene expression and up-regulated inflammatory gene expression (**Figure 4**). Canonical Pathway and Causal Network Analysis supported these pathway alterations and a pattern of simultaneous low-grade inflammation with immunosuppression.

Although G-MDSCs (granulocyte-like MDSCs) comprise the largest subpopulation of MDSCs in sepsis, expansion of M-MDSCs (monocytic MDSCs) and E-MDSCs (early MDSCs) is also observed. Importantly, different subpopulations of MDSCs are immunosuppressive through different mechanisms, and can, therefore, have different targets for intervention (53). To understand the rich "landscape" of blood MDSCs late after sepsis

(day 21), single-cell RNA sequencing and Cellular Indexing of Transcriptomes and Epitopes by Sequencing (scRNA-seq and CITE-seq) was conducted on enriched MDSCs obtained from peripheral blood mononuclear cells (PBMCs) (**Figure 5**). This was conducted to identify individual populations of MDSCs (G-, M-, and E-MDSCs) and their transcriptomic profiles in healthy and septic patients. In this case, samples were obtained at day 21 from two sepsis survivors with CCI, and samples were also obtained from two age and sex matched healthy, control subjects. Samples were first isolated on a Ficoll gradient, and then $CD11b^{+}CD33^{+}HLA\text{-}DR^{dim}$ cells were mixed 3:1 with original PBMCs to assure inclusion with all cell populations. sc-RNAseq of over 150,000 cells were conducted.

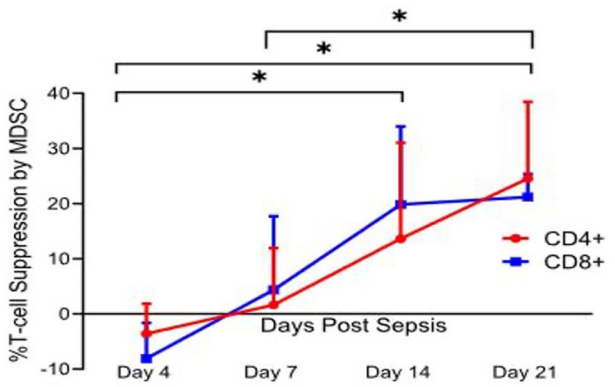


FIGURE 3 | Percent T lymphocyte suppression by MDSCs. Immature myeloid cells with the surface markers $CD33^{+}CD11b^{+}HLA\text{-}DR^{dim}$ were isolated on days 4, 7, 14, and 21 after sepsis, and from healthy control subjects. Autologous T lymphocytes were stimulated with soluble anti-CD3/28 and seeded in a co-culture with MDSCs in a 1:1 ratio. T cells were labeled with CellTrace Violet to detect proliferation, and a proliferation index (PI) was calculated for both $CD4^{+}$ and $CD8^{+}$ T cells using flow cytometric analysis. Percent suppression was calculated as the ratio of PI from stimulated T cells in the presence of MDSCs and the PI of stimulated T cells in culture medium alone. Percent suppression for both $CD4^{+}$ and $CD8^{+}$ T cells was significantly different between day 4 vs. 14 ($p = 0.0402$ and 0.0012), day 4 vs. 21 ($p = 0.0225$ and <0.0001), and day 7 vs. 21 ($p = 0.037$ and 0.045). There was no significance noted of percent suppression of $CD4^{+}$ and $CD8^{+}$ T cells between days 7 and 14 ($p = 0.17$ and 0.08). This T cell suppression was not seen in age-matched healthy control subjects. Modified from Hollen et al. (62). *indicates statistically significant intervals ($p < 0.05$).

As displayed in **Figure 5**, there was a dramatic expansion of the G-MDSC subpopulation and a less dramatic expansion of M-MDSCs in the sepsis patients. E-MDSCs, which were not detectable from healthy human subjects, were modest in sepsis survivors with CCI (detectable in only one of the sepsis subjects). G-MDSCs showed not only the greatest expansion, but also the most dramatic changes in their transcriptome. Interestingly, we did not see an increase in expression among genes that are associated with immunosuppression in cancer (such as *ARG1*, *CD274*, *COX2*, *PGE2*, and *NOS2*). While this was a small pilot study, it suggests that MDSCs present in sepsis may be inherently different from those seen in cancer-associated immunosuppression.

AGING PREDISPOSES THE DEVELOPMENT OF CCI AND PICS

The global population is rapidly aging (70, 71) with increasing healthcare resources and costs devoted to this group. The frequency of hospitalizations for sepsis in patients over 50 has increased, most dramatically in patients aged 65 years or greater (72). Advanced age is also associated with more severe organ failure, infectious complications, increased ventilator days, a longer ICU LOS, an increased 28-day mortality, and an increased likelihood of discharge to skilled nursing or long-term care facilities (73).

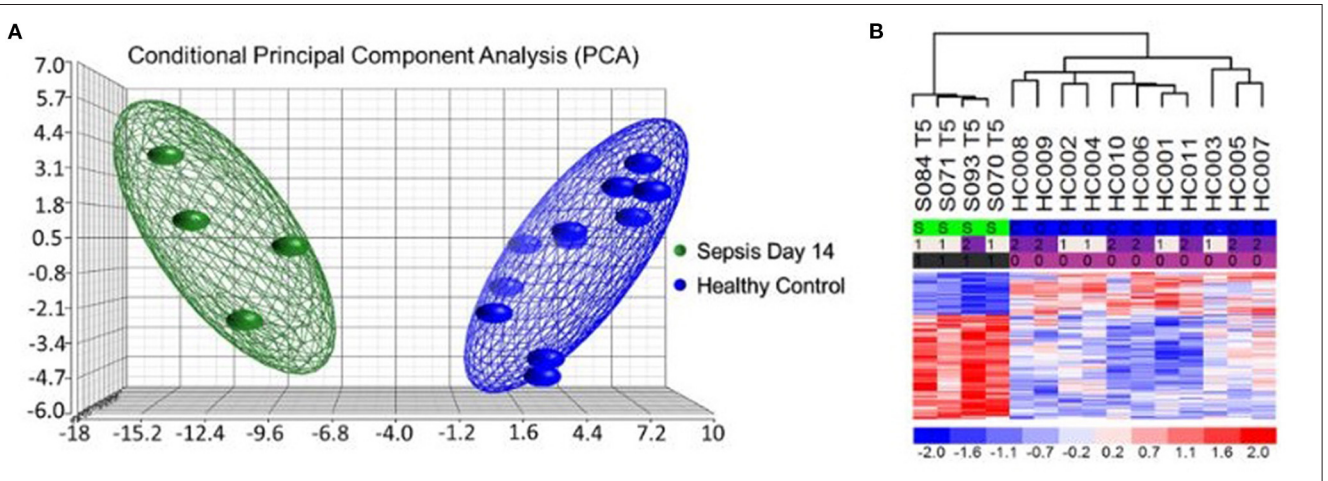


FIGURE 4 | Microarray Transcriptomic Analysis of MDSCs from Patients 14 days after Sepsis and Healthy Control Subjects. The genomic response of bulk isolated MDSC RNA in healthy controls and septic patients 14 days after initial infectious onset. **(A)** Conditional principal component analysis of septic (day 14) and healthy control MDSC gene expression patterns. **(B)** Heat map of the hierarchical clustering of MDSC gene expression patterns and variation between septic patients (S) from day 14 and healthy (H) control subjects. Modified from Mathias et al. (67).

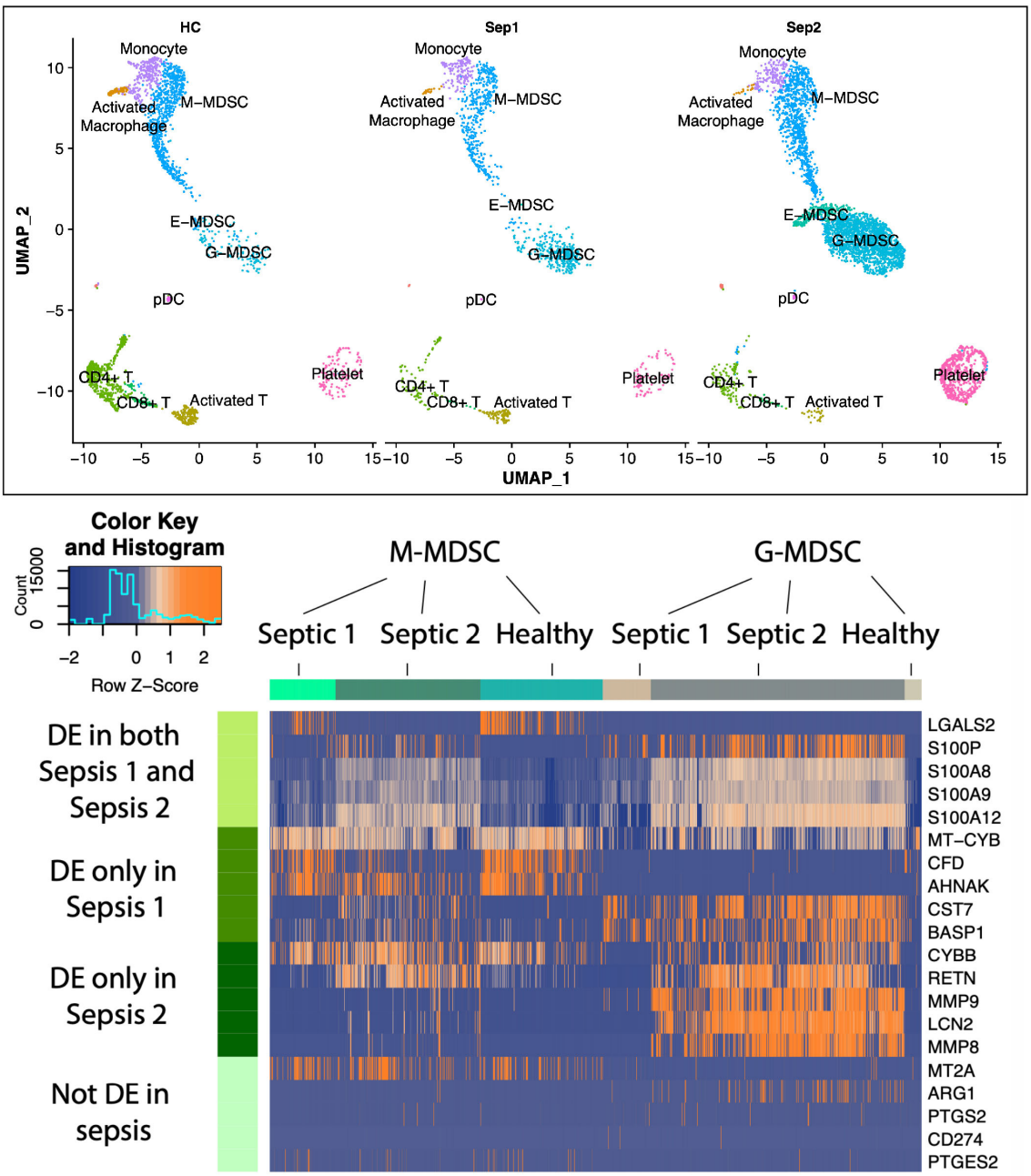


FIGURE 5 | Uniform manifold approximation and projection (UMAP) plots of cell clusters identified in healthy patients (n = 2,340 cells) vs. sepsis 1 (bacteremia, sepsis; n = 1,544 cells) and sepsis 2 (fungemia, septic shock; n = 5,587 cells) showing three distinct MDSC subsets. Heatmap illustrating expression patterns of MDSC subsets at 21 days post-sepsis vs. healthy control subjects. Rows represent the specific genes of interest differentially expressed in both sepsis patients; only sepsis patient 1; only sepsis patient 2; and genes not differentially expressed in this study, but previously determined to be important to MDSC function in cancer and autoimmunity. Number of columns represent number of cells analyzed in each group. DE, differentially expressed genes in sepsis vs. healthy controls. Colors represent mean normalized relative expression with blue representing reduced expression and orange, increased expression. Modified from Darden et al. (68).

As seen in younger populations, the in-hospital mortality from sepsis in the elderly is decreasing, but still remains significantly higher. The risk of CCI and discharge to a non-home destination is also increased in the elderly population and is multifactorial in nature (33, 72, 74). Contributing factors include senescence (normal aging), inflammaging (chronic, subclinical inflammation), comorbidities, lack of physiologic reserve, pre-existing disability, and epigenetic changes. These factors prevent older individuals from readily returning to homeostasis following critical illness and contribute to the increased risk of morbidity and mortality following sepsis (70, 71, 75, 76).

Aging has a profound role on the immune system. Immunosenescence is a state of age-associated changes in the immune system which is characterized by decreased ability to mount an effective response to pathogens (77, 78), decreased competency of the adaptive immune system (as evidenced by decreases in naïve peripheral T cells, repertoire diversity, and immunocompetent B cells) (77, 79), and dysfunctional myelopoietic effector cells (i.e., PMNs, monocytes/Mϕ, DCs, and NK cells) (78). Furthermore, the aged host's HSCs preferentially induce myelopoiesis, contributing to the substantial increase in MDSCs seen in this population as a response to the initial insult (78). "Inflammaging" is defined as chronic, low-grade inflammation that occurs with physiologic aging. It is a unique response seen in aged mammals and differs from the responses seen in the young. The "cytokine storm" seen in the younger population can be markedly attenuated or absent in the aged population, whereas immunosuppression appears to dominate (73). In these cases, early mortality is due, instead, to failure of host protective immune mechanisms to adequate microbial control.

In murine models, sepsis induces a rapid release of mature and immature myeloid cell populations from the bone marrow in response to endogenous and exogenous danger signals (55, 60). This creates niches in the bone marrow, which stimulate emergency myelopoiesis (80). Myelopoiesis predominates at the expense of lymphopoiesis and erythropoiesis (64, 80). Interestingly, elderly HSCs have this phenotype and function prior to critical illness, with myeloid-skewed cell production and a decreased ability to produce lymphoid cells (81, 82). These HSCs are also functionally inferior to their younger counterparts, with a lower functional frequency, delayed proliferative response, and reduced efficiency for short term homing (81, 82). These baseline dysfunctions are exacerbated by acute critical illness.

MUSCLE WASTING AND PROTEIN CATABOLISM SUSTAIN THE CCI RESPONSE

Skeletal muscle serves as the largest protein reserve in the body, which can be mobilized for metabolic substrates in times of stress. Critical illness is characterized by marked protein catabolism, which results from increased muscle breakdown, decreased protein synthesis, and the release of potential pro-inflammatory degradation products (83, 84). In patients who progress to CCI, this is a self-perpetuating cycle that results in profound cachexia. The exact mechanism has not been fully elucidated, but likely involves inflammation- and oxidation-associated direct mitochondrial and myocyte injury (85). Not surprisingly, muscle catabolism can result in the release of DAMPs (including mtDNA, HMGB1, and TFAM) into the systemic circulation, driving persistent inflammation (83, 86–88). In animal models, the mtDNA-TLR9-RAGE pathway, which can be activated by mtDNA or TFAM, has been shown to be involved in sepsis-induced cardiac inflammation (86, 89). As with other endogenous alarmins, these increases in both local tissue damage and systemic inflammation drives ongoing functional immunosuppression at both the level of the bone marrow (enhanced myelopoiesis) and functional lymphocyte populations (**Figure 6**). The role of MDSCs in cancer cachexia has been investigated for years, and several therapies are targeted at altering their function and have shown promise (90, 91).

Muscle wasting not only contributes to the ongoing inflammatory state seen in CCI, but also leads to a substantial functional disability (92, 93). Loss of skeletal muscle mass is associated with profound functional deficits which are most notable in the aging due to their baseline declining muscle mass (94). In these populations, even small functional changes can cause a shift from independent to dependent living (95). Decreased skeletal muscle mass is also associated with increased falls in the elderly, which are an independent predictor of 1-year mortality (96). Functional declines aren't limited to the aging population—over 80% of critical illness survivors report a reduction in physical ability (97). Physical limitations preclude patients from returning to work and contribute substantially to their reduced healthcare-related quality of life (97, 98). As seen with the self-perpetuating cycle of PICS in CCI patients, reductions in physical capabilities propagate ongoing mental health issues, economic hardship, and burden on the healthcare system.

ROLE OF ACUTE KIDNEY INJURY

Organ injury contributes to the ongoing inflammation associated with CCI. The kidney plays a crucial role in both initial and long-term survival from sepsis. AKI has been associated with increased in-hospital mortality and is more prevalent in sepsis than other critical illnesses (99). This relationship is bidirectional, as patients with acute and/or chronic kidney injury are also more likely to develop sepsis (100–103). Failure to resolve AKI is associated with both increased risk of initial mortality and progression to CCI (33, 104, 105). Chronic kidney disease (CKD) is not just a marker of CCI but perpetuates the cycle as well. Renal tubule epithelial cells are highly susceptible to oxidative stress and release large quantities of DAMPs. Urinary analysis of septic patients reveals increased levels of DAMPs and over expression of several DAMP receptors (106). These DAMPs act locally to increase secretion of chemokines by renal parenchymal and dendritic cells (DCs), which further promote local inflammation (107–109). They also have systemic effects, which are mediated by PPR toll-like receptors (TLRs) (110). TLRs are also upregulated in AKI *via* epigenetic remodeling, priming the renal tubule epithelial cells to release increased amounts of cytokines in response to antigen stimulation (111). This "hyper-responsive state," concomitant with decreased renal clearance, leads to amplified systemic inflammation and resultant organ injury (112, 113).

While the relationship between AKI and acute illness is well-established, the role of the kidney in CCI is more elusive. Its role in filtration exposes the kidney to over 30 times the blood volume daily (114), meaning renal DCs and lymph nodes are exposed to inflammatory mediators and pathogens significantly more than other tissues. This results in a positive feedback loop

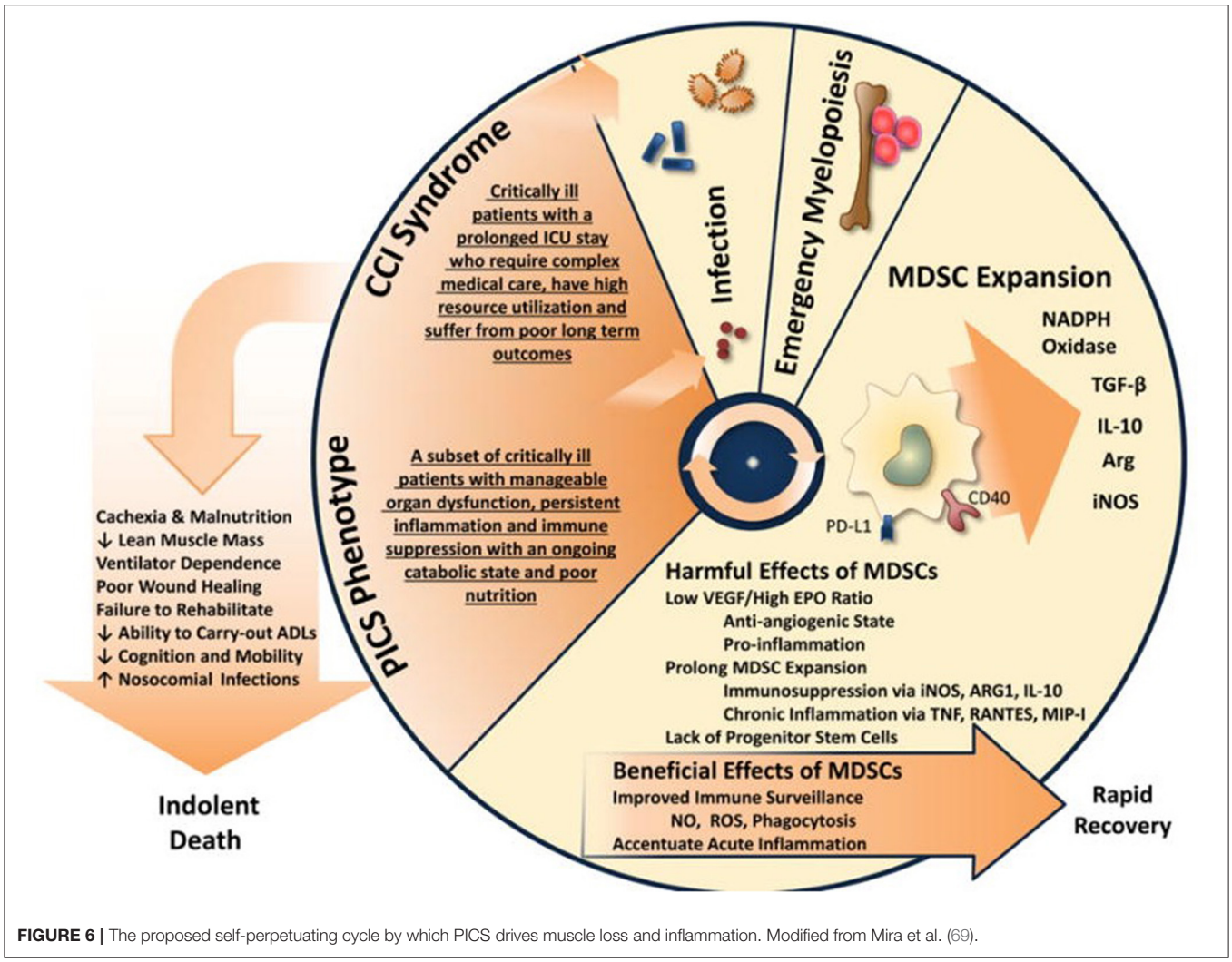


FIGURE 6 | The proposed self-perpetuating cycle by which PICS drives muscle loss and inflammation. Modified from Mira et al. (69).

in which exposure to these stimuli result in further oxidative damage and release of additional inflammatory mediators, both as a response to the filtered pathogens and to the ongoing tubular necrosis (115). As previously discussed with sepsis-associated muscle wasting, these self-perpetuating cycles of ongoing cell death and inflammation result in development of a persistent inflammatory state rather than return to homeostasis.

INVESTIGATIVE CHALLENGES

Sepsis is, at its foundation, a heterogenous disease process that occurs in a diverse patient population, especially aged and those with pre-existing comorbidities. The resulting CCI in many sepsis patients is inherently intertwined with the patient's prior comorbidities and functional status. This complex pattern of seemingly infinite variables makes both clinical decision-making and systematic investigation difficult. Human studies are difficult to standardize and depend on long-term participation of patients, many of whom are overburdened with their disease process.

Animal models have been generally successful in the investigation of early sepsis responses associated with the "genomic or cytokine storm." However, they fall short in modeling long-term processes. To better investigate these chronic mechanisms driving CCI, continued bidirectional translational research is required. Our group, along with several others, have proposed a semi-lethal cecal ligation and puncture (CLP) model with daily chronic stress to approximate the PICS endotype seen in human CCI (116–121). However, this model fails to accurately represent the sterile inflammation present in most CCI patients, and replaces it with a peritoneal abscess (122). This model also relies on young, otherwise healthy mice. It fails to capture the interplay between aging and comorbidities demonstrated in human patients. We have demonstrated that the sepsis response is notably different in aged mice when compared to their juvenile counterparts (123). Understanding these complex, interconnected mechanisms is crucial to further understanding of this disease process and development of therapeutic interventions.

MOVING FORWARD

Sepsis and CCI are immune dyscrasias at their foundation, and PICS is the predominant endotype behind CCI. Thus, a large majority of interventional studies have focused on the restoration of immune system homeostasis. Leukocyte growth factors (e.g., G-CSF) (124–128) immunomodulatory cytokines (e.g., IL-7, IL-15, and IFN-γ) (129–133) inhibitors of negative co-stimulatory pathways (e.g., anti-PD-1/PD-L1 Ab, anti-CTLA-4 Ab, anti-TIM3 Ab, and anti-LAG-3 Ab) (134–140) and the thymic peptide thymosin-α1 (141) have all been or are being investigated for potential benefits. These trials have been largely unsuccessful at finding a "silver bullet" cure, but some have shown promise in selective populations (124, 125). This is not surprising given the heterogenous disease process and highlights the importance of continued investigative efforts in endotyping these patients as a precision medicine approach. Oncology research has been successful in developing targeted therapies for specific cancer patients with PICS-like endotypes. These approaches may be applicable to sepsis-induced PICS, but further research is required.

Nutritional support is paramount in both the acute and chronic treatment of sepsis and CCI. As discussed before, the loss of skeletal muscle contributes to both the functional declines seen in CCI patients and the perpetuation of the PICS endotype. Early implementation of nutrition is clearly important, but the optimal protein and nutrient requirements remain undetermined. However, given their phenotypic similarities to cancer cachexia and aging sarcopenia, CCI patients likely have a daily protein requirement of roughly 1.5–2.0 g/kg/day (142–146). Arginine supplementation in sepsis remains controversial given its role as an intracellular substrate for nitric oxide. However, the upregulation of arginase-1 by MDSCs may result in a relative arginine deficiency (67, 147). Arginine is necessary for proper T-cell receptor function and wound healing (148, 149). Therefore, arginine supplementation may counteract the persistent arginine deficiency due to persistent MDSC expansion during PICS, promoting lymphocyte proliferation and improved tissue repair. Leucine is another amino acid that shows promising results, as it decreases muscle protein catabolism and induces protein synthesis (150). Leucine and other branched chain amino acids (BCAA) supplementation resulted in improved nutritional and immunologic parameters, such as nitrogen balance, prealbumin levels, and lymphocyte counts (151). It has also been shown to increase muscle protein synthesis through the mTOR pathway (152, 153). Studies in large burns have also shown promise using adjuncts such as insulin, oxandrolone, and propranolol to maintain an anabolic state (154–156).

Decreases in functional status are closely associated with decreases in health-related quality of life (QOL) among CCI patients. Maintaining, or improving, baseline functional status is the ultimate goal, but the prevention of unnecessary muscle loss is vital. Early ICU-based exercise and physical therapy programs have been associated with improved in-hospital outcomes, such as a reduction in the duration of mechanical ventilation and ICU length of stay. They are also associated with improved physical function after discharge (157). These programs, when combined with adequate nutritional support have demonstrated substantial improvements in muscle synthesis and functional outcomes (158). However, these have not been fully evaluated in the CCI population.

Technology and the field of medicine have developed rapidly over the past few decades. With the application of the Human Genome Project and the development of high throughput sequencing techniques, the development of individualized therapies has been made possible. These therapies have been remarkably successful in the treatment of cancer and congenital disease (159–161). Understanding the transcriptomic landscape of sepsis and CCI, and how they differ, is crucial to development of novel therapeutic agents for sepsis-induced CCI. Advances in technology have also made data collection and interpretation easier, making large, multicenter databases commonplace. The addition of biologic variables, in addition to clinical variables, will likely improve the prognostic power of these data sets and allow for early endotyping of patients (162–164).

"Big Data" is particularly useful in sepsis and CCI, as the disease process and patient population are increasingly heterogeneous. With the ability to quickly endotype a patient, accurate prognosis and optimal treatment is possible. We have shown recently that the leukocyte transcriptome within 48 h post-trauma is highly predictive of outcomes (165, 166). This technique, using regression-based prediction models, may be further improved by the use of machine-learning algorithms and deep-learning technologies (167, 168). Big data provides large sample sizes allowing for the identification of biomarker cutoff values with optimal sensitivity and specificity. However, these static thresholds fail to account for individual physiology; therefore, it is important that future efforts continue to improve upon precision medicine by integrating data from multicenter and multinational repositories with machine-learning and deep-learning technologies.

AUTHOR CONTRIBUTIONS

BF, DD, LK, PE, and LM drafted the manuscript. JR, SB, SL, and FM provided critical revisions. All authors made substantial contributions to the conception and design of the work, approved the submitted version of the manuscript, and agree to be accountable for all aspects of the work.

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REVIEW
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Adjunctive Immunotherapy With Polyclonal Ig-M Enriched Immunoglobulins for Septic Shock: From Bench to Bedside. The Rationale for a Personalized Treatment Protocol

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Septic shock still has a high mortality rate which has not hinted at decreasing in recent years. Unfortunately, randomized trials failed mainly because the septic patient was considered as a homogeneous entity. All this creates a sort of therapeutic impotence in everyday clinical practice in treating patients with septic shock. The need to customize therapy on each patient with sepsis has now become an established necessity. In this scenario, adjuvant therapies can help if interpreted as modulators of the immune system. Indeed, the host's immune response differs from patient to patient based on the virulence of the pathogen, comorbidity, infection site, and prolonged hospitalization. In this review, we summarize the rationale for using immunoglobulins as an adjunctive treatment. Furthermore, we would like to suggest a possible protocol to personalize treatment in the different clinical scenarios of the host's response to serious infectious events.

Keywords: immunoglobulins, septic shock, adjunctive treatment, sepsis, protocol

SEPTIC SHOCK: A HISTORY OF FAILING ADJUVANT TREATMENTS

Septic shock is a complex syndrome occurring when sepsis is associated with circulatory, cellular, and metabolic abnormalities to such an extent that the risk of death is substantially increased compared to sepsis alone. The clinical criteria to define this condition have recently been modified to improve its identification (1).

Despite the progressive comprehension of its pathogenesis, mortality rates are high and did not significantly change in the last 10 years. Septic shock hospital mortality was described as around 40% in a recent meta-analysis analyzing data from 71 studies from Europe and North America (2).

Another worrying aspect of septic shock is randomized clinical trials (RCTs) designed in the last years to test additional therapies that gave discouraging results.

Historically therapeutically strategies, some of them appearing promising in preclinical studies, have been developed based on septic shock pathogenesis.

One of the first targets identified and studied was endotoxin present in gram-negative bacteria, which has been blocked through different anti-lipid A antibodies without obtaining benefit in RTCs (3, 4). Similarly, the use of anti-TNF antibodies or anti-IL-1 antibodies was developed with the purpose to limit the innate immune hyperactivation responsible for tissue damage, but larger RTCs results were negative (5, 6). Endothelial dysfunction, frequently found in septic patients, was investigated trying to improve microcirculation and tissue oxygenation, but neither platelet-activating factor antagonist (7) nor activated protein C (8) reduced mortality.

Several different extracorporeal blood purification techniques have been developed in the last decades to remove inflammatory mediators. High volume hemofiltration was unable to reduce mortality in a recent meta-analysis (9), although was considered a safe technique. Hemoperfusion using filters coated with polymyxin B, aimed to remove endotoxins able to trigger the inflammatory response, displayed contrasting results in RCTs (10, 11). Concerning coupled plasma filtration and adsorption (CPFA) interesting results were obtained in the COMPACT 1 randomized study (12) but the COMPACT 2 trial was stopped earlier because of adverse events associated with CPFA. An urgent letter was sent to all CPFA users mentioning that CPFA is no longer indicated for the treatment of septic shock[1]. Among new membranes, Cytosorb a hemoperfusion cartridge able to remove broad-spectrum cytokines failed to find any decrease of IL-6 plasma levels over time (13); while a recent proof of concept pilot study demonstrated a significant effect on norepinephrine requirements (14). The new Oxyris membrane, a heparin-grafted membrane specifically designed for cytokine and endotoxin adsorption, tested on 16 patients seemed to effectively remove endotoxin and TNF-α, IL-6, IL-8, and IFNγ in patients with septic shock-associated acute renal failure (15).

Further, the unsuccessfully tested approach included immunomodulant and antioxidant therapies, aimed to reduce the overwhelming tissue damage caused by the excessive activation of the host's response. The very recent ACTS and ATESS RCTs failed to demonstrate improvement in organ function with a combination of vitamins and corticosteroids (16, 17). The promising results obtained by Meisel et al. about granulocyte-macrophage colony-stimulating factor in sepsis (18), unfortunately, have not yet found confirmation from other studies with adequate power.

As a consequence of this litany of negative studies, over the years the Recommendations provided by the Surviving Sepsis Campaign (SSC) Guidelines (19) for the management of septic shock have been progressively reduced, moreover being in most cases negative (i.e., prescribing not to do something), limiting the therapeutic space of maneuver for clinicians. Thus, although the role of the Guidelines is guided by the "not to harm" principle, stratification into subpopulations may be advisable in a heterogeneous setting.

[1]Available online at: http://www.salute.gov.it/imgs/C_17_AvvisiSicurezza_8135_azione_itemAzione0_files_itemFiles2_fileAzione.pdf

NOT ALL SEPTIC SHOCKS ARE THE SAME. IS IT POSSIBLE TO TAILOR PERSONALIZED MEDICINE?

Unfortunately, nowadays it is not easy to answer this question because sepsis is the expression of many different clinical scenarios. Patient characteristics, such as age, comorbidities, genetic factors, immune response, and previous antibiotics exposure are important factors involved in the evolution from sepsis to septic shock; thus, all these variables partly explain the difficulty of defining a unique treatment suitable for all patients.

In light of this observation, some authors developed a clinical staging system called PIRO aimed to characterize septic patients through four components: Predisposition (P), Insult/Infection (I), Response (R), and finally Organ dysfunction (O) evaluating time and number of failing organs. PIRO model can stratify infected patients in 4 stages defined by a progressive increase in mortality trying to reduce the heterogeneity of patients and to help a more tailored therapy (20, 21). Despite this attempt, complexity, and variability have been so far ignored by clinical trials enrolling patients only based on a septic shock diagnosis without considering selecting a specific and more homogeneous subgroup. The lack of patients' selection may in part explain the negative results obtained in all the RCTs so far designed.

The growing comprehension of the essential role played by the immune response in septic shock pathophysiology led to develop and test of some adjunctive treatments based on the use of immunomodulant molecules. To enlighten this fundamental point, it is useful to remember that the immune response during septic shock can be very different among patients. Some patients develop an overwhelming immune response, with a massive production of pro-inflammatory mediators such as cytokines, that are responsible for tissue damage, organ dysfunction, and early death (22). On the other side, some patients develop a condition of immune paralysis, characterized by a reduced ability of the immune system to face pathogens, leading to secondary infections and long-term mortality (22, 23). Generally, mortality in septic shock follows a biphasic curve; only a part of these patients dies during the first 3 days due to an irrepressible immune response, whereas the majority have an unfavorable outcome often a few weeks later, showing a profound impairment of the immune response (24). To confirm this, severe apoptosis-related depletion of cells of both innate and adaptive immune systems along with an increase in regulatory T cells and myeloid-derived suppressor cells, able to inhibit effector immune cell's function, have been documented (23, 25).

Although these scenarios are extreme simplifications of the complex imbalance between activation and suppression of immune response characterizing sepsis and septic shock, it appears clear that agents affecting immune function could be a strategy for implementing sepsis' treatment and improve survival rates.

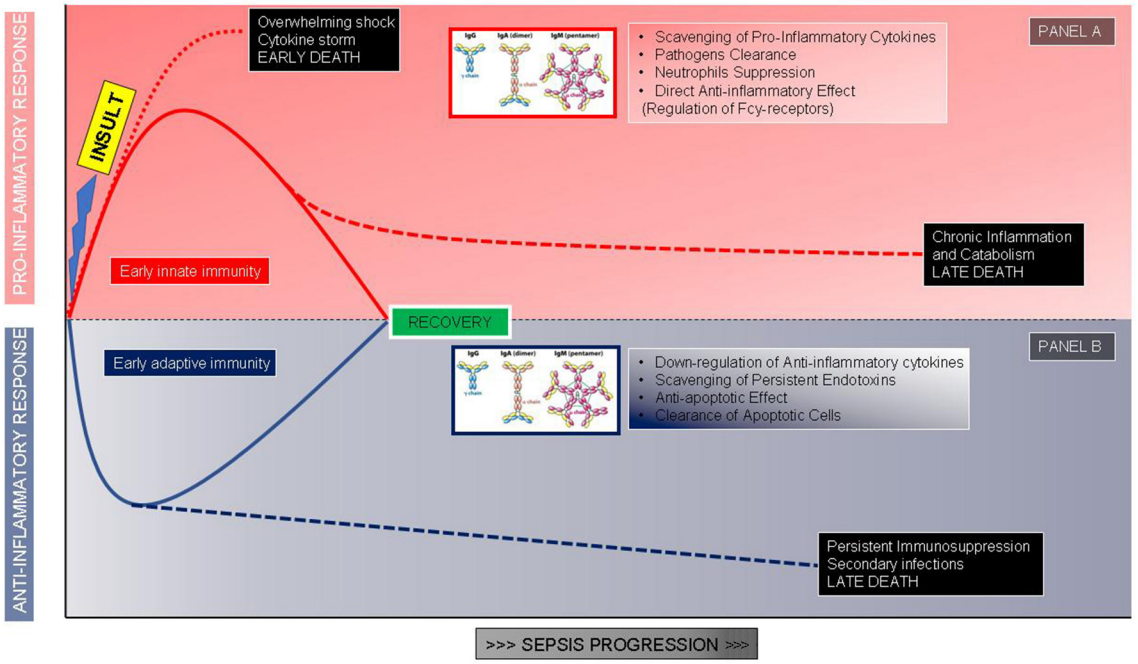


FIGURE 1 | The proposed role of immunoglobulins correlated to the phases of the host response after an infectious insult. **(A)** Hyperinflammatory response and role of immunoglobulins in modulating the proinflammatory storm. **(B)** Immunosuppressive response and role of immunoglobulins as immune system adjuvants.

THE RATIONALE OF ADJUNCTIVE THERAPY WITH POLYCLONAL INTRAVENOUS IMMUNOGLOBULINS

The evidence on Igs, here reported, was found through searches in the Medline (PubMed) and Scopus databases with the January 2000-September 2020 time limits. We narratively reviewed the efficacy and mechanisms of action of Igs, using the keywords "immunoglobulins," "mechanism of action," "efficacy" and "sepsis."

Among immune-modulatory treatments, intravenous immunoglobulins (Igs) administration may be a promising approach. The rationale for Igs use is both related to their pleiotropic effects on immune response and alterations of Igs serum levels observed in sepsis and septic shock. The potential benefits of Igs administration are related to their immune-modulating functions acting both on pathogens and immune cells: IgG and IgM can scavenge and remove toxins, to bind pathogens promoting phagocytosis, through opsonization and bactericidal activity of CD8+ lymphocytes mediated by antibody-dependent cellular cytotoxicity (26) (**Figure 1**). These molecules also display direct anti-inflammatory effects mediated by Fc receptor consisting in the modulation of dendritic cell function and reduction in response to INF-γ (27), promote the clearance of apoptotic cells, exert an anti-apoptotic action on immune cells, reduce the activity of the classical complement pathway, due prevalently to IgM, stimulate anti-inflammatory cytokines production along with a reduction of the anti-inflammatory ones favoring a balance between activation and suppression of immune response typically defective during sepsis and septic shock. Igs have also an important synergic activity with antibiotics enhancing their antibacterial efficacy (**Figure 1**). In addition to the pathophysiological rationale, several studies found a correlation between the reduction of circulating Igs concentrations in septic shock patients and poor outcomes. Venet et al. measured IgG and IgM serum titers in septic shock patients during the first 4 days after diagnosis and found out that Igs deficiency is an important predictive factor for patients' mortality (28). Another study reported an important reduction in IgM levels when patients' conditions deteriorated from severe sepsis to septic shock, underling the importance of Igs kinetic to predict patient's evolution; moreover *in vitro* stimulation with phytohemagglutinin of lymphocytes isolated by septic shock patients displays a reduced ability to produce IgM compared to healthy subjects (29). Despite the relatively high number of studies evaluating this additional therapy (30–36), the scarce number of patients included and the heterogeneity in terms of the type of preparation, dosages, durations, the severity of enrolled subjects, and type of controls impaired the significance of results (**Table 1**). Nevertheless, two recent meta-analyses revealed a reduction in mortality using polyclonal Igs compared to the control arm and suggested that the highest total dose range is probably more effective (37, 38).

The SSC Guidelines (19) suggested against the use of intravenous Igs in patients with sepsis or septic shock. This conclusion was mainly guided by the results of the only RCT on the use of intravenous Igs judged satisfying in terms of numbers, design, and risk of bias. The study revealed no difference in

TABLE 1 | Description of Igs preparation, dose prescribed, and duration of treatment in different randomized clinical trials in adults evaluating Igs efficacy as adjunctive therapy in sepsis and septic shock.

Study	Type of IG preparation	Dose	Cumulative dose (g/Kg)	Duration (days)
Toth et al. (30)	IgGAM	5 mL/kg for 3 days	0.75	3
Werdan et al. (31)	IVIG	0.6 g/kg on day 0 and 0.3 g/kg on day 1	0.90	2
Hentrich et al. (32)	IgGAM	1,300 mL for 72 h: 200 mL initially (0.5 mL/min), then 11 infusions of 100 mL every 6 h	0.30	3
Rodríguez et al. (33)	IgGAM	7 ml/Kg daily	1.75	5
Darenberg et al. (34)	IVIG	1 g/kg on day 1 then 0.5 g/kg on day 2 and day 3	2.00	3
Tugrul et al. (35)	IgGAM	5 ml/kg	0.75	3
Karatzas et al. (36)	IgGAM	5 ml/kg	0.75	3

The evaluated period was January 2000–September 2020. IgGAM, Immunoglobulins G, A, and M; IVIG, polyclonal standard IgG.

survival at 28 days, but many limitations reduced its power: long duration from 1991 to 1995, publication only 12 years later, and lack of detail of patients' severity (31). To stress the deficiency of evidence and the lack of agreement among the experts it is important to remind that the last version of the Japanese guidelines for the management of sepsis mentions Igs as a possible therapeutic option (39). At present two different types of preparations obtained from plasma of healthy donors are available: polyclonal standard IgG (IVIG) and IgM-enriched formulation (IgGAM); IVIG contains at least 96% of polyclonal IgG whereas the composition of IgGAM, consisting in IgM 12%, IgA 12%, and IgG 76%, appears to trace more accurately the physiological antibodies' production in course of infection. Both preparations can induce pathogen clearance but the higher killing on gram-negative bacteria is obtained with IgM-enriched Igs (40). The presence of IgM seems to be particularly important because of their specific roles in immune response (29). The characteristics of IgM along with the better results obtained with IgGAM in terms of the odds ratio that is a reduction of mortality's relative risk compared to IVIG in the meta-analysis (41) suggest their preferential use in septic shock. Finally, a recent pilot trial evaluating the use of IgGAM in patients with a diagnosis of sepsis or septic shock observed an improvement in sublingual microcirculation in treated patients (42) suggesting a role in the restoration of endothelial cell function as shown in previous preclinical studies (43). The authors of a recent review evaluating the use of Igs as adjunctive therapies in severe infections in ICU suggest that so far insufficient evidence is available to support their use except for streptococcal toxic shock syndrome. However, they underline the importance to identify the proper Igs type of preparation together with dose, the timing of administration, and patients' characteristics (44). Considering the importance of timing to maximize the effect of immune-modulating therapies, Berlot et al. showed that early administration of IgGAM in septic shock patients was associated with a reduction in the risk of in-ICU mortality (45). A multicenter, double-blind, randomized, controlled trial using hyperimmune Igs to treat patients affected by severe H1N1 infection found that their early use within 5 days of symptom onset was associated with reduced viral load and reduced mortality (46). More recently Yun et al. observed that the administration within 48 h of admission in ICU of intravenous Igs as adjuvant therapy in patients suffering from SARS-CoV-2 pneumonia was associated not only with a reduction in the need for mechanic ventilation but also with a reduction of mortality and in-hospital length of stay (47). Summarizing, we can conclude that there is a pressing need for more precise use of Igs in terms of patients' selection, dosage, and timing rather than excluding their benefit in treating sepsis and septic shock.

IMMUNOGLOBULINS' SIDE EFFECTS

There are no absolute contraindications to the use of Igs. Each product containing Igs is different so a patient with a life-threatening reaction to one product may have no reactions with other preparations. Thus, the contraindications are related to the particular component of the Igs product (48).

Even if in most cases Igs infusions are well-tolerated, several adverse effects have been reported having a wide range of incidence (49); these side effects are more frequently transient but rarely are serious and can lead to long term disability. Two types of risk factors for adverse effects have been identified: one related to Igs preparation and another to the patient's characteristics. Considering Igs formulation a higher concentration of IgA, anti-Rh and anti-RhD increase the prevalence of adverse reactions (50), on the other side some authors reported a greater risk of side effects in patients with primary antibody defects particularly in those with low IgA levels (51), attention should be paid also in patients with a previous history of allergies and thrombotic events. Immediate adverse effects are the majority and consist of flu-like symptoms (which are the most frequent), dermatologic reactions, arrhythmia, hypotension, and transfusion-related acute lung injury (52). On the contrary delayed adverse reactions, although affecting <1% of treated patients, can be severe and in rare cases lethal; these include renal impairment, hematological and neurological disorders, electrolytes alterations, transfusion-related infections, and thrombotic events (52). The incidence of the adverse effects is strictly related to the rate of Igs infusion, therefore during the first administration, it is recommended to start slowly in the first 30 min increasing subsequently the rate (53).

FROM BENCH TO BEDSIDE: A PERSONALIZED PROTOCOL FOR IGGAM ADJUNCTIVE TREATMENT

Based on the need to select the correct phenotype for Igs treatment we decided to use "case examples." The use of these "case examples" could be beneficial to reduce complexity and define some categories of patients uniform in terms of the immune response.

The first "example" is represented by a previously healthy young adult who develops meningococcemia or severe pneumonia sustained by Streptococcus pneumonia or toxic shock syndrome; in this patient, we could expect an overwhelming pro-inflammatory response, not balanced by an adequate anti-inflammatory response, able to eradicate bacteria but leading to tissue damages and multiorgan failure in the early phase of shock (**Figure 1A**). In this case, we could take advantage of the Igs ability in pathogen clearance, toxin, and mediators scavenging along with the anti-inflammatory effects; the administration should be early and at high dosages to block as soon as possible the hyperactivation of the efficient immune system and limit organ damages (26, 54, 55). Swedish surveillance data on Igs administered in toxic shock syndrome reported a significantly improved survival in treated patients with an odds ratio of 5.6 for Igs use (56). While a very recent prospective multicenter Scandinavian study identified as a risk factor associated with mortality the non-administration of Igs in patients with necrotizing soft-tissue infections (57).

The second "example" is a patient with persisting or breakthrough infection after a first sepsis episode: i.e., candidemia after abdominal surgery or multi-drug resistant (MDR) pathogen infection after a previous infection. The context, in this case, is a reduced pro-inflammatory response combined with a pronounced or sustained anti-inflammatory state with persisting or secondary infections. The patient may manage to limit the pathogen growth, thanks mainly to antibiotic therapy, although a complete clearance of bacteria is not reached, with the persistence of infection leading at a later time to the patient's death (**Figure 1B**). Another typical example is the appearance of breakthrough infections sustained by opportunistic relatively avirulent pathogens such as Acinetobacter or Stenotrophomonas species. In this condition Igs will be useful thanks to the anti-apoptotic effect on immune cells and the ability to clear apoptotic cells, rather than for pathogen phagocytosis; the time window of administration, in this case, could be broader. Infections caused by opportunistic pathogens, often MDR, are a clear marker of an immune-suppressed status and some evidence suggests the use of IgGAM in this specific condition (26, 55). In our experience on septic shock patients sustained by MDR, preexisting cancer, by itself a condition of immune response impairment, and *Acinetobacter baumanii* infection were independently correlated with an increased risk of 30-days mortality whereas only IgGAM administration appeared to be beneficial (58); similar results were obtained in Greece, a country where the MDR prevalence is extremely high, by Giamarellos-Bourboulis et al. showing, in severe infections by MDR gram-negative bacteria, a protective effect of IgGAM administration on 28-days mortality (59).

To categorize patients and progress toward a more personalized medicine we developed a protocol for IgGAM use in septic shock (**Figure 2**) aimed at applying this adjunctive therapy to appropriate patients at the right time and dosage.

Community and hospital-acquired septic shocks were first of all distinguished. In the context of the community-acquired infection, the cornerstone is the immediate identification of an overwhelming shock condition, such as necrotizing fasciitis or meningococcemia. Community-acquired septic shocks, developed in non-immunocompromised patients and characterized by the need for high noradrenaline dosages (>0.4 mcg/kg/min) and deep endothelial dysfunction must be promptly identified as overwhelming shock conditions. Endothelial dysfunction may be defined through the CID score, a simple score based on laboratory data: platelet count, fibrinogen, and fibrin markers (i.e., D-dimer or fibrin split products) plasma concentration and prothrombin time, used to identify endothelial alteration and risk of disseminated intravascular coagulation (60). In these conditions, IgGAM therapy should be administered as soon as possible, in any case within 3 h, starting the first day with a higher dose of 500 mg/kg/day (doubling the standard dose) then continuing with 250 mg/kg/day for 3–5 days or up to clinical improvement (**Figure 2**). The aim is to use IgGAM to scavenge toxins and to improve the clearance of bacteria boosting antibiotic effect and promoting phagocytosis.

If an overwhelming shock is not present, patients coming from the community should be evaluated to identify a possible immune-suppressed status: a previous antibiotic exposure in the last 30 days, neutropenia, defined by a neutrophil count $<1{,}000/mm^3$, or on immunosuppressive therapy, including long term use of corticosteroids are even individually sufficient to define a condition of immune-depression. In this case, Igs administration should be started within 6–12 h from shock occurrence, when noradrenaline dosages are higher than 0.1 mcg/Kg/min. IgGAM in that instance should be used at a standard dose of 250 mg/Kg/day for 3–5 days (**Figure 2**); we don't need a high dose to control an excessive cytokine release or to reduce the bacterial burden, but we take advantage of the immune support given by the treatment, being usually endogenous Igs reduced. In this case, the goal is to favor the balance between pro and anti-inflammatory response thanks to the Igs' role in modulation of pattern recognition receptors (inflammasomes), signaling pathways (NF-kB), and effector molecules (cytokines) and their direct anti-apoptotic effect on immune cells.

If a septic shock patient coming from the community does not show any signs of overwhelming shock nor of immune suppression the administration of a standard dose of 250 mg/Kg/day for 3–5 days should be considered after 12–24 h (**Figure 2**), when noradrenaline administration >0.1 mcg/Kg/min is persistently necessary to maintain target pressure and/or in case of a significant worsening of the CID score (60) indicating a non-positive evolution of the patient's status.

Hospitalized patients often have an impaired immune function due to multiple predisposing factors including multiple

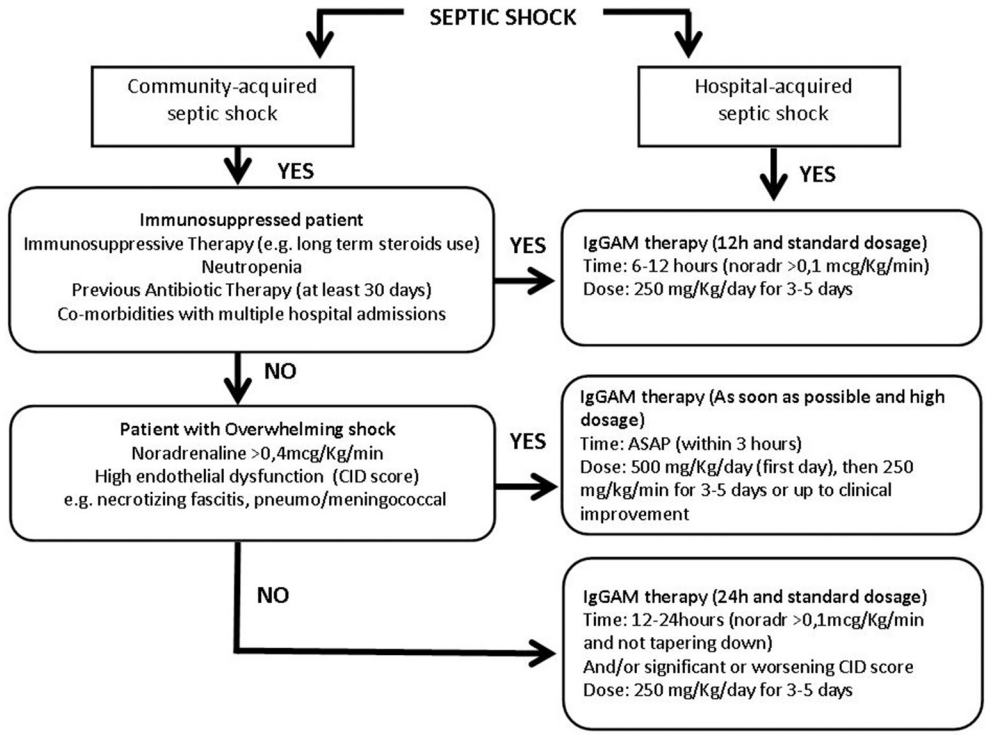


FIGURE 2 | Flow chart of the immunoglobulins' treatment protocol in patients with different septic shock phenotypes. More details in the text. IgGAM, Immunoglobulins G, A, and M; ASAP, as soon as possible; Noradr, Noradrenaline.

comorbidities, frequent exposition to antibiotics, high risk of MDR pathogens colonization/infection and, therefore, these patients should be a priori considered as immune-compromised and should be treated in the same way of immuno-suppressed patients coming from the community: within 6–12 h from shock occurrence, when noradrenaline dosages are higher than 0,1 mcg/Kg/min, at the standard dose of 250 mg/Kg/day for 3–5 days (**Figure 2**).

This simple protocol could help to roughly divide patients based on pre-existent conditions and shock characteristics waiting for further studies aimed to better clarify the efficacy of Igs administrations in this setting.

Alongside this suggested clinical protocol, we are running a multicenter RCT "Efficacy and Safety of Adjunctive IgM-enriched Immunoglobulin Therapy with a Personalized Dose Based on Serum IgM-titers vs. Standard Dose in Patients with Septic Shock," which aims to compare whether a personalized dosage of IgGAM, to achieve and maintain serum titers above 100 mg/dl, could show a different impact on 28-day mortality than a standard dose (250 mg/kg for 3 days) *(NCT04182737).*

CONCLUSIONS

Septic shock requires further studies on the use of adjuvant therapies. To date, a process of selecting potentially profitable patients in terms of personalized medicine is preferable. To this purpose, we believe that a protocolized use of Igs therapy may be an option in the treatment of septic shock, however any type of protocol must be validated by a RCT before extensive clinical use.

AUTHOR CONTRIBUTIONS

SB, ER, MT, EB, and IC have written the initial draft. MM, SD, LB, RG, and MG reviewed the final version. ER, MT, EB, and IC also helped to draw the figures. All authors have written and reviewed the manuscript.

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REVIEW
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Current Challenges in the Management of Infective Endocarditis

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Infective endocarditis is a relatively rare, but deadly cause of sepsis, with an overall mortality ranging from 20 to 25% in most series. Although the classic clinical classification into syndromes of acute or subacute endocarditis have not completely lost their usefulness, current clinical forms have changed according to the profound epidemiological changes observed in developed countries. In this review, we aim to address the changing epidemiology of endocarditis, several recent advances in the understanding of the pathophysiology of endocarditis and endocarditis-triggered sepsis, new useful diagnostic tools as well as current concepts in the medical and surgical management of this disease. Given its complexity, the management of infective endocarditis requires the close collaboration of multidisciplinary endocarditis teams that must decide on the diagnostic approach; the appropriate initial treatment in the critical phase; the detection of patients needing surgery and the timing of this intervention; and finally the accurate selection of patients for out-of-hospital treatment, either at home hospitalization or with oral antibiotic treatment.

Keywords: infective endocarditis, transesophageal echocardiography - three-dimensional echocardiography, positron - emission tomography, *Staphycoccus aureus*, viridans group streptococci, *Enterococcus*

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INTRODUCTION

First described by the French physician Lazare Rivière more than 350 years ago, the clinicopathological manifestations of the infection of heart valves were better characterized through the enormous contributions of William Osler at the end of the 19th century (1). The infection affects the endocardial surface of the heart, most commonly the valves, but also may occur on mural endocardium, on cardiac septal defects, on arteriovenous or arterioarterial shunts and on intravascular devices. The current name of this infection, infective endocarditis, was popularized in the 1960s by Lerner and Weinstein to cover other possible, but infrequent etiologies in addition to bacterial infections (2).

Although the incidence of infective endocarditis seems to be slightly increasing (3), this disease continues to be a relatively rare, but severe cause of sepsis. Currently, up to 40–50% of affected patients require valve surgery at some point during the clinical course, with overall mortality remaining around 20–25% per year in most published series (4). Although the classic clinical syndromes of acute or subacute endocarditis are still observed to a certain degree, current clinical

forms have changed. Furthermore, there have been profound epidemiological changes in high-income countries, with a clear and progressive increase in the number of cases associated with prosthetic valves and intravascular devices (3).

In this narrative review, we will address: (1) the epidemiological changes mentioned above; (2) several new advances in the understanding of the pathophysiology of infective endocarditis and in endocarditis-triggered sepsis; (3) new diagnostic tools; and finally (4) therapeutic aspects such as the relevance of early surgical treatment in selected cases, new available drugs and new useful treatment strategies.

The PubMed database was used to search medical literature published in English from 1st January 2010 to 30th October 2020, using the search terms "infective endocarditis" AND "epidemiology" OR "pathophysiology" OR "diagnosis" OR "treatment" OR "management." We reviewed both original and review articles, excluding case reports and editorial articles. Some earlier published articles were also included due to their relevance to this review.

EPIDEMIOLOGY

Infective endocarditis is considered an infrequent disease, with an annual incidence ranging from 1.5 to 15 cases per 100,000 inhabitants. It displays significant international variation (5, 6). The highest rates have been found in the United States, while the incidence is lower in Denmark. There is a significant lack of epidemiological information from Asia, Oceania and Latin America. Furthermore, the incidences can vary significantly even within the same country (7). Even though the scarcity of epidemiological data, particularly from low-income countries, a slight increase in the incidence of infective endocarditis has been noted since 2000. In this regard, controversy remains about the true impact of restricting antibiotic prophylaxis in high-risk patients, as recommended by NICE guidelines (8), with some investigations showing an increasing trend of infective endocarditis afterwards. The efficacy and impact of this strategy on antibiotic resistance are yet to be fully addressed (9).

Toward a Nosocomial Profile of Infective Endocarditis

A predisposing condition, such as rheumatic heart disease, is nowadays less commonly detected among cases of infective endocarditis, although the importance of such predisposing conditions persists in low-income countries (10). Cases associated with intravenous drug use have decreased globally, but a dramatic increase of this habit continues to affect the epidemiology of endocarditis in North America (11) and in some Eastern European countries (12). Other risk factors are being increasingly detected in high-income countries, such as degenerative valve disease, intracardiac devices (both cardiovascular implantable electronic devices as well as left ventricular assist devices), indwelling catheters and immunosuppression. This explains why the latest analyses of the demographics of endocarditis cases show a trend toward nosocomial characteristics in high-income countries: older patients, staphylococcal (both *Staphylococcus aureus* and coagulase-negative staphylococci) (13, 14) and enterococcal cases (15), and the involvement of prosthetic valves and cardiovascular implantable electronic devices (CIEDs) (3, 4, 11, 16, 17). By contrast, the oral streptococcal (so-called "subacute") endocarditis classically associated with rheumatic heart disease has become less frequent (5, 18).

Infrequent Etiologies and Culture-Negative Endocarditis

Other etiologies of endocarditis are infrequent: 2–5% of cases can be produced by Gram-negative bacilli (both aerobic Gram-negative bacilli or by the known HACEK group: *Haemophilus* spp., *Aggregatibacter actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens*, *Kingella kingae*). Also uncommon are fungal endocarditis that can represent <2% of cases, mostly produced by yeasts of the *Candida* spp. genus or rarely by other yeasts or filamentous fungi.

Finally, a variable proportion (up to 10–20% of cases) without documented etiology are considered "culture-negative endocarditis," mostly as a consequence of prior administration of antibiotics or caused either by fastidious slow-growing microorganisms or by truly non-cultivable intracellular bacteria (e.g., *Coxiella burnetii*, *Chlamydophila* spp, *Bartonella* spp, *Tropheryma whippelii*) (4, 19).

Infective Endocarditis Associated With Transcatheter Aortic Valve Implantation

Transcatheter aortic valve implantation (TAVI) has led to a revolution in the management of valvular heart disease. This technique has become a suitable alternative to surgery in elderly patients with aortic valve stenosis who carry a high or moderate surgical risk (20). Therefore, the number of TAVI procedures has dramatically increased in recent years (21). The incidence of endocarditis associated with TAVI has been estimated to be 0.8–1.4% (22, 23). A meta-analysis comparing endocarditis following TAVI vs. surgical replacement found no difference in the overall incidence (24). Endocarditis after TAVI displays characteristics of healthcare-associated infections, with a high predominance of staphylococcal and enterococcal infections (23, 25, 26). In-hospital mortality of TAVI-associated endocarditis is elevated, strongly influenced by the epidemiological profile of the patients (27).

Endocarditis and Sepsis

Sepsis and septic shock are severe complications that may arise from any type of infection, with poor early and late prognoses in the patients affected. A study on 894 episodes of infective endocarditis showed that 17.4% of the patients had septic shock at any time during hospitalization (28). A multivariate analysis suggested that *S. aureus* and signs of a persistent infection were independent predictors for the development of septic shock, alongside a previous diagnosis of diabetes mellitus and other systemic complications such as acute kidney injury and supraventricular tachycardia. Furthermore, the multivariate study indicated that the development of septic shock at any time during hospitalization was strongly associated with in-hospital

mortality. Similar results were obtained in a study comparing endocarditis diagnosed "early" or "late" after the development of first symptoms. That study showed that the so-called "acute endocarditis" was more frequently presented as or complicated with septic shock (29). Sepsis and septic shock are associated with a 4-fold increase in the probability of death (28, 30).

Morbidity and Mortality

Despite advances in diagnosis and therapeutics, infective endocarditis presents a significant morbidity burden and a remarkably high overall mortality (20–25% of cases). Endocarditis-related mortality has remained steadily high since 2000 and is strongly associated with several risk factors, such as advanced age, a high Charlson comorbidity index, non-community acquisition, prosthetic valves, staphylococcal infections, perivalvular complications, stroke, and the non-performance of surgery when indicated (3, 4, 11, 16, 31, 32). Of note, studies on psychological outcomes in survivors after an episode of endocarditis have demonstrated a reduction in quality of life and the occurrence of posttraumatic stress disorder (33).

INFECTIVE ENDOCARDITIS PATHOPHYSIOLOGY

The concurrence of several pathogenic events is required for the development of infective endocarditis, which partly explains the relatively low incidence of this disease.

From the seminal studies performed in animal models, it has been well known that inducing infective endocarditis in the absence of pre-existing endothelial damage is extremely difficult (34, 35). Important predisposing conditions are prior valvular involvement, classically rheumatic or currently due to degenerative disease (as well as the presence of prostheses or endovascular devices). These structural alterations induce turbulent blood flow that causes mechanical stress on the vascular wall, ultimately producing endothelial injury. More recently, the ability to induce experimental endocarditis in structurally healthy, but inflamed valves has been demonstrated (36). This mechanism could explain the development of endocarditis in previously normal hearts in patients with infections caused by aggressive microorganisms (e.g., *S. aureus*) and with endothelial inflammation caused by sepsis itself or by other agents that cause vascular damage.

After the initial endothelial damage or inflammation, the second key pathogenic event is the deposition of sterile fibrin-platelet aggregates in these injured areas. These lesions, leading to what is known as "non-bacterial thrombotic endocarditis," have been described in up to 2.4% of the autopsies performed in patients with certain underlying diseases (37) and are the ideal niche for the subsequent anchoring of the bacteria seeded in the bloodstream. Not all bacteria from the bloodstream, however, have the same ability to colonize these lesions (38). Gram-negative bacilli, for example, are particularly susceptible to humoral innate immune responses (39). Furthermore, certain Gram-positive microorganisms, particularly some species of streptococci and *S. aureus*, have specific molecules on their surfaces called adhesins, such as "microbial surface components recognizing adhesive matrix molecules" (MSCRAMMs) and "secretable expanded repertoire adhesive molecules" (SERAMs). These adhesins recognize integrins, specific ligands located on the injured or inflamed endothelial surface. Since the 1980s to 1990s, a repertoire of molecules of the MSCRAMM and SERAM type have been described in detail for streptococci and *S. aureus* (40–44), which interact not only with the endothelium, but also with platelets and key proteins of the clotting cascade (45–47).

The last important pathogenic event is the maturation and growth of the fibrin-platelet aggregates, which clump together with bacteria at a high inoculum (more than 10^9 colony-forming units per gram of vegetation) in what is known as "vegetation." Within this vegetation, bacterial communities are partly organized into complex biofilms, embedded in the fibrin-platelet aggregate and in a matrix of macromolecules produced by them. The arrival of phagocytes and antibiotics can be compromised in these structures, inside which bacteria can also modify their metabolism toward persistent phenotypes, with greater tolerance to antibiotics (48–50). The vegetation represents the pathological hallmark of infective endocarditis and determines its main clinical manifestations, namely: (1) the growth of this "full bacterial lesion" causes continuous bacteremia at a high inoculum that can seed distant septic metastases, (2) the invasion of the structures to which this vegetation is anchored can cause valvular destruction, negatively affecting the patient's hemodynamics, and finally (3) this friable mass can detach pieces that cause distant embolisms, which can significantly affect the function and prognosis of the affected patients. In recent years, intensive studies have been carried out in this field. Through pharmacological manipulation, successful attempts have been made to hinder the interactions between bacteria and the endothelium, effectively preventing the development or modulating the severity of infective endocarditis in animal models treated with antiplatelet and anticoagulant drugs (51, 52). Although the possible prophylactic role of some antiplatelet or anticoagulant drugs has been reinforced by observational clinical studies (53–55), the prophylactic use of these drugs has not been effectively transferred to clinical practice yet (56).

Endocarditis-Triggered Sepsis and Septic Shock

As described previously, a non-negligible proportion of patients with endocarditis may present with severe sepsis or septic shock (28), which can eventually lead to multi-organ failure. This complication appears to be associated with some characteristics of the patients (57) as well as with particularly virulent microorganisms such as *S. aureus* and beta-hemolytic streptococci (58). In addition to their invasive and destructive effects on the affected anatomical structures, these bacteria can seed distant septic metastases. Moreover, they display a repertoire of other virulence mechanisms, including the excretion of exotoxins that can act as superantigens, which overactivate the immune system (59–61). The systemic inflammation that is consequently triggered has an important hemodynamic impact,

with generalized endothelial dysfunction and a drop in vascular resistance. Any increases in the compensatory cardiac output may be hampered by sepsis itself, a phenomenon known as "septic cardiomyopathy" (62), or by the destruction of the valve as a result of the infection. This extremely serious situation explains why the presence of septic shock is associated with a significant increase in the risk of mortality.

DIAGNOSTIC TOOLS

The diagnosis of infective endocarditis relies on a combination of clinical, microbiological and imaging information, as specified by the modified Duke criteria (63, 64). The classic combination of clinical features of infective endocarditis remains a critical feature in diagnosis, particularly for subacute or chronic endocarditis. The mainstay of diagnosis involves the information provided by blood cultures and different imaging techniques that can detect anatomical changes such as valve vegetations or associated complications. However, a shift toward more acute infections and the involvement of prosthetic materials have decreased, to some extent, the usefulness of applying these classic clinical features in diagnosis. The performance of the modified Duke criteria has been thus compromised in the era of non-community acquired infective endocarditis. Therefore, modifications have been proposed to improve their sensitivity (65, 66). Nowadays, the diagnostic accuracy of these criteria seems to largely rely on the development of new and more sensitive non-invasive imaging techniques (**Table 1**).

Echocardiography

Transthoracic echocardiography (TTE) is the main imaging method in the diagnosis of endocarditis, with varying sensitivity rates for valvular and paravalvular abnormalities such as vegetations (sensitivity around 65%), a new regurgitation or dehiscence of a prosthetic valve, perforations, abscesses and fistulae (64). Transesophageal echocardiography (TOE) provides a better detection and characterization of local abnormalities (sensitivity for intracardiac vegetations of ∼95%), particularly when TTE is negative, in the case of valvular or paravalvular complications as well as in prosthetic valve endocarditis (PVE) and endocarditis associated with CIEDs (67).

Three-dimensional TOE (3D-TOE) may complement conventional TOE. Although its contribution to the diagnosis of endocarditis is not clearly established (68, 69), its main value is in providing a detailed description of vegetations, regurgitations and abscesses in both native (70) and prosthetic valve endocarditis (71). This technique can also differentiate vegetations from thrombi (72) and can be used in surgical planning. Intraoperative TOE has been demonstrated to be useful in the surgeries for endocarditis and has been proposed for use in routine exploration (73, 74).

Cardiac Computed Tomography

Although TOE remains the mainstay in the diagnosis of infective endocarditis, there is growing interest in the application of cardiac computed tomography (CT). This tool shows good anatomical correlation, especially when diagnosing a perivalvular abscess of the aortomitral intervalvular fibrous body and other structures surrounding the aortic root, thus overcoming the limitations of TOE (75–77). Recent data support cardiac CT as an adjuvant exploration technique when a better depiction of valvular complications is needed or when echocardiography proves to be insufficient in both native (77) and prosthetic valve endocarditis (78–80). Furthermore, cardiac CT is frequently used to preoperatively assess the presence of coronary artery disease in aortic endocarditis where performing a coronary angiography carries a prohibitive high risk of the dislodgment of vegetations (74). A recent study from Wang and colleagues added a prognostic value to cardiac CT, suggesting a synergistic role with TOE in surgery planning and in predicting early and late mortality (81).

Positron Emission Tomography/Computed Tomography (PET/CT)

To complement the detection of anatomic abnormalities, progress has been made in measuring biological activity through ^{18}F-fluorodeoxyglucose positron emission/computed tomography (FDG-PET/CT) and radiolabeled white blood cell single-photon emission CT/CT (WBC-SPECT/CT). Their use has been recommended by the European Society of Cardiology in patients with suspected PVE for valves implanted for more than 3 months (74), with a positive result included as a major criterion for the diagnosis of prosthetic valve and device-related endocarditis. Prospective studies of patients with suspected PVE have revealed a remarkable performance of FDG-PET/CT and WBC-SPECT/CT in the diagnosis of PVE (82, 83). They suggest that these two imaging techniques can be used in a stepwise fashion when evaluating the presence of endocarditis. FDG-PET/CT should be used first, since it has higher sensitivity, and when the results are not conclusive, WBC-SPECT/CT may be performed. Similar results have been obtained with both FDG-PET/CT (84, 85) and WBC-SPECT/CT (86–89) for suspected CIEDs endocarditis. Controversy remains on the use of FDG-PET/CT in patients with aortic root grafts with a prosthetic valve, since a high rate of false positives has been observed in relation to surgical adhesives (82, 90). FDG-PET/CT performs very well in the diagnosis of PVE when adjusting for confounders such as the low inflammatory activity caused by the initiation of antibiotic treatment (82, 90, 91), suggesting that FDG-PET/CT should be performed as soon as possible when infective endocarditis is suspected. Furthermore, FDG-PET/CT has proven prognostic value in PVE by correlating with major cardiac events (92).

The role of FDG-PET/CT in the diagnosis of native valve endocarditis has not been fully established and may be limited to cases where endocarditis is strongly suspected but the Duke criteria are not totally met. Studies on the use of FDG-PET/CT in native valve endocarditis are mostly retrospective and might overestimate the sensitivity of this technique (93, 94). In such cases, FDG-PET/CT may have an impact on diagnosis by detecting extracardiac complications of endocarditis (95).

The increasing use of TAVI and left ventricular assist devices (LVAD) have led to challenges in diagnosing the infective complications associated with their use. A recent study in

TABLE 1 | Imaging and laboratory techniques used in the diagnosis of infective endocarditis.

Transthoracic echocardiography (TTE)	Mainstay of diagnosis. Limited sensitivity in prosthetic valve endocarditis and paravalvular complications.
Transesophageal echocardiography (TOE)	Mainstay of diagnosis. Indicated in prosthetic valve endocarditis and for the detection and characterization of valvular and paravalvular complications.
Three-dimensional transesophageal echocardiography (3D-TOE)	Complement to TOE when characterizing valvular and paravalvular complications. Surgical planning
Intraoperative transesophageal echocardiography	Routine exploration when surgery is performed. Assessment of post-operative anatomy and ventricular function.
Cardiac computed tomography (CT)	Better characterization of complications involving an aortomitral fibrous body and the aortic root. Alternative to coronary angiography for exploration in patients with aortic endocarditis.
Positron emission tomography/computed tomography (PET/CT)	Major Duke criteria for diagnosis of prosthetic valve endocarditis and device-associated endocarditis. Increasing use in infective endocarditis associated with TAVI and LVAD. Detection of extracardiac complications of endocarditis or alternative sources of infection.
Radiolabeled white blood cell single-photon emission computed tomography/computed tomography (WBC-SPECT/CT)	Complement to PET/CT when results are non-conclusive. Similar performance to that of PET/CT.
Brain magnetic resonance imaging (MRI)	Detection and characterization of silent or clinically evident intracranial complications. Evaluation of evolving changes in intracranial complications that may influence the timing for surgery.
Thoracoabdominopelvic computed tomography	Not recommended as a routine exploration in asymptomatic patients.
Contrast-enhanced ultrasound	Promising results on detecting intraabdominal emboli as an alternative to CT.
Colonoscopy	Detection of colorectal disease or neoplastic processes in *Streptococcus gallolyticus* or *Enterococcus faecalis* endocarditis.
Culture and polymerase chain reaction of samples from explanted heart valves	Improves the diagnostic performance of the Duke criteria.
Serum cytokine profiles and bacteria-targeting tracers	Under research; not routinely used.

TAVI, transcatheter aortic valve implantation; LVAD, left-ventricle assistance devices.

patients with suspected TAVI-related endocarditis showed that the inclusion of FDG-PET/CT led to the reclassification of 36% of the patients diagnosed with "possible endocarditis" by the modified Duke criteria (96). Moreover, FGD-PET/CT and WBC-SPECT/CT have been used in cases of endocarditis associated with LVADs, showing variable rates of sensitivity and specificity (97–100). WBC-SPECT/CT and FDG-PET/CT show similar sensitivity and specificity, but the former may be more challenging to perform since it uses a more difficult protocol and requires the manipulation of blood specimens.

FDG-PET/CT is also useful in revealing unexpected sources of primary infections and detecting extracardiac complications of endocarditis. Thus, its use can lead to changes in treatment plans (101). However, no recommendations have been made in international guidelines regarding the detection of extracardiac complications.

Cerebral Imaging

Recommendations for neuroimaging in infective endocarditis remain unclear. Brain CT is often used when neurological symptoms are present, although brain magnetic resonance imaging (MRI) has better sensitivity in defining lesions. Clinically silent complications of the central nervous system, such as embolisms, may occur in up to 60% of patients (102). Some centers routinely perform brain MRI when infective endocarditis is diagnosed since it may provide additional diagnostic findings for fulfilling the modified Duke criteria and may change therapeutic plans (103) or the timing for surgery (104). However, while major intracranial hemorrhages and extensive ischemic stroke worsen prognosis after valve surgery, brain MRI findings of clinically silent complications do not affect postoperative mortality (105). In this sense, the clinical significance of cerebral microbleeds, one of the most frequently encountered silent lesions, remains to be elucidated (106, 107).

Other Diagnostic Tools

Systematic thoracoabdominopelvic CT has not demonstrated a clear utility in the diagnosis of left-sided endocarditis in asymptomatic patients. Furthermore, it increases the risk of kidney toxicity (108). Nevertheless, the finding of pulmonary embolisms in chest CT may be useful in the diagnostic workup of right-sided endocarditis either on the tricuspid native valve in intravenous drug addicts or associated with pacemakers (109). Contrast-enhanced ultrasound may be useful as an alternative for the detection of abdominal complications, mostly spleen infarctions (110).

Regarding the detection of the portals of bacterial entry, colonoscopy has proved to be very useful. The relationship between *Streptococcus gallolyticus* and colon cancer has been well known since the 1950s (111). Two observational studies on infective endocarditis caused by *Enterococcus faecalis* found a high rate of colorectal disease when a colonoscopy was performed, with a high incidence of neoplastic disease

particularly in those with an unknown source of bacteremia (112, 113). A systematic and multidisciplinary search for portals of bacterial entry has been proposed, suggesting that a meticulous physical examination should be performed when evaluating patients with infective endocarditis (114).

Procedures involving polymerase chain reaction (PCR) using samples from explanted heart valves could improve diagnostic performance (115) in comparison with conventional cultures. Although interesting from a theoretical point of view, those molecular techniques applied to the detection of genetic material in blood samples for cases of culture-negative endocarditis have shown low sensitivity so far (116). Finally, there is an increasing interest in finding new predictors of mortality in patients with endocarditis through the use of serum cytokine profiles (117) or bacteria-targeting tracers alongside diverse nuclear imaging techniques (118). Regarding acute-phase reactants and biomarkers of severe infection, procalcitonin levels are significantly increased in the cases of endocarditis complicated with sepsis or septic shock when compared to cases without these complications (119). No other biomarker has demonstrated a good predictive value in this setting. Current research of biomarkers in endocarditis is focused on proteomic analysis of some molecules that are not yet in routine clinical use (120).

To conclude, progress has been made through the development of new diagnostic techniques and improvements in known ones. However, for a complex and systemic disease such as infective endocarditis, it seems that diagnosis will be further improved by using a refined combination of clinical, microbiological and multimodal imaging information.

MANAGEMENT

The available evidence to guide antibiotic treatment of infective endocarditis is composed mostly of data from observational studies and some from experimental animal models. A recent Cochrane review analyzed the evidence from six small clinical trials evaluating various antibiotic regimens for endocarditis with a range of etiologies. After an in-depth analysis, the authors of the review concluded that given the high risk of bias, insufficient data, or underpowered designs, the evidence offered by these trials did not support or reject any of the regimens evaluated (121). This poor evidence may partly explain the significant heterogeneity in the management of this disease observed in some surveys (122) and the lack of adherence to some recommendations of the European guidelines even among those who developed them (123).

With these caveats in mind, when treating this infection, the pathophysiological peculiarities of infective endocarditis must be taken into account, such as the presence of very high concentrations of bacteria protected from immune responses within vegetations and the potential existence of bacteria with reduced metabolism embedded in biofilms. For these reasons, it has been postulated that to achieve microbiological eradication, the treatment of infective endocarditis must involve bactericidal antibiotics administered parenterally in high doses and for prolonged periods (124).

The selection of the antibiotic regimen for a particular case of endocarditis is often complex and beyond the scope of this review. All the details on this are available in the published guidelines (74, 125). However, in the next few paragraphs, we will offer a brief overview of the common regimens that are used against the most frequent microorganisms that cause infective endocarditis.

Antibiotic Therapy

Cell Wall-Active Antibiotic Treatment

The cornerstone of the antibiotic treatment of endocarditis is the use of beta-lactams in high doses: penicillin or ceftriaxone for the viridans group streptococci, ampicillin for *E. faecalis*, and antistaphylococcal penicillins or first-generation cephalosporins for methicillin-sensitive *S. aureus* or coagulase-negative staphylococci. In allergic patients or in those with infections caused by strains resistant to beta-lactams, the alternative is usually another cell wall-active agent, such as vancomycin for the viridans group streptococci and *E. faecalis* or vancomycin or daptomycin for staphylococci. Due to the limited amount of evidence, treatment regimens for endocarditis caused by coagulase-negative staphylococci are usually extrapolated from those recommended for treating endocarditis caused by *S. aureus* (126).

Antibiotic Combinations

The synergistic combination of aminoglycosides and beta-lactams demonstrates rapid bactericidal action, allowing treatment to be shortened to 2 weeks for native valve endocarditis caused by susceptible viridans group streptococci (127). This antibiotic combination is also recommended for treating infections caused by the viridans group streptococci that are partially or fully resistant to penicillin, although this recommendation is based on less robust evidence (128, 129).

The combination of ampicillin and aminoglycosides is essential for at least the first 2 weeks of treatment (130) to reduce the risk of relapse in endocarditis caused by *E. faecalis*, given the relative tolerance of these bacteria to beta-lactams (which is also observed in other species such as *Granulicatella adiacens* and *Abiotrophia defectiva*). When this combination cannot be used due to high-level aminoglycoside resistance or an unacceptable risk of toxicity, combination with ceftriaxone (so-called "double beta-lactam") is recommended, which achieves bactericidal action presumably through the complementary saturation of penicillin-binding proteins (PBPs) (131, 132).

In endocarditis caused by *S. aureus*, the combination of beta-lactams and aminoglycosides is currently not recommended for native valve infections due to the increased risk of renal toxicity without any relevant clinical benefit (133, 134). It is only recommended for PVE according to experimental data, although some retrospective series question its usefulness even in that scenario (135).

The use of rifampicin in a combination treatment for native valve endocarditis caused by *S. aureus* is discouraged (136), but could be useful in PVE, given its potent activity in infections involving biofilms. Guidelines recommend this regimen for PVE, but only after clearing blood cultures to avoid the emergence of

resistant mutants during treatment (136). In any case, this drug may not be essential after valve replacement surgery in patients operated during the active phase of treatment (137).

There is enormous interest in the potential utility of the combination of beta-lactams with daptomycin or fosfomycin in endocarditis caused by methicillin-susceptible *S. aureus*. Experimental evidence shows that combinations of both drugs with cloxacillin produce greater sterilization of vegetations on the left-sided valves in animal models (138). However, this potential beneficial effect of the combination with daptomycin was not observed in a retrospective study conducted in our center (139) or in a recent clinical trial of bacteremia caused by susceptible *S. aureus* (140), although it is worth mentioning that there was an underrepresentation of infective endocarditis as a cause of bacteremia in both studies (9 and 10% of patients, respectively). The combination of cloxacillin with fosfomycin in bacteremia and endocarditis caused by susceptible *S. aureus* is an attractive alternative. In an ongoing multicenter clinical trial (SAFO trial) we are testing this combination for bacteremia caused by methicillin-susceptible *S. aureus* (141).

In the case of endocarditis caused by methicillin-resistant *S. aureus* (MRSA), combination therapy is likely to be more effective given the relatively poor results of both vancomycin and daptomycin monotherapy. The possible synergistic effect of vancomycin and beta-lactams in MRSA bacteremia suggested by *in vitro* studies, retrospective studies (142, 143) and a pilot clinical trial (144) could not be confirmed in the international multicenter CAMERA 2 clinical trial. This trial had to be prematurely discontinued due to greater renal toxicity in the combination arm, which was associated with a shorter duration of bacteremia, but no differences in mortality between the groups (145).

The combination of daptomycin and fosfomycin has produced promising results in animal models of MRSA endocarditis (146). This combination has also shown good microbiological results and clinical benefits in patients aged under 70 years in our recent clinical trial of MRSA bacteremia (BACSARM trial) (147), which again had a poor representation of endocarditis cases (diagnosed in 12% of the cases).

Finally, combinations of daptomycin and beta-lactams without and with intrinsic anti-MRSA activity for the treatment of MRSA bacteremia have been shown to be effective in a retrospective study (148) and in a pilot clinical trial (149), respectively, although with little specific information for endocarditis cases.

Length of Antibiotic Therapy

Even in infections caused by susceptible microorganisms, the risk of microbiological relapse is plausible in endocarditis. For this reason, long treatments are typically recommended, generally 4 weeks for native valve endocarditis and 6 weeks for PVE. Exceptions to this general rule are the abbreviated 2-week treatments that have proved to be effective for right-sided endocarditis (150) and the combination treatment of beta-lactams and aminoglycosides for native valve endocarditis caused by penicillin-susceptible viridans group streptococci (127). Furthermore, there are clinical trials currently underway that aim to demonstrate the efficacy of shorter-than-standard therapeutic regimens (151).

In native valve endocarditis caused by *E. faecalis*, 4-week aminoglycoside treatment courses are recommended for cases with a clinical evolution shorter than 3 months (152). In cases with a longer symptomatic duration and in those using the double beta-lactam combination, a 6-week treatment course is recommended (153).

The decision of the total duration of antibiotic therapy in patients undergoing surgery during the active phase of treatment is affected by the result of the valve culture (154). According to the findings of a retrospective study, an antibiotic treatment duration of 2 weeks after surgery may be sufficient for valve culture-negative streptococcal endocarditis (155). Management guidelines recommend completing the pre-stipulated duration of the antibiotic treatment course when the valve culture is negative and, conversely, restarting a complete cycle if the valve is not sterile at the time of surgery.

Outpatient Parenteral Antibiotic Treatment

Infective endocarditis requires long and expensive hospitalizations for its management given the need to administer parenteral antibiotics for several weeks. Outpatient parenteral antibiotic treatment (OPAT) has been shown to be efficient and cost-effective in the management of endocarditis. A series of specific criteria must be met by the patients for them to be considered safe candidates for OPAT (156). Generally, this option is considered suitable for patients who have overcome the critical phase of the disease (first 2 weeks), provided that they remain stable. Recent studies have demonstrated the safety of this strategy, even expanding the restrictive criteria proposed in the guidelines (157).

New Antibiotic Molecules

In recent years, new antibiotics targeting Gram-positive microorganisms (and, therefore, potentially useful for treating endocarditis) have been incorporated. The fifth-generation cephalosporins ceftaroline and ceftobiprole represent the first beta-lactams with intrinsic anti-MRSA activity. Although infective endocarditis is not among the approved indications based on pivotal clinical trials, there are case series that have reported that its off-label use is an effective salvage treatment in patients with endocarditis (158, 159). Ceftaroline and, in particular, ceftobiprole also have anti-enterococcal activity (160), which makes them attractive as candidates for combination treatments with ampicillin or daptomycin in managing enterococcal infective endocarditis (161).

Another interesting new molecule is the lipoglycopeptide dalbavancin, a bactericidal drug with a chemical structure and antibacterial spectrum similar to those of teicoplanin, but with a very long half-life that allows its administration every week or every 2 weeks. Dalbavancin is a potentially useful option for OPAT in selected patients. *In vitro* studies have demonstrated its potent activity against endocarditis-producing strains (162). Furthermore, recently published series of cases indicate that it

shows efficacy as a continuation drug in endocarditis cases of diverse etiology (163, 164).

Oral Antibiotic Treatment

The effectiveness of oral treatment for endocarditis has been demonstrated for cases involving right-sided valves (165), as stated in the management guidelines. For left-sided endocarditis, retrospective studies (166), a small clinical trial of endocarditis caused by the viridans group streptococci (167) and a recent randomized clinical trial (168) of endocarditis with diverse etiologies (streptococci, enterococci, and staphylococci) have demonstrated that partial oral regimens are effective in treating selected patients who have overcome the critical phase.

In that recent randomized clinical trial, the POET trial, 1,954 patients were evaluated, of whom 400 were finally selected. After a minimum of 10 days of intravenous antibiotic therapy, the patients were randomized to continue intravenous therapy or to complete treatment with a combination of oral drugs. The clinical endpoint was a composite of all-cause mortality, unplanned cardiac surgery, embolic events, and relapse of bacteremia with the primary pathogen from the time of randomization until 6 months after the completion of antibiotic treatment. This endpoint was found to be similar between the groups. Due to the diverse etiologies included in the trial and the various combinations of oral antibiotics administered, the results of the POET trial cannot be used to make specific therapeutic recommendations. However, this trial has raised the possibility of including oral sequential treatment as a suitable option for treatment completion in selected patients.

Antimicrobial Treatment for Fungal and Culture-Negative Endocarditis

Antifungal treatment for *Candida* spp. endocarditis is usually based on the use of drugs with fungicidal activity, either based on regimens with liposomal amphotericin B (or other lipid formulations) with or without flucytosine or on regimens based on echinocandins in high doses. Although the evidence is scarce, based mostly on retrospective experiences and expert consensus, the use of combined treatments and long-term azole suppressive treatments is frequent. A high number of cases will require surgical intervention as part of the management (169). On the other hand, in the rare cases of endocarditis due to *Aspergillus* species (often responsible for culture-negative endocarditis), the most commonly used antifungal is voriconazole and the surgery requirements is also high (74, 125).

The appropriate selection of an empirical antibiotic treatment for patients with culture-negative endocarditis is a difficult task and so expert consultation is recommended. The decision is often conditioned by the clinical presentation of the disease, the presence or absence of prosthetic material and other epidemiological data such as other comorbidities, dental hygiene, alcoholism, contact with animals, etc. Thus, for patients with native valves and subacute presentation, empirical treatment should cover viridans group streptococci, enterococci and HACEK (e.g., ampicillin plus either gentamicin or ceftriaxone) whereas in cases with acute clinical presentation and/or presence of prosthetic valves it seems reasonable to cover *Staphylococcus aureus*, coagulase-negative staphylococci, β-hemolytic streptococci and aerobic gram-negative bacilli (e.g., cloxacillin plus ceftriaxone plus either vancomycin or daptomycin) (170, 171) The details on directed treatment options for specific pathogens are available in the published guidelines (74, 125).

Surgical Treatment

More than 50% of patients with endocarditis will need valve surgery. Among these, a significant proportion will require it during the active phase (that is, during the initial hospitalization and before the end of antibiotic treatment), which is known as early valve surgery (EVS). Although both the American and European guidelines have the same indications for EVS (**Table 2**) (74, 125), the latter guideline goes further in terms of recommending the timing of surgery. It suggests emergent surgery (within the first 24–48 h) in the case of refractory heart failure secondary to valve regurgitation, and an urgent surgery (during the first week) in almost all other clinical settings.

This contentious issue of the timing of surgery in the guidelines is partly due to the limited and conflicting evidence published to date that is difficult to interpret. There is only one randomized clinical trial that has demonstrated a clinical benefit in terms of a reduction in embolic events for "very" EVS in young patients (median age of 48 years) with native mitral valve endocarditis and mostly caused by streptococcal bacteria (172). It is unclear to what extent this evidence can be extrapolated to older patients and to endocarditis with other etiologies.

Most of the available evidence consists of findings from retrospective studies that require laborious statistical adjustments in order to mitigate indication and survival biases and draw valid conclusions. According to these studies, the benefit of EVS can only be demonstrated for native valve endocarditis with surgical requirement (173). The benefit is less evident in the case of PVE (only demonstrated in patients who are more likely to require surgery) (174) and could be present in some carefully selected patients with PVE caused by *S. aureus* (175).

It seems reasonable to conclude that the decision to proceed with an emergency or urgent valve surgery during the active phase of endocarditis cannot be recommended systematically

TABLE 2 | Indications for surgery in left-sided valve endocarditis (74).

Heart failure

- Severe acute regurgitation, obstruction or fistula causing refractory pulmonary edema or cardiogenic shock.

Uncontrolled infection

- Locally uncontrolled infection (abscess, false aneurysm, fistula, or enlarging vegetation).
- Persistent positive blood cultures despite appropriate antibiotic therapy.
- Infection caused by fungi or multidrug-resistant organisms or prosthetic valve endocarditis caused by staphylococci or non-HACEK Gram-negative bacteria.

Prevention of embolism

- Persistent vegetations >10 mm after one or more embolic episode despite appropriate antibiotic therapy or severe valve stenosis or regurgitation.
- Isolated very large vegetations (>30 mm).

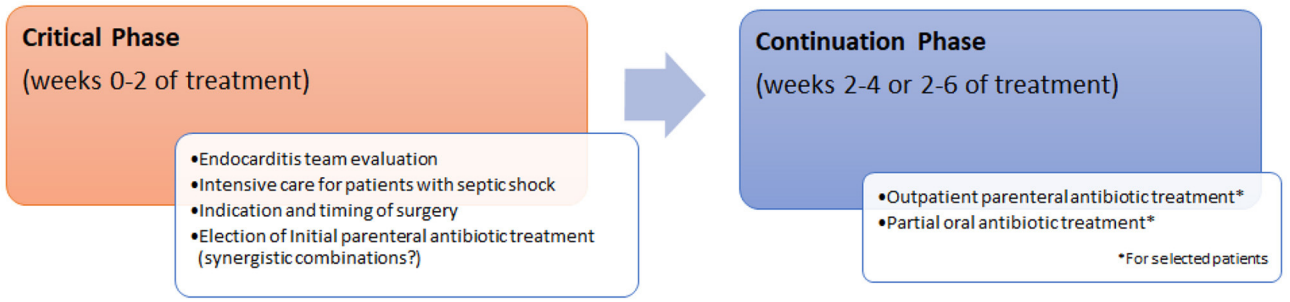


FIGURE 1 | Management of infective endocarditis.

or routinely. Instead, this decision should be based on an individualized assessment (176).

Management of Sepsis in Endocarditis

The management of septic shock in patients with endocarditis does not seem to differ from that recommended for sepsis from other sources (59). In addition to volume expansion, vasoactive drugs in some cases and the early initiation of appropriate bactericidal antibiotic treatment, there is still no clinical evidence about the possible effectiveness of additional immunomodulatory treatments in cases of endocarditis and suspected toxic shock (61). It seems appropriate to point out that early valve surgery may be necessary in some cases as a source control measure (30), such as in cases with uncontrolled infection despite correct antibiotic treatment.

CONCLUSIONS AND PERSPECTIVE

It is evident that the management of infective endocarditis requires the close collaboration of a multidisciplinary team that includes experts in critical care for patients with severe sepsis or septic shock. The training of endocarditis teams is recommended by the guidelines, while some studies have demonstrated the beneficial impact of their performance on patient outcomes (177, 178).

It seems reasonable to conclude that patients with endocarditis should be evaluated preferentially by these multidisciplinary teams in the critical phase of the disease, at which time the most appropriate initial intravenous antibiotic therapy should be chosen. New therapeutics and possible new synergistic combinations of antibiotics are of very high interest. After a thorough clinical and anatomical cardiac evaluation, patients eligible for surgical treatment should be selected and the timing of the intervention decided. It is possible that in coming years, an increasing proportion of appropriately selected patients will be able to continue their antibiotic treatment in home hospitalization regimens with OPAT, using long half-life parenteral antibiotics or combinations of oral antibiotics (**Figure 1**).

There is a need for international and multi-center working groups to establish a common work agenda in order to scientifically address all the unresolved diagnostic and therapeutic aspects of infective endocarditis.

AUTHOR CONTRIBUTIONS

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

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Dynamic Changes in Microbial Composition During Necrotizing Soft-Tissue Infections in ICU Patients

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Introduction: Recent studies described the threat of emerging multidrug-resistant (MDR) bacteria in intensive care unit (ICU) patients, but few data are available for necrotizing skin and soft tissue infections (NSTI). In a cohort of ICU patients admitted for NSTI, we describe the dynamic changes of microbial population during repeated surgeries.

Materials and Methods: This retrospective study compiled consecutive cases admitted for the management of severe NSTI. Clinical characteristics, NSTI features, morbidity and mortality data were collected. The microbiological characteristics of surgical samples obtained during initial surgery were compared with those obtained during the first reoperation, including persistence of initial pathogens and/or emergence of microorganisms. Risk factors for emergence of microorganisms and MDR bacteria were assessed by univariable and multivariable analyses.

Results: Among 100 patients {63% male, 58 years old [interquartile ratio (IQR) 50–68]} admitted for NSTI, 54 underwent reoperation with a median [IQR] delay of 3 (1–7) days. Decreased proportions of susceptible strains and emergence of Gram-negative bacteria, including *Pseudomonas aeruginosa*, staphylococci and enterococci strains, were reported based on the cultures of surgical specimen collected on reoperation. On reoperation, 22 (27%) of the isolated strains were MDR ($p < 0.0001$ vs. MDR bacteria cultured from the first samples). Broad-spectrum antibiotic therapy as first-line therapy was significantly associated with a decreased emergence of microorganisms. Adequate antibiotic therapy from the initial surgery did not modify the frequency of emergence of microorganisms ($p = 0.79$) and MDR bacteria ($p = 1.0$) or the 1-year survival rate.

Conclusion: The emergence of microorganisms, including MDR bacteria, is frequently noted in NSTI without affecting mortality.

Keywords: necrotizing soft-tissue infections (NSTI), intensive care unit (ICU), sepsis, multidrug-resistant (MDR) bacteria, outcome, antimicrobial therapy

INTRODUCTION

Necrotizing soft-tissue infections (NSTI) require early diagnosis, adequate early surgical source control and appropriate antibiotic management according to recent international recommendations (1, 2). However, despite early management of NSTI, morbidity and mortality remain high (3). Recent reports underlined the emergence of multidrug resistant (MDR) bacteria in the ICU (4, 5), but few data focused on NSTI (6). Risk stratification for antibiotic resistance in skin and soft tissue infections remains poorly investigated, and few specific risk factors have been identified for specific bacterial species or MDR pathogens (7). Repeated inspection and additional debridement are recommended, which could help to re-assess antibiotic therapy through additional microbiological samples (1, 8–13). Many patients require repeated procedures and prolonged anti-infective treatments that might increase the risk of emergence of MDR bacteria. The timeline of the changes of such pathogens has been minimally addressed in NSTI. A better understanding of dynamic changes of microbial population in ICU patients managed for NSTIs could be helpful for optimizing anti-infective therapy.

The goals of the present study were to describe an ICU cohort of patients admitted for NSTI, define the dynamic changes of microbial population during repeated surgeries, and assess the risk factors for emergence of MDR bacteria and the prognosis of these microbiological issues.

MATERIALS AND METHODS

Study Population

This retrospective study compiled all consecutive patients admitted to our University Hospital ICU for the management of NSTI from April 2009 to March 2019. The identification of the cases was made through the database of the health information system of our hospital. The ICD-10 scores were used, initially including a search of all skin and subcutaneous lesions reported in patients admitted to the ICU. Then, the analysis of the medical reports allowed to select those with a diagnosis of NSTI. Patients with uncertain diagnosis or without NSTI were excluded.

The retrospective nature of our study waived the need for signed informed consent. This study was declared to the French Data Protection Authority (CNIL: 2096382v0) and was approved by the French Institutional Review Board (Comité d'Éthique de la Recherche en Anesthésie-Réanimation, IRB number 00010254-2020-153).

Surgical Procedures

The index surgical exploration was performed to confirm the diagnosis and to achieve source control through aggressive debridement of infected necrotic tissue. Surgical samples were obtained for microbiological analysis. Wound care, including cleaning and trimming, was performed every 24 or 48 h at bedside according to the local aspect. In case of unfavorable systemic and/or local evolution, a second surgical exploration was performed in the operating theater to ensure the adequacy of source control and to collect additional microbiological samples (2, 14). Only the first two procedures were analyzed (only two patients had subsequent samples).

Microbiological Data

Blood cultures were collected at the time of ICU admission and repeated in clinical situations evocative of sepsis or bacteraemia. Fine needle aspiration specimens were taken at the time of admission before the source control procedure. Direct needle aspiration was focused on the leading edge, mid-lesion or bullae according to conventional recommendations (15, 16). Peroperative specimens were obtained from deep tissues collected during initial surgical source control or at the time of reoperation (17, 18). Swabs and samples from non-sterile sites (such as open bullaes) were not considered. Cases of NSTI based on inconsistent samples (swabs, non-sterile sites, open skin injury, etc.) were excluded.

Microbiological samples were immediately sent to the laboratory for bacterial and fungal cultures. Samples were processed according to the laboratory standard methods. Plates were incubated for 48 h at 35°C. All morphologically distinct colonies were identified by standard bacteriologic techniques and tested for antibiotic susceptibility by the disk diffusion method according to EUCAST[1]. The carbapenem resistant Gram-negative bacilli strains were screened for bla_{KPC}, bla_{VIM}, bla_{IMP}, bla_{NDM}, and bla_{OXA-48} genes by home-made PCR then using the Cepheid Xpert® Carba-R assay (Cepheid, Sunnyvale, USA).

Results of microbiological cultures of samples collected during first and second operations were compared in terms of species and susceptibility profile. A persisting micro-organism was defined as the same microorganism (bacteria or fungi) isolated from one sample to the next on the basis of identification and susceptibility profile without analysis of genetic relatedness (19). Emerging microorganisms were defined as microorganisms (bacteria or fungi) isolated from the sample of the second operation and not from the first one on the basis of identification and/or susceptibility profile (19). MDR and extensively drug resistant (XDR) bacteria were defined according to international definitions (20).

[1]EUCAST: Clinical breakpoints and dosing of antibiotics. Available online at: https://eucast.org/clinical_breakpoints/ (accessed June 22, 2020).

Empirical Therapy

Empirical anti-infective therapy (EAT) taking into account clinical severity was systematically initiated on the first operation and targeted Gram-positive and Gram-negative aerobic and anaerobic microorganisms. The conventional regimen was based on beta-lactams with anti-anaerobic activity (amoxicillin-clavulanate, piperacillin/tazobactam or imipenem/cilastatin) or third-generation cephalosporin associated with metronidazole combined ± aminoglycosides and ± anti-Gram-positive agents in case of suspicion of resistant bacteria (2, 21). Documented anti-infective therapy was adapted to the results of identification and susceptibility testing (≥48 h) and was defined as adequate when targeting all the cultured microorganisms. The same rule was applied to the first reoperation.

Data Collection

Demographic data and severity (SAPS-II and SOFA) scores were recorded on ICU admission (22, 23). The severity of the underlying medical condition [malignancy, obesity, diabetes mellitus, vascular disease, alcohol use, active smoking, and immunosuppression (immune deficiency, HIV, or chronic corticosteroids or antineoplastic medication)] was assessed. The Charlson comorbidity index and the Laboratory Risk Indicator for Necrotizing Fasciitis score (LRINEC) were calculated (24, 25). Characteristics of the NSTI were assessed [site (cephalic, trunk, pelvis, and limbs), multiple locations, amputation, and stoma]. The proportion of injured skin surface was assessed according to the nine rules for burns (26). This estimate was calculated independently by two authors (MT and ST) based on the anatomic descriptions of the operative reports and the pictures taken by the surgeons and intensivists. Clinical and therapeutic features were recorded on admission and during the ICU stay, including sepsis shock, renal failure, and need for mechanical ventilation, vasoactive support, renal replacement therapy and reoperation. The length of ICU and hospital stay and the mortality rates at day 28, day 90, and 1 year after admission were collected.

Statistical Analysis

Results are expressed in medians and IQR as well as absolute numbers and proportions for categorical data. Continuous data were compared using the Mann-Whitney U test. Categorical data were analyzed using the Chi2 or Fisher exact test. All reported statistical tests were 2-sided, and p values <0.05 were considered significant.

Risk factors for emerging microorganisms and emerging MDR bacteria were analyzed in univariable analysis with univariable logistic regression or with non-parametric tests corresponding to unpaired Wilcoxon test for continuous variables and Fisher's exact test for discrete variables when univariable logistic models did not converge (27, 28). Survival curves were estimated by the Kaplan–Meier method, and differences in survival between groups were assessed by the log-rank test. For multivariable analyses, a logistic regression model was used due to a high number of predictors compared with the number of events/non-events (29). Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. For comparison, we have reported results from normal univariable and adjusted logistic regression. Variables with a p-value <0.1 in univariable analysis were entered in a multivariable logistic regression analysis with stepwise selection. The logistic regression models analyzed separately the variables available on admission and those collected during ICU stay. Statistical analyses were performed using the R software version 3.6.2.

RESULTS

Study Population

Overall, 100 consecutive patients [63% men, 58 (IQR 50–68) years old] were admitted to our ICU for the diagnosis of NSTI with a median delay of 2 (0–5) days between hospital admission and index surgery. The general characteristics of the patients are presented in **Table 1**. The median body surface area of the lesions was 4.5% (4.5–9), and limbs, pelvis, cephalic and trunk areas were involved in 52, 28, 23, and 13% of the patients, respectively. Multiple locations were observed in 14% of the patients.

Initial surgery was always performed under general anesthesia with intubation and mechanical ventilation. The median duration of mechanical ventilation was 3 days [0–13]. All patients received EAT at the time of surgery, mainly with piperacillin + tazobactam ($n = 33$), cephalosporin ($n = 21$), amoxicillin-clavulanic acid ($n = 25$), aminoglycoside ($n = 24$), carbapenem ($n = 18$) and vancomycin ($n = 7$). EAT with antitoxin activity was used in 40% of the cases [clindamycin ($n = 26$) and linezolid ($n = 12$)]. EAT was not adequate for 12 patients (12%). Patients with *Enterobacterales* isolated from surgical samples were more severe than other patients with an increased frequency of vasoactive support (71 vs. 43% of patients with other microorganisms, $p = 0.018$) and renal replacement therapy (38 vs. 16%, $p = 0.032$).

A second procedure was required in 54 patients with a median delay of 3 (1–7) days after index surgery. No significant difference was found between the patients who underwent one single surgical procedure and patients who underwent at least a second operation except age ($p = 0.016$) and vasoactive support on admission ($p = 0.031$) (**Table 1**). The main indications for re-interventions were the persistence of necrosis in the lesions, worsening of local or systemic clinical signs, and worsening of biological parameters. At the time of this second procedure, the median SOFA score was 4 (2–8) with a median SOFA score difference between the index surgery and the day of reoperation of 0 [−3; +3] and a median hyperleukocytosis of 15 (10–23) G/L with a median difference in leucocytosis of 1 (−2; +5) G/L.

Microbiological Findings Over Time

The cultures of surgical samples collected on the initial surgery yielded 174 microorganisms (168 bacteria and 6 fungi) (**Table 2**). The predominant pathogens were Gram-positive bacteria [$n = 87$ (50%)] with a majority of streptococci [$n = 45$ (27%)]. During the initial procedure, 9 (5%) MDR strains were isolated in the samples of 7 (7%) patients (**Table 2**). The microbial combinations obtained during the first surgery are presented in **Supplementary Table 1**. Bacteraemias were reported in 24 patients and mostly related to Gram-positive cocci.

TABLE 1 | Characteristics and comparison of patients who underwent one surgical procedure with the patients who underwent two surgical procedures.

***n* = 100**	**Overall**	**One surgical procedure**	**Two surgical procedures**	
Variables	**100**	**46**	**54**	***p***
Demographic data				
Age (years), median (IQR)	58 (50–68)	62 (55–69)	56 (48–63)	0.016
Male gender, *n* (%)	63 (63)	27 (59)	36 (67)	0.539
Weight (kg), median (IQR)	82 (67–95)	85 (69–101)	80 (66–92)	0.309
BMI (kg/m^2), median (IQR)	28 (23–34)	29 (23–37)	27 (22–31)	0.194
Underlying diseases				
Chronic obstructive pulmonary disease, *n* (%)	15 (15)	6 (13)	9 (17)	0.822
Cardiovascular disease, *n* (%)	34 (34)	12 (27)	22 (41)	0.238
Diabetes mellitus, type I or II, *n* (%)	40 (40)	18 (39)	22 (41)	1.000
Peripheral vascular disease, *n* (%)	17 (17)	9 (20)	8 (15)	0.716
Immunosuppression, *n* (%)	10 (10)	5 (11)	5 (9)	1.000
Cancer, *n* (%)	23 (23)	11 (24)	12 (22)	1.000
Active smoking	54 (54)	27 (59)	27 (50)	0.504
Alcohol use, *n* (%)	20 (20)	10 (22)	10 (19)	0.880
Use of steroids or NSAI drugs, *n* (%)	7 (7)	2 (40)	5 (42)	1.000
Charlson score median (IQR)	4 (3–7)	3 (2–5)	3 (2–7)	0.943
CT scan on the first examination, *n* (%)	49 (49)	24 (52)	25 (49)	0.915
Median delay from first exam to CT scan, days (IQR)	1 (0–4)	2 (0–4)	1 (0–4)	0.981
Deep abscess, *n* (%)	31 (31)	10 (39)	21 (60)	0.160
Bullae, *n* (%)	27 (27)	11 (42)	16 (46)	0.997
Fasciitis signs, *n* (%)	21 (21)	9 (35)	12 (34)	1.000
Osteitis signs, *n* (%)	6 (6)	3 (12)	3 (9)	1.000
Severity criteria				
SAPS II score on admission, median (IQR)	28 (23–37)	34 (21–52)	37 (27–46)	0.547
SOFA score on admission, median (IQR)	5 (3–6)	4 (3–6)	5 (4–7)	0.161
LRINEC score on admission, median (IQR)	2 (1–4)	2 (1–4)	2 (1–5)	0.552
Treatment				
Vaso-active support on admission, *n* (%)	53 (53)	18 (41)	35 (65)	0.031
Renal replacement therapy, *n* (%)	22 (22)	6 (14)	16 (31)	0.081
Length of mechanical ventilation, median (IQR)	3 (0–13)	1 (0–7)	9 (1–15)	0.018
Antibiotic duration, median (IQR)	15 (11–18)	13 (7–15)	16 (15–28)	0.001
Vacuum-assisted closure device, *n* (%)	28 (28)	11 (24)	17 (32)	0.504
Skin graft, *n* (%)	24 (24)	6 (13)	18 (33)	0.020
Amputation, *n* (%)	13 (13)	7 (15)	6 (11)	0.566
Outcomes				
Length of hospital stay in days, median (IQR)	40 (18–59)	35 (9–58)	43 (27–57)	0.112
Length of ICU stay in days, median (IQR)	7 (2–19)	3 (1–9)	11 (6–27)	<0.001
Death, *n* (%)	25 (25)	10 (22)	15 (28)	0.644

The second surgical procedure performed in 54 (54%) patients led to the isolation of 95 microorganisms (89 bacteria and 6 fungi) (**Table 2**). The predominant pathogens were Gram-negative bacteria [$n = 47$ (52%)] with a majority of *Enterobacterales* [$n = 33$ (35%)] and *Pseudomonas aeruginosa* [$n = 14$ (17%)]. On reoperation, 22 (27%) of the isolated strains were MDR ($p < 0.0001$ vs. MDR bacteria cultured from the first samples). The emergence of two carbapenemase-producing bacteria (*Klebsiella pneumoniae* NDM) was reported during an outbreak in our ICU. Proportions of microorganisms, MDR bacteria and microbiological comparisons between the two surgical procedures are displayed in **Table 2**. The incidence of enterococci, *E. coli*, and non-fermenting Gram-negative bacilli were significantly different based on the NSTI location (**Supplementary Table 2**). The highest proportions of anaerobes were cultured during the first surgery for pelvic NSTIs reaching 20% of the isolates, while the highest proportions of Gram-positive bacteria were observed in cephalic NSTIs (65% of the isolates).

Persisting Microorganisms

Among the microorganisms isolated during the first surgery, 30 (18%) were persistent on reoperation (**Table 2**), including *P. aeruginosa* [$n = 4$ (10%)] and *Enterobacterales* [$n = 17$ (43%)].

TABLE 2 | Microorganisms isolated from the first and second surgery.

	First surgery	Reoperation	Emerging organisms	Persisting organisms
Aerobes, *n* (%)	146 (84)	84 (88)	56	28
Gram-positive bacteria, *n* (%)	87 (50)	37 (39)*	20	17
Enterococci, *n* (%)	16 (9)	7 (7)	4	3
E. faecalis, *n* (%)	12 (7)	3 (4)	2	1
E. faecium, *n* (%)	1 (1)	3 (4)	2	1
Streptococci, *n* (%)	45 (27)	10 (12)*	2	8
GAS, *n* (%)	12 (7)	1 (1)	0	1
Staphylococci, *n* (%)	26 (15)	20 (21)	14	6
Staphylococcus aureus, *n* (%)	15 (9)	8 (10)	6	2
Coagulase-negative staphylococci, *n* (%)	11 (7)	10 (12)	8	2
Gram-negative bacteria, *n* (%)	59 (35)	47 (52)*	36	11
Enterobacterales, *n* (%)	48 (28)	33 (35)	26	17
Escherichia coli, *n* (%)	21 (12)	15 (18)	10	5
Enterobacter spp., *n* (%)	2 (1)	6 (7)*	6	0
Klebsiella spp., *n* (%)	9 (5)	12 (15)*	10	2
Non-fermenting Gram-negative bacilli, *n* (%)	11 (7)	14 (17)*	10	4
Pseudomonas aeruginosa, *n* (%)	9 (5)	14 (17)*	10	4
Anaerobes, *n* (%)	22 (13)	5 (6)	3	2
Bacteroides spp., *n* (%)	9 (5)	4 (5)	3	1
Fungi, *n* (%)	6 (4)	6 (7)	6	0
Candida albicans, *n* (%)	1 (1)	2 (2)	2	0
Total number of strains, *n* (%)	174 (100)	95 (100)	65	30
Total number of MDR bacteria, *n* (%)	9 (5)	22 (27)*	19	3
MRSA	2	3	3	0
Overproduction of intrinsic or plasmid-encoded AmpC	2	5*	3	2
ESBL producing *Enterobacterales*	4	9*	8	1
Carbapenemase producing *Enterobacterales*	1	2	2	0
XDR *Pseudomonas aeruginosa*	0	3*	3	0

*GAS, group A streptococci; MRSA, methicillin-resistant Staphylococcus aureus; AmpC, cephalosporinase; ESBL, extended spectrum betalactamase; XDR, extensively drug resistant. The variables followed by an asterisk * indicate those with a significant difference between 1st surgery and re-intervention among patients.*

Lower limbs and pelvic region were the two main sites in which persisting bacteria were identified. No clinical characteristics or co-morbidities were significantly associated with persistence of microorganisms.

In univariable analysis, a significant association was observed between persisting microorganisms and a post-operative NSTI; the surface of altered skin; the SAPS II score on admission; the need for renal replacement therapy; the number of nosocomial infections, especially hospital-acquired pneumonia; the polymicrobial nature of initial infection; the presence of anaerobes, *Enterobacterales* or *P. aeruginosa* in the first surgical samples. Adequacy of EAT, its duration and its delay of initiation were not associated with persisting microorganisms. In multivariable analysis, the nosocomial context of NSTI [OR 4.4 (95% CI 1.4–14.2), $p = 0.01$], the presence of hospital-acquired pneumonia [OR 7.8 (95% CI 1.4–67), $p = 0.03$] and the polymicrobial nature of the initial infection [OR 3.8 (95% CI 1.2–13.5), $p = 0.03$] were independent factors for persisting microorganisms on reoperation.

Emerging Microorganisms

Overall, 65 emerging microorganisms were reported in 30 patients (30%) between the first and second operations. A total of 19 emerging MDR strains were cultured in 15 patients (28%) on the second surgery, involving both Gram-positive and Gram-negative organisms (**Table 2**, **Figure 1**). The different mechanisms of resistance are presented in **Supplementary Table 3** and **Figure 1**. Among MDR bacteria, overproduction of intrinsic or plasmid-encoded AmpC cephalosporinases were the most frequent mechanisms of resistance. Cephalosporinase- and ESBL-production were the most frequent emerging mechanisms of resistance.

We expected an increased proportion of emerging MDR during pelvic NSTIs related to the proximity to the bowel flora. However, the emergence of bacteria ($p = 0.44$), persistence of bacteria ($p = 0.74$), and emergence of MDR ($p = 0.9$) were not significantly different in NSTI involving the pelvis area vs. other areas. A comparison of the clinical data of patients with/without the emergence of MDR bacteria is presented in **Supplementary Table 4**.

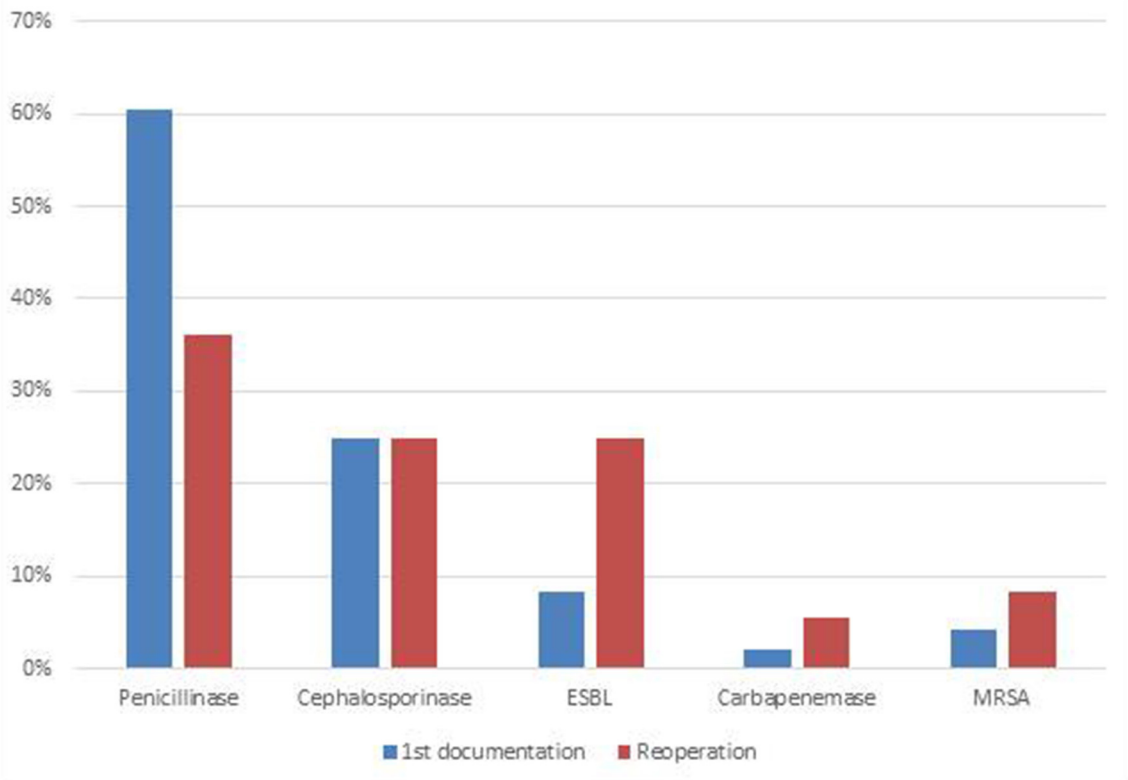


FIGURE 1 | Proportions of identified antibiotic resistances among MDR bacteria during initial surgery and at the time of reoperation. ESBL, extended-spectrum betalactamase; MRSA, methicillin-resistant *Staphylococcus aureus*.

The risk factors for emergence of microorganisms at the time of reoperation analyzed in multivariate analysis are presented in **Table 3**. When considering the variables collected at the time of ICU admission, initial administration of broad-spectrum antibiotic therapy (piperacillin/tazobactam and/or aminoglycosides) was protective factor observed against the emergence of microorganisms on reoperation (**Table 3**). Concerning the variables collected during the ICU stay, hospital-acquired pneumonia increased significantly the risk of emergence of bacteria.

We did not observe any link between the clinical criteria collected on ICU admission and emerging MDR bacteria (**Table 4**). In multivariable analysis, the only identified risk factor for emergence of MDR bacteria on the second surgical procedure was a prolonged delay before initial source control (**Table 4**).

Outcomes

The total median duration of antibiotic therapy was 14 (11–15) days. Overall, surgical source control was successful in 90% of the cases. However, 11% of the patients required an amputation, and 13% of them needed a colostomy. A vacuum-assisted closure (VAC) device was used in 30% of the patients, and skin wound repair required skin graft in 24% of patients. Twenty-two patients underwent renal replacement therapy. The median length of ICU and hospital stay were 7 (2–19) and 39 (17–58) days, respectively.

Mortality rates at Day 28, Day 90, and 1 year were 18, 23, and 25%, respectively. Kaplan-Meier estimates of survival among patients [with or without persisting microorganisms (panel A), with or without emerging microorganisms (panel B) and with or without MDR bacteria acquisition (panel C)] are displayed on **Supplementary Figure 1**. The mortality rate in the entire cohort did not change over the study period ($p = 0.786$, **Supplementary Figure 2**). The type of microorganisms cultured from the initial surgery did not modify the ICU/hospital length of stay or mortality rate. Persistence of microorganisms was significantly associated with a longer ICU length of stay [17 (7–31) days vs. 5 (1–12) days in patients free of persisting microorganisms, $p = 0.001$], but these persisting microorganisms did not influence the survival rate at 1 year ($p = 0.135$). Similarly, emergence of microorganisms was associated with a longer ICU length of stay [12 (4–35) days vs. 6 (1–18) days in patients free of emerging microorganisms, $p = 0.052$] but was not associated with the survival rate at 1 year ($p = 0.805$). The acquisition of MDR bacteria was not associated with increased ICU or hospital length of stay or survival rate at 1 year.

DISCUSSION

To the best of our knowledge, this is the first study to focus on the microbiological changes (persistence and emergence of

TABLE 3 | Risk factors for emergence of bacteria.

Variables	Univariable analysis			Multivariable analysis			
	Patients without any emerging microorganisms	Patients with emerging microorganisms	*p*-value	Odds ratio	2.5% CI	97.5% CI	*p*-value
On admission							
Age median (IQR)	59 (50–68)	56 (50–64)	0.253				
Male gender, *n* (%)	47 (67)	16 (53)	0.258				
BMI, median (IQR)	27 (22–31)	28 (24–36)	0.410				
Anterior SSTI history, *n* (%)	4 (6)	3 (10)	0.430				
Chronic skin disease, *n* (%)	8 (11)	4 (13)	0.749				
Smoker, *n* (%)	43 (61)	11 (37)	0.029	0.35	0.12	0.96	0.04
Anterior antibiotherapy <6 months, *n* (%)	16 (23)	7 (23)	1				
Cardiovascular disease, *n* (%)	27 (40)	7 (23)	0.167				
SOFA score, median (IQR)	5 (3–7)	4 (3–6)	0.175				
SAPS II score, median (IQR)	27 (23–37)	28 (22–38)	0.557				
Haemodynamic failure, *n* (%)	54 (77)	16 (53.3)	0.031	0.26	0.08	0.75	0.01
LRINEC score, median (IQR)	2 (1–4)	2 (1–5)	0.921				
% of corporeal surface, median (IQR)	4.5 (4.5–9)	7 (4.5–17)	0.08	1.02	0.95	1.10	0.6
Adequate empiric antibiotherapy, *n* (%)	56 (80)	23 (77)	0.790				
1st line - aminoglycosides, *n* (%)	21 (30)	3 (10.0)	0.041	0.17	0.03	0.65	0.008
1st line - piperacillin-tazobactam, *n* (%)	29 (41)	4 (13.3)	0.006	0.22	0.05	0.71	0.01
During ICU stay							
Antibiotic de-escalation, *n* (%)	41 (62)	16 (57.1)	0.653				
Delay to diagnosis in days, median (IQR)	1 (0–3)	1 (0–2)	0.064	1.00	1.00	1.00	0.07
Total antibiotherapy duration, median (IQR)	14 (10–15)	15 (14–15)	0.476				
Hospital-acquired pneumonia, *n* (%)	3 (4)	8 (27)	0.003	2.04	1.32	3.16	0.005
ICU length of stay, median (IQR)	6 (1–17)	12 (4–35)	0.030	1.00	1.00	1.01	0.37
Delay to reoperation, median (IQR)	3 (1–6)	3 (2–7)	0.47				

microorganisms) in ICU patients with NSTI who required a reoperation. We observed a shift of Gram-positive to Gram-negative bacteria between the first and the second operation combined with a significant increase in the proportion of MDR bacteria at the time of the second surgery. A broad spectrum first-line antibiotic therapy was associated with reduced emergence of microorganisms. However, adequate antibiotic therapy from the initial surgery did not modify the frequency of emergence of microorganisms or MDR bacteria or the 1-year survival rate of the patients harboring these MDR bacteria.

Our patients are comparable to those described in the most recent epidemiological cohort collected in ICU patients with a significant proportion of NSTI of the limbs and pelvis (30). There is a large variability in terms of the source of NSTI between centers depending on the case mix and the environment. The pelvic site of infection increases the proportions of Gram-negative bacteria among the cultured microorganisms. Most recent and largest studies found between 21 and 34% of NSTI concerning pelvis area (30–32). The proportion of pelvic NSTI in our cohort was slightly more frequent than what was reported in the older literature (33–35). Interestingly, the incidence of Gram-negative isolates in these specific cases was not different from our observations in limb infection, an issue not generalizable to other centers where Gram-positive bacteria are usually the predominant pathogens. The severity scores on admission were quite low in our population despite mechanical ventilation in all the cases and septic shock in half of the patients. Similarly to others, we observed prolonged ICU and hospital stays, which are probably related to the prolonged care required by this specific population (36). The type and susceptibility profiles of the microorganisms cultured from surgical samples and blood cultures on admission are consistent with previous studies with a predominance of Gram-positive isolates in the first operation samples (37–40).

The number of the reoperations was high in our population. Other authors also reported important rates of repeated surgical debridement with a dramatic impact of a delayed initial surgery (>24 h) on mortality (39, 41, 42). Aggressive surgical treatment, especially in patients without favorable evolution, could explain why we did not identify a significant impact of this surgical delay in our cohort. The large proportion of reoperations allowed to make microbiological comparisons. The cultures of the second surgical samples revealed a microbiological shift with decreased proportions of Gram-positive cocci associated with the emergence of Gram-negative bacteria, including *P. aeruginosa*. The microbiological course of NSTI seems to be influenced by the type of EAT. Criteria explaining the persistence of microorganisms were not expected. The observed risk factors for persisting pathogens highlighted various criteria poorly described in the literature, such as the post-operative NSTI,

TABLE 4 | Risk factors for emergence of MDR bacteria.

Variables	Univariable analysis			Multivariable analysis			
	No emergence of MDR strains	Emerging MDR strains	*p*-value	Odds ratio	2.5% CI	97.5% CI	*p*-value
On admission							
Age median (IQR)	56 (46–67)	62 (56–67)	0.333				
Male gender, *n* (%)	19 (58)	7 (47)	0.543				
BMI, median (IQR)	27 (22–29)	28 (20–43)	0.611				
Anterior SSTI history, *n* (%)	3 (9.1)	2 (13.3)	0.642				
Chronic skin disease, *n* (%)	8 (9)	4 (27)	0.079	2.94	0.68	11.39	0.14
Smoker, *n* (%)	14 (42)	8 (53)	0.543				
Anterior antibiotherapy <6 months, *n* (%)	21 (25)	2 (13)	0.510				
Cardiovascular disease, *n* (%)	29 (35)	5 (33)	1.000				
SOFA score, median (IQR)	5 (3–6)	4 (3–7)	0.849				
SAPS II score, median (IQR)	26 (22–37)	32 (27–37)	0.133				
Haemodynamic failure, *n* (%)	59 (69)	11 (73)	1.000				
LRINEC score, median (IQR)	2 (1–5)	1 (0–2)	0.041	0.84	0.62	1.08	0.19
% of corporeal surface, median (IQR)	4.5 (4.5–9)	9 (4.5–11)	0.201				
Adequate empiric antibiotherapy, *n* (%)	75 (90.4)	16 (94.1)	0.978				
1st line—aminoglycosides, *n* (%)	22 (26)	2 (13)	0.512				
1st line—piperacillin-tazobactam, *n* (%)	29 (34)	4 (27)	0.768				
During ICU stay							
Antibiotic de-escalation, *n* (%)	51 (64)	6 (43)	0.152				
Delay to diagnosis in days, median (IQR)	0.5 (0–2)	3.5 (1–6)	0.071	1.21	1.02	1.53	0.002
Total antibiotherapy duration, median (IQR)	14 (12–15)	14 (11–15)	0.644				
Hospital-acquired pneumonia, *n* (%)	7 (8)	4 (27)	0.062	9.01	0.39	130.20	0.145
ICU length of stay, median (IQR)	7 (2–19)	8 (6–19)	0.428				
Delay to reoperation, median (IQR)	4 (1–7)	2 (2–3)	0.192				

polymicrobial nature of infection or combined hospital acquired pneumonia. These factors could be easily detected and might be useful for detection of patients at risk of reoperation.

A first-line broad-spectrum antibiotic therapy seems to play a protective role in the emergence of microorganisms between the two surgeries. This unexpected observation is in contradiction with the current trend of limiting antibiotic selection pressure. However, the 2018 WSES/SIS-E consensus conference stressed the need for aggressive broad-spectrum EAT to target Gram-positive, Gram-negative and anaerobic bacteria (2). Paradoxically, adequacy of antibiotic treatment did not change the frequency of emergence of microorganisms regardless of the period analyzed or the outcomes of the patients. Concomitantly, we observed a progressive emergence of MDR strains over time. These findings are consistent with the results found in other ICU surgical populations, such as patients treated for intra-abdominal infections in whom an emergence of MDR bacteria was gradually observed with the number of reoperations (19). According to our data, the use of broad-spectrum antibiotic therapies, such as piperacillin-tazobactam and aminoglycoside or carbapenems, did not seem to influence the emergence of MDR bacteria. Only delayed diagnosis and antibiotic therapy appear to be independent factors associated with the emergence of MDR bacteria. This finding could reinforce the need for early and aggressive management from the initial phase of anti-infective therapy.

We observed some variabilities in terms of emerging bacteria between the different location of NSTI, which are potentially related to inhomogeneous diffusion of antibiotics or local microbiota issues. The quality of source control and its delay can also be variable (43). Persistence and emergence of microorganisms were not significantly associated with survival rates but were associated with prolonged ICU length of stay. Interestingly, emerging MDR bacteria were frequently reported in our cohort without significant changes in survival rates or ICU/hospital length of stay.

Our work has some limitations. First, it is a retrospective, monocentric and observational study with a relatively small sample conferring a low level of evidence. We consider this study to be strictly exploratory. Thus, it is not possible to draw definite conclusions from such a limited number of cases and our observations need to be confirmed in a larger multicentric approach. This comment is relevant for the analysis of the relationship between antibiotic selection pressure and emergence of MDR microorganism which needs a specific investigation. Second, the long period of inclusion, which was necessary regarding the low incidence of NSTI in ICU patients, is questionable. However, we observed that the 1-year mortality was stable throughout the inclusion period among patients with NSTI. In addition, we cannot exclude that confounding factors for mortality may not have been included, mitigating the multivariate analysis results. Third,

the surgical samples were examined only by culture-based techniques, which have some limitations. All the pathogens involved in NSTI could be not detected, especially if antibiotic therapy was initiated before sampling (44), or in case of hardly or uncultivable microorganisms like anaerobes. Culture-free techniques (16S rRNA amplicon and metagenomics shotgun sequencing) could have provided a deeper insight into the global taxonomic and resistance profiles of the microbial population. Indeed, these techniques are highly effective in characterizing microbial population and very complementary to culture-based techniques (45). In addition, persistence of the microorganisms was assessed according to a phenotypic description without any genotypic confirmation. Another point to consider is the impossible differentiation between wound colonization and authentic infection. Indeed, focusing on bacterial species *per se* may cause inclusion of irrelevant cases and thereby over-interpretation of observations. The only clinical surrogates of the pathogenic role of the microorganisms isolated on the second surgery are the persistent organ dysfunctions and high white blood cell counts. This issue prompts cautious consideration of these results and the need for antibiotic escalation in case of abatement of clinical signs of infection. The emergence of MDR bacteria was only assessed for the surgical wound. However, a broader analysis of the emergence of MDR bacteria could also be of interest, especially in terms of digestive carriage. Finally, the pharmacokinetic characteristics of anti-infective agents and their tissue diffusion could play a role in the mechanisms of persistence or emergence of microorganisms, but this issue was not monitored on a routine basis, representing a limitation in the interpretation of the results. However, the pharmacokinetic characteristics of antibiotics have rarely been reported in the management of NSTI.

The concept of emergence and persistence of microorganisms encourages us to take a closer look to the issue of colonization, which is similar to the practice used in burned patients. A combination of close clinical evaluation, repeated biomarkers and microbiological mapping during the first days of management of NSTIs could be interesting to evaluate the decreased duration of anti-infective therapy and consequently antibiotic selection pressure (34, 35).

CONCLUSION

In our cohort of ICU patients admitted for NSTI, we highlighted the high frequency of persisting and emerging microorganisms and their important consequences in terms of morbi-mortality with a prolonged ICU length of stay. Broad-spectrum first-line therapy was associated with a decreased emergence of microorganisms. We showed a shift of Gram-positive to Gram-negative bacteria between the first and the second operation combined with a significant increase in the proportion of MDR bacteria at the time of the second surgery. However, use of an adequate antibiotic therapy from the initial surgery did not modify the frequency of emergence of microorganisms or MDR bacteria or the 1-year survival rate of the patients facing these MDR bacteria.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by a French Institutional Review Board (Comité d'Éthique de la Recherche en Anesthésie-Réanimation, IRB number 00010254-2020-153). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

MT participated in study design, acquisition of data, analysis and interpretation of data, performed the statistical analysis, and drafted the manuscript. ST and PM participated in study design, acquisition of data, analysis and interpretation of data, and drafted the manuscript. AT-D, LR, BL-J, JD, NZ, MB-R, PT, AS, and EA were involved in the analysis and interpretation of data. NG participated in the design of the study, were involved in the analysis and interpretation of data and drafted the manuscript. All authors have read and approved the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.609497/full#supplementary-material

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REVIEW
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Management of *Staphylococcus aureus* Bloodstream Infections

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Staphylococcus aureus bloodstream infections are associated with a high morbidity and mortality. Nevertheless, significance of a positive blood culture with this pathogen is often underestimated or findings are misinterpreted as contamination, which can result in inadequate diagnostic and therapeutic consequences. We here review and discuss current diagnostic and therapeutic key elements and open questions for the management of *Staphylococcus aureus* bloodstream infections.

Keywords: *Staphylococcus aureus* blood stream infections, endocarditis, diagnostic and therapeutic algorithm, current recommendations, open questions

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INTRODUCTION

Staphylococcus aureus (*S. aureus*) is one of the leading pathogens causing community-acquired and hospital-acquired bloodstream infections ranking second after *Escherichia coli*. Incidences were estimated between 10 to 30 cases per 100,000 person-years (1) and hospital mortality is high, ranging between 15 and 40% (2, 3).

The gram-positive pathogen has developed several strategies to adapt to the infected host by evading the hosts immune system, e.g., it can form biofilms, adhering to intravascular catheters and implantable medical devices (4). Furthermore, intracellular persistence in different kind of host cells such as epithelial and endothelial cells or osteoblasts, has been described (5). Inside these biofilms and host cells, *S. aureus* can form slow growing subpopulation, so called small colony variants (SCV). These colonies display a lower metabolic activity and have an increased tolerance against antibiotics, which can result in refractory or chronic infections and relapses (6, 7).

In 8–15% of the patients, hematogenous spread may also lead to later secondary complications such as endocarditis, vertebral osteomyelitis, abscesses, and implant associated infections of prosthetic joints, electronic cardiac devices etc., which can occur up to weeks or months after the primary infection. Notably, patients with community-acquired SA-BSI and patients with prolonged bacteremia have an increased risk for secondary foci (8). Further risk factors for complications are inadequate antibiotic treatment, an unknown primary focus of infection or insufficient source control (9).

Given the high rates of mortality and morbidity associated with SA-BSI the management differs from bloodstream infections with other bacteria. A structured management in diagnostic and treatment is crucial for an optimal outcome. Several studies have shown that an adherence to treatment guidelines and infectious disease bedside-consultation can lead to a reduction of mortality by up to 50% (10–12).

CURRENT DIAGNOSTIC AND TREATMENT STANDARDS

Diagnostic Key Principles

As a principle, blood cultures positive for *S. aureus* always need to be respected as a clinically significant finding and should result in an appropriate treatment. Blood culture contamination with *S. aureus* is a very rare event (<5%) and due to the high mortality and the high risk of serious complications associated with *S. aureus* bloodstream infections (SA-BSI), a prompt therapy is generally recommended (12–14).

S. aureus detection in urine culture (*S. aureus* bacteriuria) should lead to the search of an underlying bloodstream infection as *S. aureus* rarely causes genuine urinary tract infection, but is most likely filtrated through the kidneys (15, 16). An exception are patients with urinary tract foreign bodies and/or after urological interventions. In these patients, the urinary tract can be the primary focus of a bloodstream infection with the pathogen. *S. aureus* bacteriuria in patients with a SA-BSI has been associated with a worse outcome (17). Careful patient history and thorough physical examination with a special emphasis on potential foci are mandatory. Most frequent sources of SA-BSI are intravascular catheters and soft tissue infections (18). Further diagnostics have to be performed depending on clinical findings. In up to one third of all cases, however, septic embolism remains inapparent in the clinical examination and will be diagnosed solely in an extended diagnostic work-up imaging (19).

In order to prevent further spreading of *S. aureus* causing secondary septic metastases, source control must be carried out as quickly as possible. Infected foreign bodies incl. vascular catheters or cardiac electronic devices have to be removed quickly and completely in addition to an adequate antibiotic treatment (20, 21). If vascular catheters have been *in situ* during bacteremia, a removal should be considered even if another site is suspected as focus of the SA-BSI since catheters remain the most frequent primary source of infection and moreover

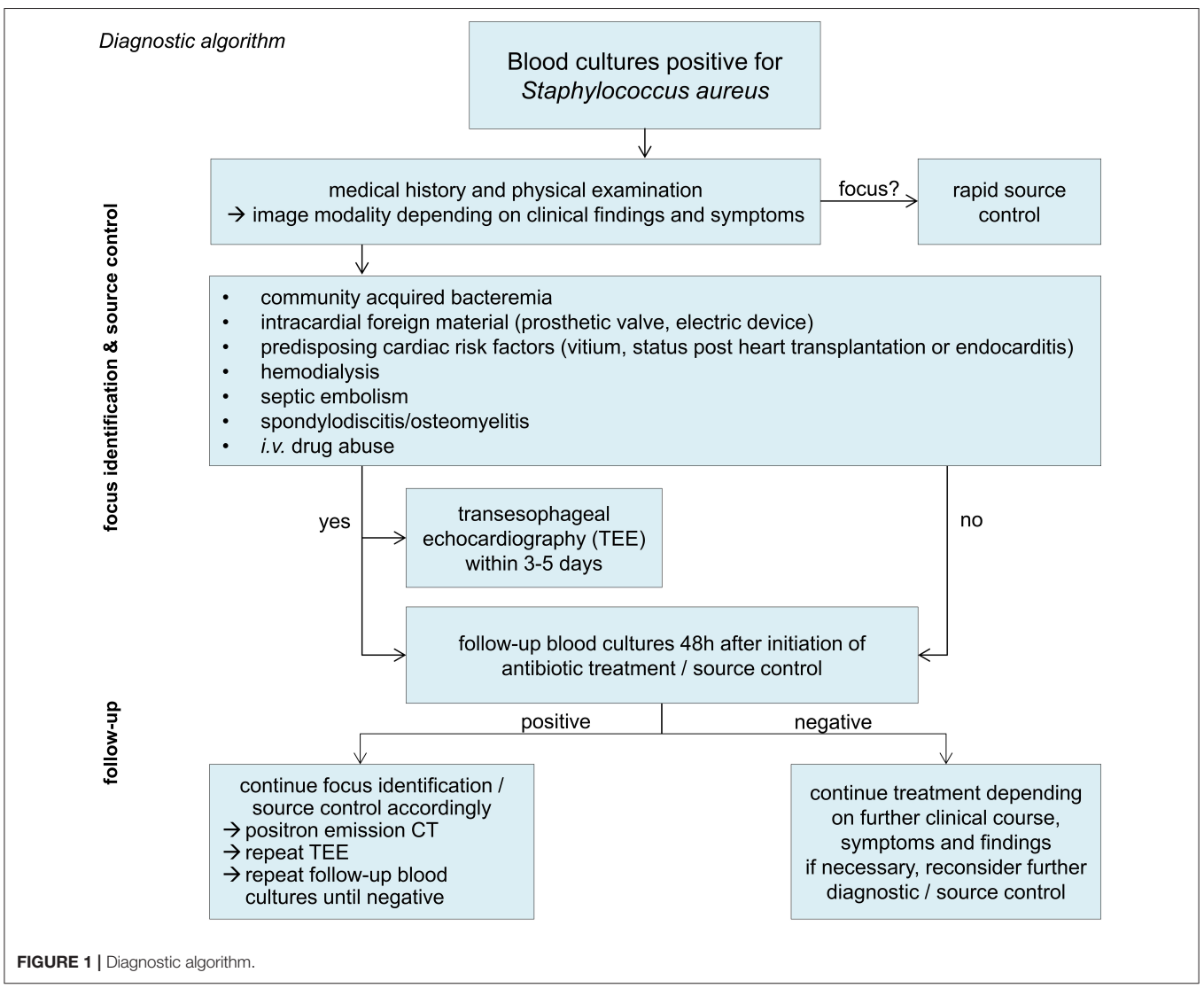


FIGURE 1 | Diagnostic algorithm.

there is a high risk of secondary catheter colonization (22–25). Endocarditis occurs in about 10–20% of patients with SA-BSI and worsens the patient's prognosis (26, 27). The diagnostic sensitivity of transesophageal echocardiography (TEE) is twice as high as the one of a transthoracic echocardiography (TTE) and should thus be used preferably (28). Ideally, TEE is performed within a few days (3–5 days) after diagnosis of a SA-BSI. In patients with persisting clinical suspicion of endocarditis and/or positive follow-up blood cultures, a repeated TEE after about 7 days is recommended (29, 30).

Twenty-four hours (up to 72 h at the latest) after the initiation of therapy, follow-up blood cultures are required to evaluate therapy success (31). Positive blood cultures at this point are associated with the presence of septic metastasis, insufficient source control, and consequently with a poorer outcome and therefore require further investigation (31, 32). It has been recommended to take at least two blood culture pairs at each time of collection as the sensitivity of blood culture depends on the number sampled (33, 34). However, at least for further follow-up blood cultures and particularly in intensive care units patients, this has to be weighed with the aims of "patient blood management" (35).

In patients with positive follow-up blood cultures, a fluorodeoxyglucose positron emission computed tomography scan (FDG-PET CT) should be considered. In a cohort study on 115 patients with gram-positive bacteremia (56% with *S. aureus*), PET-CT imaging had a very high sensitivity and specificity and detected twice as many septic embolisms compared to conventional diagnostic methods (19). An overview of the diagnostic key priciples is shown in **Figure 1**.

Therapeutic Key Principles

Antibiotics of choice in the therapy of bloodstream infections by methicillin (oxacillin) sensitive *S. aureus* (MSSA) are beta-lactam antibiotics with high activity against *S. aureus*. Best outcomes are being achieved with anti-staphylococcal penicillins (e.g., flucloxacillin) and first generation cephalosporins (cefazolin). A recent meta-analysis showed that cefazolin is not inferior to a therapy with anti-staphylococcal penicillins in the therapy of MSSA bloodstream infections (36). Moreover, cefazolin treatment was associated with a significant lower risk for drug side effects (nephrotoxicity, hepatotoxicity, venous irritation) and was associated with a numerically higher survival rate.

Piperacillin/tazobactam, ceftriaxon, cefuroxim and other broad spectrum beta-lactams should not be used for definite treatment of SA-BSI despite *in vitro* confirmed susceptibility, because they are not only associated with a higher likelihood to select multi-drug resistant pathogens but also with an increased mortality according to retrospective studies (37).

The relevance of penicillin allergy has recently been discussed (38). In case of an IgE-mediated (immediate type) penicillin allergy, daptomycin is recommended as an alternative to β-lactam antibiotics (39). Vancomycin application was associated with increased mortality compared to β-lactam antibiotics and therefore is not recommended for the definite treatment of MSSA bloodstream infections (40).

Antibiotics of choice for treating bloodstream infections due to methicillin (oxacillin) resistant *S. aureus* isolates (MRSA) are vancomycin and daptomycin (41). The reference range for vancomycin trough levels is 15–20 mg/l (12, 42). Lower trough levels have been associated with treatment failure (43). Another reason for treatment failure and poorer outcome is a reduced vancomycin susceptibility (44–46). Vancomycin resistance is defined by a minimum inhibitory concentration (MIC) of ≥16 μg/ml, strains with a MIC of 4–8 μg/ml are so called "vancomycin intermediate susceptible *S. aureus*" (VISA) (47). The term "hetero-resistant" VISA (hVISA) refers to *S. aureus* strains that are primarily within the susceptible range but contain subpopulations which can develop a reduced susceptibility under exposure to vancomycin (48). Patients with hVISA BSI are at higher risk of having a persistent bacteremia, which is associated with higher mortality in SA-BSI (49, 50). Altogether, occurrence of isolates with reduced susceptibility to vancomycin has increased over the last years probably also due to more frequent use of vancomycin in patients with MRSA infections (51). However, prevalence is still low with under 5% and daptomycin remains an effective treatment option in these isolates (52, 53).

Daptomycin monotherapy (with 8–12 mg/kg ideal bodyweight) is considered an equivalent alternative. Daptomycin has been shown to be inactive in patients with pneumonia, probably because it is inactivated by pulmonary surfactant and therefore unsuitable in patients with pneumogenic infection (54). Linezolid should not be used for MRSA bloodstream infection due to its bacteriostatic effect (55).

The new MRSA effective cephalosporins (ceftarolin, ceftobiprole) and lipoglycopeptides (dalbavancin) should not yet be used as first choice as there are no randomized controlled trials in patients with MRSA bloodstream infections (56–58).

Treatment duration depends on the clinical course and classification of infection as "complicated" or "uncomplicated"

BOX 1 | "Uncomplicated" *Staphylococcus aureus* bloodstream infection (41).

no evidence of endocarditis in the physical examination or echocardiography
no implanted foreign bodies *in situ* (e.g., prosthetic valves, cardiac electronic device, prosthetic joints)
negative blood cultures 48–96 h after initiation of therapy
no evidence of deep-seated focus or septic metastases (e.g., vertebral osteomyelitis)
defervescence within 48–72 h after initiation of therapy

BOX 2 | Prerequisite criteria for an oral sequential therapy (59).

adequate reduction of inflammatory parameters
clinically satisfactory response to treatment
no evidence of abscess, insufficient source control or endocarditis
temperature <38.0°C/100.4°F for >48 h
negative follow-up blood cultures

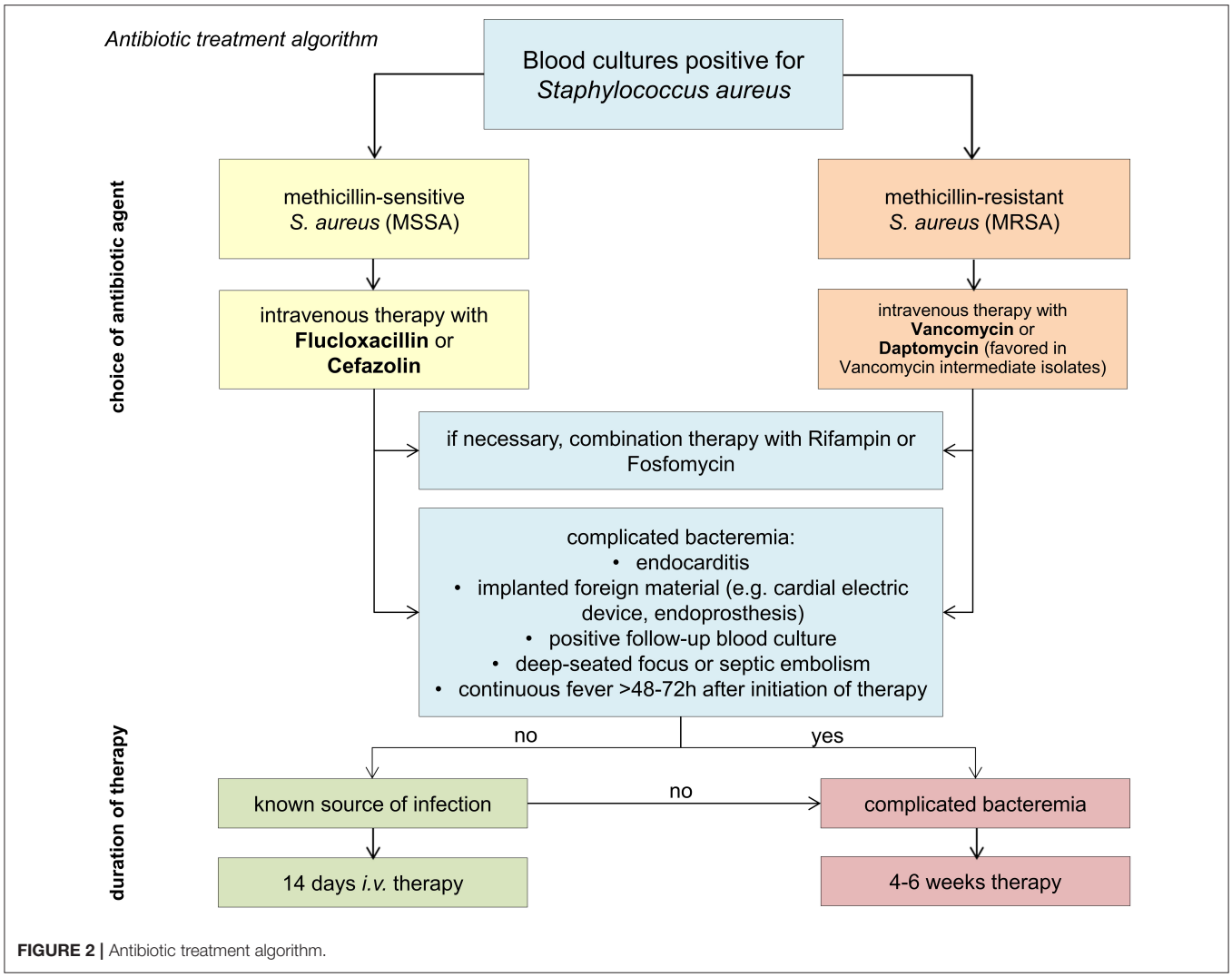


FIGURE 2 | Antibiotic treatment algorithm.

SA-BSI (**Box 1**). For patients with uncomplicated bloodstream infection an intravenous antibiotic treatment is recommended for at least 14 days. SA-BSI classified as complicated or without known source of infection require a minimum of 4–6 weeks of therapy. After completion of at least 14 days i.v. antibiotic therapy, an oral sequential therapy can be considered (**Box 2**). For an overview of the therapeutic principles (see **Figure 2**).

Pertinent Open Questions

Despite its frequency, the quality of evidence for the treatment of patients with SA-BSI is insufficient with only few randomized controlled trials and only a handful of larger multicenter retrospective studies available (3, 26, 60–62). The following chapter summarizes open questions and associated recent data.

CURRENT QUALITY EVIDENCE AND OPEN QUESTIONS

Who Needs Echocardiography?

Endocarditis is a dreaded complication of SA-BSI. Recently published studies show, that indication for an echocardiography should be risk-adjusted as not all patients have the same risk of acquiring an endocarditis (30, 63–65). Currently, a TEE is recommended in the following situations: community-acquired SA-BSI, prolonged bacteremia, history of intravenous drug abuse or dialysis, cardiac risk factors (foreign bodies, valve defects, history of endocarditis, heart transplant), septic embolism, e.g., vertebral osteomyelitis or cerebral embolism (30, 63, 65). Since clinical presentation of symptoms and findings are not specific, scores may be a helpful tool to identify patients who need transesophageal echocardiography. However, so far only retrospective studies have been performed on this topic (30, 65). Moreover, a large number of risk factors for endocarditis have been described in the literature to date (27, 65, 66) and hence, they apply to a large number of patients in daily clinical routine.

How Long Do We Have to Treat?

It is recommended to treat an uncomplicated SA-BSI for at least 14 days to avoid relapse (67). Among others, a prospective observational cohort study showed that relapse occurred in up to 8 vs. 0% of patients with short-course therapy <14 days (68).

In complicated SA-BSI and episodes with unknown primary focus of infection international experts recommend at least 28 days of antibiotic therapy (67). However, underlying evidence is not satisfactory (69) and guidelines and recommended quality indicators are mainly based on retrospective data (62, 70–73). Moreover, recommendations regarding treatment standards mostly refer to studies showing an improvement of outcomes by means of adherence to a bundle of measures or infectious specialist consultations (67). Randomized trials are needed to provide more evidence to what extend respective treatment elements such as duration of antibiotic therapy contribute to an improvement of patient outcomes.

Can We Switch to Oral Antibiotics?

Currently at least 14 days of parenteral antibiotic therapy are recommended in patients with a SA-BSI. At what point, or whether a switch to oral therapy is safe at all, is a matter of debate. In a recently published randomized multicenter trial by Iversen et al. changing to oral antibiotic combination treatment (e.g., dicloxacillin or linezolid plus rifampcin) was noninferior to continued intravenous treatment for patients with left side endocarditis including patients with *S. aureus* endocarditis (59). However, the outcome of the study was mainly carried by patients with native valve endocarditis due to streptococci (196 out of 400 patients). Therefore, it is unclear whether the conclusion of the study also applies to patients with *S. aureus* endocarditis. Comparable evidence for other foci of SA-BSI is lacking. Obviously, a sufficient oral bioavailability of the applied antibiotic is essential in any case. Possibly, the SABATO trial which finished recruitment recently will provide new aspects (61). The major objective of the randomized, parallel-group, observer-blinded, clinical non-inferiority trial is to demonstrate that in patients with low-risk SA-BSI a switch from intravenous to oral antimicrobial therapy is non-inferior to a conventional course of intravenous therapy.

What Is the Role of Combination Therapy?

The role of combination therapy, particularly with rifampicin or fosfomycin, in addition to a ß-lactam antibiotic, in MSSA, or vancomycin, in MRSA, SA-BSI, is a matter of debate. Theoretically, combination therapy could lead to a higher bactericidal activity compared to antibiotic monotherapy and synergistic effects could occur. Combination therapy may be superior in the eradication of intracellular staphylococci and biofilms on foreign materials and thus reduce the risk of secondary late infection and recurrence (74, 75).

However, disadvantages of combination therapy, such as drug-related side effects and interactions need to be considered in the benefit-risk assessment. Clinical studies have not shown a benefit of routine combination therapy for all patients with SA-BSI (3, 76–79). The multicenter randomized ARREST trial by Thwaites et al. (3) found no significant effect of additional rifampicin on treatment failure, disease recurrence, or death.

The Role of Computerized Decision Systems and Phone Consultations

A structured management in diagnostic and treatment is crucial for an optimal outcome. Several studies have shown that an adherence to treatment guidelines and particularly infectious disease bedside-consultation can lead to a reduction of mortality by up to 50% (10–12). Given the limited availability of infectious diseases physicians, who are usually based in larger hospitals and the standardized management outlined above, novel approaches are currently under investigation, such as computerized decision support systems and phone consultations, to improve outcome by providing respective expertise also in smaller hospitals (80, 81). However, it has yet to be proven whether these approaches are as effective as bedside-consultations by infectious diseases physicians.

CONCLUSION

Management of patients with SA-BSI remains challenging as mortality and complication rates are high and we still lack sufficient high-quality evidence addressing the most pertinent questions. A structured management preferably provided by an antibiotic stewardship team or infectious consultation including a standardized diagnostic work-up and therapeutic approach is prerequisite for all patients with SA-BSI to improve treatment quality and patient outcomes.

AUTHOR CONTRIBUTIONS

AK, SH, SW, CB, and MP: drafting of the manuscript. All authors: critical revision of the manuscript and additional important intellectual content.

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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.

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Extensively Drug-Resistant Hypervirulent *Klebsiella pneumoniae* From a Series of Neonatal Sepsis in a Tertiary Care Hospital, India

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The recent emergence of multidrug-resistant (MDR) *Klebsiella pneumoniae* with hypervirulent traits causing severe infections and considerable mortality is a global cause for concern. The challenges posed by these hypermucoviscous strains of *K. pneumoniae* with regard to their optimal treatment, management, and control policies are yet to be answered. We studied a series of extensively drug-resistant (XDR) and hypervirulent *K. pneumoniae ST5235 isolates* with resistance to carbapenems and polymyxins causing neonatal sepsis in a tertiary care hospital in India. A total of 9 *K. pneumoniae* isolates from 9 cases of neonatal sepsis were studied with respect to their clinical relevance, antimicrobial susceptibility profile, presence of extended spectrum β lactamase (ESBL) production, and responsible genes, carbapenemases (classes A, B, and D), and aminoglycoside-resistant genes. Hypervirulence genes encoding hypermucoid nature, iron uptake, and siderophores were detected by multiplex PCR. The plasmid profile was studied by replicon typing. Isolates were typed by multilocus sequence typing (MLST) and enterobacterial repetitive intergenic consensus (ERIC) PCR to study the sequence types (STs) and clonal relation, respectively. The neonates in the studied cases had history of pre-maturity or low birth weight with maternal complications. All the cases were empirically treated with piperacillin–tazobactam and amikacin followed by imipenem/meropenem and vancomycin and polymyxin B as a last resort. However, all the neonates finally succumbed to the condition (100%). The studied isolates were XDR including resistance to polymyxins harboring multiple ESBL genes and carbapenemase genes (bla_{NDM} and bla_{OXA-48}). Hypervirulence genes were present in various combinations with *rmpA/A2* genes present in all the isolates. IncFI plasmids were detected in these isolates. All belonged to ST5235. In ERIC PCR, 6 different clusters were seen. The study highlighted the emergence and burden of XDR hypervirulent isolates of *K. pneumoniae* causing neonatal sepsis in a tertiary care hospital.

Keywords: polymyxins, hypermucoviscous, MDR, carbapenems, fatal, ST5325

INTRODUCTION

Drug resistance in *Klebsiella pneumoniae* is ever increasing with adoption of several evolutionary routes by this "World Health Organization (WHO) critical priority pathogen" (1). With tremendous ability to acquire resistant determinants and hypervirulent elements, *K. pneumoniae* is now a global threat causing considerable mortality in the affected patients. Evolving gradually from multidrug-resistant (MDR) to extensively drug-resistant (XDR), *K. pneumoniae* isolates had not exhibited simultaneous drug resistance and hypervirulence for long until recently with the emergence of carbapenem-resistant hypervirulent *K. pneumoniae* (CR-hvKP) (2). The primary reason for this delayed emergence has been the successive convergence of two distinct traits of drug resistance and virulence through recombination of several plasmids, resulting in MDR-virulent strains (3). While hvKP has been associated with community-acquired infections, increasing reports of healthcare-associated infections are also being reported (4).

Over the years, hvKP has spread like an epidemic in Asian countries like China, South Korea, and Japan, with the first case being reported from Taiwan in 1986 (5). Widely prevalent and diverse convergent MDR-virulent strains of *K. pneumoniae* have already been reported from the South Asian region including India (6). There have been sporadic reports from India on CR-hvKP in invasive infections revealing the seriousness of the situation. The first report on CR-hvKP in sepsis from the subcontinent came in 2018 followed by a single case of neonatal sepsis recently (7, 8). We report a series of neonatal sepsis due to CR-hvKP along with simultaneous resistance to polymyxins causing high mortality from a tertiary care center.

MATERIALS AND METHODS

Study Site and Design

The study was conducted at the Institute of Medical Sciences, Banaras Hindu University, in Varanasi, and Assam University, Silchar, Assam, India. During a retrospective audit on mortality in the neonatal intensive care unit (NICU), a cluster of cases of neonatal sepsis due to MDR *K. pneumonia* with significant mortality was found during the months of April to June 2017. The isolates were revived from stock cultures and studied as detailed below. The study was approved by the Institute's ethical committee.

Bacterial Strains and Identification

A total of 9 isolates that were biochemically identified as *K. pneumoniae* were revived by subculture on MacConkey agar and were re-identified by a VITEK® 2 compact system (bioMerieux, USA).

Antimicrobial Susceptibility Testing

Susceptibility testing was done using both disk diffusion method and VITEK® 2 compact system on the following antibiotics: amoxicillin/clavulanic acid, piperacillin/tazobactam, cefuroxime, ceftriaxone, cefoperazone, cefepime, ertapenem, imipenem, meropenem, amikacin, gentamicin, ciprofloxacin, levofloxacin, and trimethoprim/sulfamethoxazole. For susceptibility against polymyxin B and colistin, broth microdilution was performed. Results were interpreted based on CLSI 2020 guidelines (9). For quality control, *Escherichia coli* ATCC 25922 was used.

Molecular Characterization of Extended Spectrum β Lactamases, Carbapenemases, and Aminoglycoside-Resistant Determinants

All the isolates were tested for the presence of ESBL genes, as well as carbapenemase and aminoglycoside resistance-encoding genes. Briefly, multiplex PCR was performed for the amplification of ESBL genes (bla_{TEM}, bla_{SHV}, $bla_{CTXM1,2,9)}$ along with class A (*bla*$_{SME}$, *bla*$_{NMC}$, *bla*$_{GES}$, *bla*$_{KPC}$), class B (*bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{NDM}$), and class D (*bla*$_{OXA-48}$), carbapenemase genes using primers and reaction conditions as described elsewhere (10, 11). The isolates were further screened for the presence of aminoglycoside resistance genes by three multiplex PCR assays targeting different aminoglycoside resistance genes, viz., *ant(2″)-Ia*, *ant(3″)-I*, *ant(4′)-Ia*, *aac(3)-I*, *aac(3)-IIc*, *aac(6′)-Ib*, *aac(6′)-II*, *aph(2″)-Ib*, *aph(2″)-Ic*, *aph(2″)-Id*, *aph(3′)-I*, *aph(3′)-IIb*, *aph(3′)-IIIa*, *aph(3′)-VI a*, and *aph(4)-Ia* (12).

Determination of Hypervirulence Genes in *K. pneumoniae* Isolates

All the isolates were selected for the characterization of different virulence genes by five different multiplex PCR assays. For amplification and characterization of hypervirulence genes, a set of 29 primers was designed in-house using the NCBI primer blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/); from the nucleotide sequence of virulence genes, the amplified products were further sequenced to confirm the presence of virulence genes. The primer sequences and running conditions are summarized in **Supplementary Table 1**.

Replicon Typing

For performing PCR-based replicon typing, plasmids from the *K. pneumoniae* isolates were extracted (QIAprep Spin Miniprep Kit, QIAGEN, Germany) and were transformed into *E. coli* JM107. Transformants were selected in the media containing 100 μg/ml of ampicillin. Plasmids isolated from transformants were subjected to PCR assay. Eighteen pairs of primers were used to perform five multiplex and three simplex PCR assays recognizing FIA, FIB, FIC, HI1, HI2, I1-Ig, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA (13).

Multilocus Sequence Typing

Multilocus sequence typing (MLST) was carried out for all *K. pneumoniae* isolates. Genomic DNA was extracted using QIAamp® DNA mini kit as per manufacturer instruction. Amplification of the seven housekeeping genes *rpoB*, *gapA*, *mdh*, *pgi*, *PhoE*, *infB*, and *tonB* was done using the primer pairs and conditions as described earlier (14). The amplified product from all nine isolates was sequenced by Sanger sequencing (EzeDiagnon Healthcare PVT., LTD, India). The obtained

sequences were assembled by BioEdit v7.2.5 software. The sequence types were determined after analyzing with the help of the Institut Pasteur *K. pneumoniae* MLST database (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html).

Typing of the Isolates by ERIC-PCR

The heterogeneity of the isolates was determined by enterobacterial repetitive intergenic consensus (ERIC) PCR using the universal primer ERIC-F (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC-R (5′-AAGTAAGTGACTGGGGTGAGCG-3′). Amplification was done under previously described reaction conditions, and the bands patterns were analyzed by agarose gel electrophoresis (15). Based on the band patterns, the dendrogram was constructed by using computer program NTSYS-pc version 2.0.

RESULTS

A total of 9 isolates of *K. pneumoniae* subspecies *pneumoniae* were characterized. The characteristics of the patients who were the source of these isolates are shown in **Table 1**. Majority of the neonates were of very low birth weight (<1,500 g, 66.6%), were pre-term/pre-mature (55.5%), and presented with respiratory distress (6, 66.6%). All but 1 (88.8%) mother of these cases of neonatal sepsis had complications during or prior to delivery in form of preeclampsia, heart disease, and abnormal vaginal discharges. In majority of the cases (6, 66.6%), the mode of delivery was by lower-segment Cesarean section (LSCS). All the cases were empirically treated with piperacillin–tazobactam and amikacin followed by imipenem/meropenem and vancomycin. As a last resort, treatment with polymyxin B was also given. However, all the neonates finally succumbed to the condition (100%).

All the isolates were extensively drug resistant (XDR) including resistance to polymyxins as per the resistance profile shown in **Table 2**. The MIC range noted for polymyxin B was 4–32 μg/mL whereas that for colistin was 4–16 μg/mL. Presence of ESBL genes and carbapenemase- and aminoglycoside-encoding genes is shown in **Table 3**. All the isolates harbored bla_{SHV}, bla_{CTXM}, and *aac(6′)-Ib*. Among the class B carbapenemases, bla_{NDM} and/or bla_{OXA-48} were detected in 4 isolates while 5 isolates did not harbor these genes. None of the isolates had class A carbapenemase genes.

Among the hypervirulence genes, *rmpA2* was present in 8 isolates while 1 isolate harbored the *rmpA* gene. Iron uptake-encoding genes (*fyuA*/*kfuA*, *kfuB*) were present in 7 isolates while the gene for allantoin metabolism (*allB*) was seen in a single isolate. Siderophore-encoding genes (*iroC*, *ybt*, *irp*, *iucA*) were present in all the isolates. The distribution of these hypervirulence genes is shown in **Table 3**.

All the isolates harbored the IncFIc plasmid. Strain typing by ERIC-PCR revealed 100% similarity in 2 isolates each in groups B, E, and F (**Figure 1**). All the isolates belonged to ST5235.

DISCUSSION

Carbapenem resistance in *K. pneumoniae* is of critical importance owing to its widespread dissemination. Concurrently, hypervirulence in *K. pneumoniae* is also a serious public health threat. In this study, we report a series of neonatal sepsis due to carbapenem and polymyxin (polymyxin B and colistin)-resistant hvKP causing 100% mortality. To the best of our knowledge, this is the first extensive series of neonatal sepsis caused by XDR-hvKP from the subcontinent.

Hypervirulence has been reported to be associated with very high mortality with rates ranging from 3 to 31%, often increasing up to 35% in case of sepsis (4). This rate further increases when hvKP additionally presents with drug resistance, where the rate as high as 100% has been noted in cases of sepsis due to CR-hvKP (16). Extremely high mortality (84.2%) associated with meropenem-resistant phenotypic and genotypic hvKP-causing sepsis has also been reported from India (6). There has been another single case study on neonatal sepsis from Kolkata, India, with CR-hvKP with inconclusive data on survival as the neonate was lost to follow-up (7). Unfortunately,

TABLE 1 | Details of neonates and *K. pneumoniae* isolates in the study.

S. no.	Isolate identity	Source	DoA	DoI	Risk factors				
					VIbw	Respiratory distress	Pre-term/ pre-mature	Complications in mother	Mode of delivery
1	BK1	Blood	20 April 17	24 April 17	N	Y	N	Y	LSCS
2	BK2	Blood	05 April 17	28 April 17	Y	N	Y	Y	LSCS
3	BK3	Blood	27 April 17	01 May 17	N	N	Y	Y	LSCS
4	BK4	Blood	27 April 17	01 May 17	Y	Y	Y	Y	LSCS
5	BK5	Blood	02 May 17	02 May 17	Y	Y	N	Y	SVD
6	BK6	Blood	07 May 17	09 May 17	N	N	Y	N	LSCS
7	BK7	Blood	08 May 17	14 May 17	Y	Y	N	Y	SVD
8	BK9	Blood	21 May 17	24 May 17	Y	Y	N	Y	SVD
9	BK14	Blood	30 May 17	03 June 17	Y	Y	Y	Y	LSCS

DoA, date of admission; DoI, date of isolation; VIbw, very low birth weight; N, no; Y, yes; LSCS, lower-segment cesarean section; SVD, spontaneous vaginal delivery.

TABLE 2 | Resistance profile of *K. pneumoniae* isolates.

Isolates	Antimicrobial resistance profile															
	AMC	PTZ	CXM	CRO	CFP	CPM	LVX	ETP	IPM	MEM	AMK	GEN	CIP	SXT	CL*	PB*
BK1	R	R	R	R	R	R	R	R	R	R	I	R	R	S	R	R
BK2	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
BK3	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
BK4	R	R	R	R	R	R	R	R	R	R	I	R	R	S	R	R
BK5	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
BK6	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
BK7	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
BK9	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
BK14	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

*AMC, amoxicillin/clavulanic; PTZ, piperacillin/tazobactam; CXM, cefuroxime; CRO, ceftriaxone; CFP, cefoperazone; CPM, cefepime; LVX, levofloxacin; ETP, ertapenem; IPM, imipenem; MEM, meropenem; AMK, amikacin; GEN, gentamicin; CIP, ciprofloxacin; SXT, trimethoprim/sulfamethoxazole; CL, colistin; PB, polymyxin B; R, resistant; I, intermediate; S, susceptible. *Microbroth dilution method.*

TABLE 3 | Summary of *K. pneumoniae* isolates showing resistance genes, ERIC, MLST and plasmid profiles, and virulence genes.

S. no	Isolate identity	ERIC type (group)	Sequence type (ST)	Plasmid	AMR genes	Virulence genes			
						Hypermucoid	Iron uptake	Allantoin metabolism	Siderophores
1	BK1	A	5235	IncFIC	bla_{SHV}, bla_{CTXM}, bla_{NDM}, bla_{OXA-48}, *aac(6')-Ib*	*rmpA2*	-	-	*ybtQ*, *ybtA*, *ybtE*, *ybtS*, *iroC*, *irp1*, *irp2*
2	BK2	B	5235		bla_{SHV}, bla_{CTXM}, *aac(6')-Ib*	*rmpA2*	-	-	*ybtQ*, *ybtA*, *ybtE*, *ybtS*, *iroC*, *irp1*, *irp2*
3	BK3	B	5235	IncFIC	bla_{SHV}, bla_{CTXM}, bla_{NDM}, bla_{OXA-48}, *aac(6')-Ib*	*rmpA2*	*fyuA*	-	*ybtQ*, *ybtA*, *ybtE*, *ybtS*, *irp1*, *irp2*
4	BK4	C	5235	IncFIC	bla_{SHV}, bla_{CTXM}, bla_{NDM}, bla_{OXA-48}, *aac(6')-Ib*	*rmpA2*	*fyuA*	-	*ybtQ*, *ybtA*, *ybtE*, *ybtS*, *ybtU*, *ybtXirp1*, *irp2*
5	BK5	D	5235	IncFIC	bla_{SHV}, bla_{CTXM}, *aac(6')-Ib*	*rmpA2*	*fyuA*	*allB*	*ybtQ*, *ybtE*, *ybtS*, *irp1*, *irp2*
6	BK6	E	5235	IncFIC	bla_{SHV}, bla_{CTXM}, *aac(6')-Ib*	*rmpA2*	*fyuA*	-	*ybtQ*, *ybtA*, *ybtE*, *ybtS*, *irp1*, *irp2*
7	BK7	E	5235	IncFIC	bla_{SHV}, bla_{CTXM}, *aac(6')-Ib*	*rmpA2*	*fyuA*	-	*ybtQ*, *ybtA*, *ybtE*, *ybtS*, *irp1*, *irp2*
8	BK9	F	5235	IncFIC	bla_{SHV}, bla_{CTXM}, *aac(6')-Ib*	*rmpA2*	*fyuA*	-	*ybtQ*, *ybtA*, *ybtE*, *ybtS*, *iroC*, *irp1*, *irp2*
9	BK14	F	5235	IncFIC	bla_{SHV}, bla_{CTXM}, bla_{OXA-48}, *aac(6')-Ib*	*rmpA*	*kfuA*, *kfuB*,	-	*iucA*, *irp1*, *irp2*

100% mortality was seen in the neonates in this study where isolates were also resistant to polymyxins. There have been sporadic reports of colistin-resistant hvKP where isolates retained susceptibility to aminoglycosides, ciprofloxacin, piperacillin–tazobactam, imipenem, and tigecycline despite colistin resistance (17). Consequently, mortality rates were low due to available treatment options.

For long, it was thought and observed that hvKP isolates remained susceptible to antimicrobials until the recent introduction of "convergent" *K. pneumoniae* strains that are both drug resistant and hypervirulent (5). Few of the previous studies have mentioned low drug resistance potential of the hvKP isolates based on either their susceptibilities to the first line of antimicrobials or due to the sporadic presence of ESBL genes not limiting therapeutic options to a greater extent (18). On the other hand, there has been a noticeable upsurge in reports on MDR hvKP especially from developing countries like China, India, Iran, and Brazil where tackling of these isolates has been a big challenge from the treatment point of view (6, 16, 17, 19, 20). The situation is exemplified in the

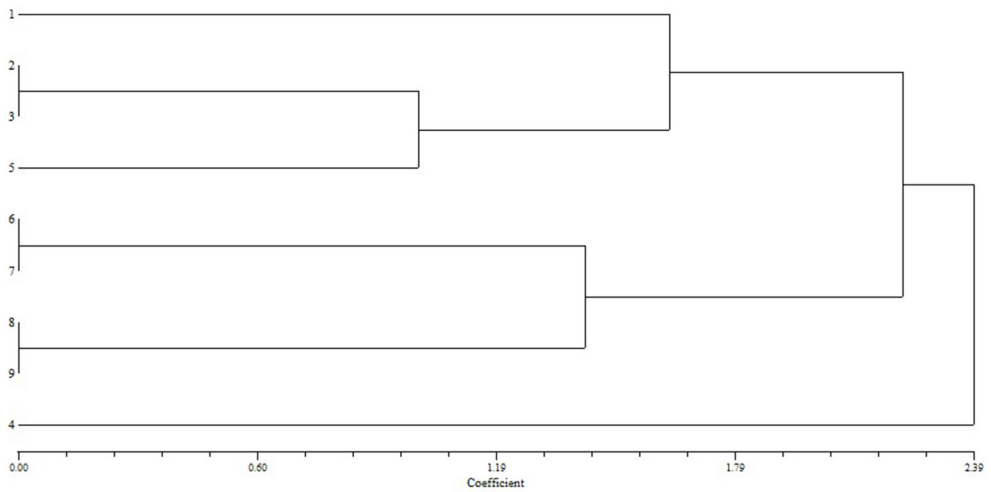


FIGURE 1 | Dendrogram constructed using the ERIC-PCR profile of *K. pneumoniae* isolates.

current study where XDR isolates with resistance to last-resort drugs like carbapenems and polymyxins from cases of neonatal sepsis displayed hypervirulence, posing a huge challenge for control. Management of such infections encompasses both adequate source control and active antimicrobial treatment (4). However, it should be mentioned that the neonatal unit in the study followed strict disinfection policy and appropriate infection control practices and even after thorough routine environmental surveillance, no such isolates have been found in the environment or in any of the surveillance cultures from the neonates.

There has been dearth of data and absence of any trial assessing the antimicrobials best suited for treatment of infections caused by hvKP. In contrast, prevalence of MDR in hvKP has been increasing in parallel with the increase in healthcare-associated infections. Suggestions on the use of newer antimicrobial combinations like ceftazidime–avibactam, meropenem–vaborbactam, and imipenem–relebactam for effective treatment of infections by CR-KP have been made (4). However, these antimicrobials have a limited role in infections due to class B carbapenemases, which is one of the major mechanisms of carbapenem resistance in these isolates as revealed in this study and those from developing countries. The bla_{NDM} and $bla_{\mathrm{OXA-48}}$ genes have been often reported to be associated with the hvKP isolates in most of the studies from Asian countries (6, 19). Against antibiotic selection pressure, studies have shown that hvKP isolates can harbor resistance gene plasmids including ESBL, carbapenemases, and those for colistin resistance (5).

It is a well-known fact that several virulence factors of *K. pneumoniae* have facilitated its spread and emergence as a global threat. Among the several genetic markers to define the hypervirulence in *K. pneumoniae*, presence of regulators of the mucoid phenotype, *rmpA* and *rmpA2* genes, produced siderophores like aerobactin, enterobactin, yersiniabactin, and salmochelin, and genes involved in iron uptake and allantoin metabolism are the major ones (18, 21). There has been evidence that *rmpA*/*rmpA2* genes along with siderophores are closely associated with invasive infections (22). All the XDR hvKP isolates in the present study were phenotypically and genotypically hypermucoviscous with multiple types of siderophore-encoding genes. The highly invasive profile of these isolates was reflected in their ability to cause 100% mortality in the neonatal unit.

Emergence of new sequence types among hvKP strains has been noted in few of the recently conducted studies. Though ST23 has been proposed as the major dominant clone of hvKP in Asia (5); most of the infections caused by this clone are community acquired (18). All the XDR hvKP isolates in this study belonged to ST5235, the significance of which could not be discussed owing to paucity of literature on this sequence type.

Despite being an addition to the scarce data on XDR hvKP in neonatal sepsis, this study was not without limitations. While the sources of these isolates could not be traced, classification of the nature of the infections as community or hospital acquired could not be made. Whole-genome sequences of the isolates could have provided better insight into the genome of these isolates. Nevertheless, this study puts forward several yet-to-be-answered issues related to the emergence of XDR hvKP in hospitals. While the origin of these isolates still remains debated, their exact control measures are yet to be determined. More importantly, consensus decisions on optimal therapy against these XDR *K. pneumoniae* isolates with hypervirulence could be the best armament, thus changing the epidemiology of these infections.

CONCLUSION

The study analyzed a fatal case series of neonatal sepsis caused by hvKP with extensive drug resistance to carbapenems and polymyxins in a tertiary-care hospital in India, thus

revealing the challenges posed by these emerging pathogens in developing countries.

DATA AVAILABILITY STATEMENT

The datasets generated in this article are not readily available because it is a collaborative study and requires administrative approval for availability. Requests to access the datasets should be directed to ab0404@gmail.com.

AUTHOR CONTRIBUTIONS

TB designed the concept and prepared the manuscript. JW performed the experimental work, data collection, and analysis and preparation of the manuscript. SS collected samples and carried out susceptibility profiling. AK helped in clinical analysis of the study. AB supervised the whole work. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.645955/full#supplementary-material

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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.

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REVIEW
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Rapid Antimicrobial Susceptibility Testing Methods for Blood Cultures and Their Clinical Impact

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Antimicrobial susceptibility testing (AST) of bacteria isolated in blood cultures is critical for optimal management of patients with sepsis. This review describes new and emerging phenotypic and genotypic AST methods and summarizes the evidence that implementation of these methods can impact clinical outcomes of patients with bloodstream infections.

Keywords: antimicrobial susceptibility testing, phenotypic, genotypic, sepsis, bloodstream infection

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INTRODUCTION

Sepsis, defined as an infection with dysregulated host response leading to life-threatening organ dysfunction, occurred in nearly 49 million incident cases and accounted for 19% of all deaths worldwide in 2017 (1, 2). The burden of sepsis and its attributable mortality vary greatly by geographic region and patient age (3, 4). In the USA, sepsis is the most common cause of in-hospital death and costs greater than $24 billion annually (1, 5). Mortality rates due to bloodstream infection (BSI) range between 12 and 32% in North America and Europe and are even higher in low- and middle-income countries. Mortality is due in part to increasing rates of antimicrobial-resistant pathogens (5–10). Patients infected with resistant pathogens are more likely to receive ineffective empiric antibiotic therapy, which is associated with poor outcomes, including death (11–14). Conversely, treatment with overly broad antibiotics increases risk of adverse drug events and drives further development of resistance (15, 16).

Sepsis is frequently caused by BSIs. In a retrospective analysis of nearly 3 million adult encounters across 409 US hospitals, a positive blood culture was found in 17% of patients with sepsis (17). In a Swiss prospective population-based study, blood culture-proven pediatric sepsis accounted for 66% of all pediatric hospital admissions for sepsis (18). Knowledge of the antimicrobial susceptibility profile of a blood isolate as soon as possible is often critical for optimal management and outcomes of patients with sepsis, enabling de-escalation or escalation of antibiotics to appropriate definitive therapy (19–22). While standard turnaround time for clinical microbiology laboratories to isolate, identify, and perform antimicrobial susceptibility testing (AST) of bacterial isolates is 48–96 h from the time a blood culture turns positive (23), many rapid testing methods provide results within 6–24 h (24, 25). These novel diagnostics are routinely used in many hospitals; however, the clinical benefit of these methods has not been well-quantified. In this review, we describe currently available rapid AST methods along with the data that support their clinical benefit to patients with sepsis and bacteremia. Fungemia is not discussed, as to date, rapid methods focus on identification of *Candida* in blood cultures, but not susceptibility testing.

ANTIMICROBIAL SUSCEPTIBILITY TESTING METHODS

US and European regulatory agencies require novel AST methods to generate results that are substantially equivalent to those of the international reference method, broth microdilution (BMD) (26). BMD is fraught with technical limitations that make these correlations challenging, not the least of which include need for a bacterium isolated in pure culture (which may artificially select out a subpopulation of microorganism that grows best *in vitro*), use of culture media that are a poor mimic of the physiological environment of the body, and use of an inoculum size that is infrequently observed in clinical specimens [i.e., 10^8 colony-forming units (CFU)/ml] (27–30). Despite these limitations, BMD is thought to provide a reasonable, albeit imperfect, correlation to treatment outcomes (31) with decades of data. It is important to recognize that results obtained from new AST methods are "fit" to match those obtained for the reference BMD during test development, which prohibits discovery of outputs that may be better predictors of clinical outcome than the minimum inhibitory concentration (MIC). As such, novel methods for determination of AST have focused primarily on developing a more rapid result—by two primary approaches: those that evaluate a microorganism's phenotype and those that evaluate its genotype (32). In general, rapid phenotypic methods are replacement technology for traditional AST tests used in the clinical laboratory, as these can predict both susceptibility and resistance to an antimicrobial and correlate reasonably well with reference BMD (32). In contrast, full correlation between the genotype and BMD has remained elusive (33–35), and genotypic methods are universally backed up with a phenotypic susceptibility test that is performed upon isolation of the microorganism from blood cultures. A combination of genotypic and phenotypic methods may provide both prediction of susceptibility (if no resistance gene is detected) and AST results for antimicrobials with multifactorial and unclear resistance mechanisms (for example, daptomycin) (32, 36–40).

NOVEL PHENOTYPIC METHODS FOR RAPID ANTIMICROBIAL SUSCEPTIBILITY TESTING FROM POSITIVE BLOOD CULTURES

Historically, off-label use of positive blood culture broth as the inoculum for disk diffusion or automated AST methods (e.g., Vitek 2) was widely performed in clinical laboratories to expedite time to results for AST from positive blood cultures (41). This was possible, as most blood cultures are monomicrobial and the concentration of bacteria in a positive blood culture approximates a 0.5 McFarland, the inoculum concentration used for traditional AST tests (42). However, the practice became less widespread in the USA following implementation of more stringent laboratory regulation in the form of the Clinical Laboratory Improvement Amendments, which placed stricter controls over laboratory-developed tests. To help with this dilemma, standardization of a direct-from-blood culture disk diffusion method has been undertaken by two laboratory standards groups: the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI). The EUCAST rapid AST method includes use of positive blood culture as the inoculum for a standard disk diffusion test, which is incubated for 4–8 or 16–18 h. Alternative interpretive criteria are provided for some organisms/antimicrobials at 4–8 h of incubation, which include a "area of technical uncertainty," i.e., an indeterminate result for some antimicrobials and organisms (43). CLSI's method is similar to that of EUCAST, with plans to publish in 2021 (Audrey Schuetz, personal communication to RMH). While more rapid, these methods remain manual and labor-intensive. A large international evaluation of the EUCAST method demonstrated that 88% of results could be read and 70% interpreted at the 4-h timepoint, which improved to 99 and 85%, respectively, by 6 h (44). Total laboratory automation (TLA) instrumentation may allow for automated setup and reading of disk diffusion zones, providing increased consistency and throughput, although application of this method has not been widely done (45).

More sophisticated approaches to rapid AST from blood cultures use alternative approaches to evaluate the phenotype by applying approaches such as microscopic evaluation of antimicrobial-induced changes to cell morphology, evaluation of division rates, or gene expression (37) (**Table 1**). Two rapid AST methods are currently approved by regulatory agencies and clinically in use for rapid phenotypic AST from positive blood cultures: the Accelerate PhenoTest BC® (Tucson, AZ), which is both US Food and Drug Administration (FDA) cleared and Conformite-Europeenne *in vitro* Diagnostic (CE-IVD) approved, and the Alfred 60AST (Alifax, Italy), which is CE-IVD. The PhenoTest BC performs rapid identification (ID) (1.5 h) and AST (∼7 h) from positive blood cultures by performing fluorescence *in situ* hybridization and time-lapse imaging of bacteria under dark-field microscopy, respectively (39, 46). A variety of morphological and kinetic changes in the bacteria compared with no-antimicrobial controls are used to determine MICs. The PhenoTest has been widely evaluated in the literature and has rapid turnaround time and good performance in US and European studies (46, 49–54). In contrast, the Alfred 60AST utilizes light scattering to detect bacterial growth in a liquid-based culture broth, determining results within 3–5 h. Organism identification is not performed with this latter method and must be determined using alternative methods (47).

In addition to these methods, a number of novel technologies are in late-phase development, which seeks to increase the speed of phenotypic testing. These technologies evaluate morphological and/or physiological responses earlier in the course of antimicrobial exposure than the traditional 16–20 h. Examples of responses include changes to cell size, mass, membrane integrity, metabolism, and DNA transcription; these approaches are reviewed in detail elsewhere (37, 38). Among the many methods in development, several have achieved CE-IVD, although are not yet in distribution. Among these, the dRAST (QuantaMatrix, Inc., Seoul, Republic of Korea) (55) and ASTar® (Q-linea, Sweden) (56) methods both evaluate morphological changes by time-lapsed microscopic imaging of

TABLE 1 | Select rapid phenotypic AST methods that are approved for testing positive blood cultures.

Test	AST technology	TTR	Regulatory status	References
PhenoTest BC (Accelerate Diagnostics)	Time-lapse imaging of bacterial cells under dark-field microscopy. Morphological and kinetic changes analyzed.	7 h	US FDA cleared, CE-IVD	(46)
Alfred (AliFAX)	Light scattering to detect bacterial growth in liquid culture broth.	3–5 h	CE-IVD	(47)
dRAST (QuantaMatrix)	Time-lapse imaging of bacterial cells on micropatterned plastic microchips.	6 h	CE-IVD	(48)
Reveal AST (Specific Diagnostics)	Sensor array for volatile organic compounds emitted during microorganism growth.	4.5 h	CE-IVD	[1]
ASTar (Q-linea)	Time-lapse imaging of bacterial growth in broth.	3–6 h	CE-IVD	[2]
Fastinov	Flow cytometry applying fluorescent dyes that reveal cell damage during treatment.	80 min	CE-IVD	[3]
LifeScale (Affinity Biosensors)	Mass measurement using a microcantilever.	4 h	CE-IVD	[4]

AST, antimicrobial susceptibility testing; TTR, time to result.
[1]*https://specificdx.com/reveal-ast (accessed November 30, 2020).*
[2]*https://www.qlinea.com/our-products/astar/astar-instrument/ (accessed November 30, 2020).*
[3]*http://www.fastinov.com/ (accessed November 30, 2020).*
[4]*http://www.lifescaleinstruments.com/Products/Clinical (accessed November 30, 2020).*

bacterial cells exposed to antimicrobials. Both yields results in ∼6 h and requires off-line identification of the bacteria. More novel approaches include those taken by LifeScale (Affinity Biosensors, Santa Barbara, CA), which measures impact of antimicrobials to bacterial cell mass via a microcantilever (57), Fastinov (Portugal), which evaluates cell by flow cytometry (58), and Reveal™ AST (Specific Diagnostics, Mountain View, CA) (see table footnote 1), which utilizes sensor arrays to measure changes to volatile organic compounds emitted during bacterial growth. The extent to which these methods may correlate with BMD in full-scale clinical trials remains to be determined, but early results are promising (37, 38).

GENOTYPIC METHODS

Genotypic methods in clinical use today are supplemental, not replacement, technology to traditional AST (32). These methods detect the presence/absence of one or more resistance genes, which predict antimicrobial resistance to a single class of antimicrobials. As an example, detection of *mecA* in a blood culture that also harbors *Staphylococcus aureus* predicts methicillin resistance, but additional testing is required to confirm susceptibility to other antimicrobial agents, such as vancomycin, daptomycin, or linezolid. Furthermore, it is rare for complete correlation between genotype and phenotype to be observed—key examples are the presence/absence of *mecA* or *vanA/B*, which predicts resistance/susceptibility to oxacillin in staphylococci or vancomycin in enterococci, respectively. Outside these two examples, antimicrobial resistance is almost always multifactorial and ever-evolving, making such determinations challenging, even with whole-genome sequencing (34, 35), which is poorly standardized (33). Nonetheless, genotypic methods are of value to determine antimicrobial resistance, allowing a more rapid escalation of therapy, which is particularly valuable for patients with sepsis.

Many assays based on the detection of one or more antimicrobial resistance genes present in a positive blood culture are available commercially and routinely used in the clinical laboratory. Most of these are based on polymerase chain reaction (PCR) (**Table 2**). Only a limited number of genes and their variants are typically queried (i.e., *mecA*/*mecC* for staphylococci, *vanA*/*vanB* for enterococci, and bla_{CTX-M} and carbapenemase genes for Gram-negative bacteria). Genetic tests cannot assign a detected resistance gene to a specific bacterium in a polymicrobial specimen, which may result in overcalling resistance. For example, *mecA* detection in a specimen harboring both *S. aureus* and *Staphylococcus epidermidis* could lead to incorrect assigning of this gene to the pathogen, and not the contaminant. Alternatively, rapid detection of a resistance gene in a culture containing multiple Enterobacterales species with differing susceptibilities may reduce undercalling resistance. Furthermore, new mutations and resistance mechanisms are continually evolving, which may limit the ability of certain genetic tests to predict resistance, particularly if a mutation occurs in primer complementary regions (66) or involves overexpression of a gene, like *AmpC*-associated inducible resistance. It is important to note that genotypic methods, in addition to detecting resistance genes, also provide organism identification, and knowledge of select genus or species alone can sometimes guide antimicrobial therapy, particularly for Gram-positive organisms. For example, detection of *Streptococcus pyogenes* should prompt treatment with penicillin. Organism identification without AST is less useful for treatment of Gram-negative organisms, which have diverse and complex resistance mechanisms.

CLINICAL IMPACT OF RAPID ANTIMICROBIAL SUSCEPTIBILITY TESTING METHODS

The impact of rapid blood culture diagnostic methods on clinical and economic outcomes has been mixed. The majority of outcomes studies to date are single-center, observational studies that evaluate the impact of rapid organism identification

TABLE 2 | Select genotypic tests that are approved for rapid detection of resistance markers in positive blood cultures.

Test	Organisms identified	Resistance genes	References
Biofire BCID2 (Biofire, Salt Lake City, UT)	9 Gram-positive bacterial targets 14 Gram-negative bacterial targets 7 yeast targets	Carbapenemases *bla*$_{IMP}$ *bla*$_{KPC}$ *bla*$_{OXA-48-like}$ *bla*$_{NDM}$ *bla*$_{VIM}$ Colistin resistance *mcr-1* ESBL *bla*$_{CTX-M}$ Methicillin *mecA/C* MREJ Vancomycin *vanA/B*	(59)
Verigene BC-GN (Luminex, Austin, TX)	9 Gram-negative bacterial targets	Carbapenemases *bla*$_{IMP}$ *bla*$_{KPC}$ *bla*$_{OXA-48-like}$ *bla*$_{NDM}$ *bla*$_{VIM}$ ESBL *bla*$_{CTX-M}$	(60)
Verigene BC-GP (Luminex)	13 Gram-positive bacterial targets	Methicillin *mecA* MREJ Vancomycin *vanA/B*	(61)
ePlex® BCID-GP (GenMark, Carlsbad, CA)	20 Gram-positive bacterial targets "pan" Gram-negative target "pan" Candida target	Methicillin *mecA* MREJ Vancomycin *vanA/B*	(62)
ePlex® BCID-GN (GenMark)	21 Gram-negative targets "pan" Gram-positive target "pan" Candida target	Carbapenemases *bla*$_{IMP}$ *bla*$_{KPC}$ *bla*$_{OXA-48/OXA-23}$ *bla*$_{NDM}$ *bla*$_{VIM}$ ESBL *bla*$_{CTX-M}$	(63)
ePlex® BCID-FP (GenMark)	15 Fungal targets	None	(64)
Xpert® MRSA/SA BC (Cepheid, Sunnydale CA)	1 Gram-positive target	Methicillin *mecA*	(65)

with or without AST. Some of these studies have demonstrated decreased time to appropriate antibiotics, lower mortality, shorter durations of hospital and intensive care unit (ICU) stay, and reduced costs, although these are not consistent findings. These studies have been previously summarized elsewhere and have been incorporated in meta-analyses (67–69). Limitations of these studies include their varying quality; small sample sizes; single-center, retrospective, and observational designs; and historical or absent control groups (70–73).

A few studies have used the more rigorous study design of a prospective randomized controlled trial (RCT) to evaluate outcomes of rapid blood culture AST methods and are summarized in **Table 3**. In general, these RCTs have shown less dramatic benefits than observational studies. The first RCT evaluating the impact of rapid AST on clinical outcomes was published in 1994 and was a single-center trial using the Baxter MicroScan WalkAway-96 system with provision of AST results on either the same day using blood culture broth to inoculate the test (intervention) or the day after using isolated colonies to inoculate the test (control) (74). Patients in the same-day AST group had significantly lower mortality, fewer ancillary tests, and lower health-care costs than those in the control group (74). Unfortunately, more recent studies using newer technologies have not shown such dramatic mortality benefits. A single-center RCT conducted in the Netherlands evaluated the impact of a laboratory-developed semi-molecular AST method combining culture in the presence of antibiotics plus real-time 16S rRNA PCR and found that the rapid test resulted in no appreciable benefits, in terms of either antimicrobial utilization or clinical outcomes, as compared with conventional testing (75). However, in this study, clinicians did not appear to act upon the rapid AST results, perhaps explaining the lack of difference between the study arms. A single-center, randomized, controlled trial evaluated the impact of the FilmArray Blood Culture Identification (BCID) test, which can detect species of bacteria and *Candida* as well as select resistance markers (*mecA*, *vanA*, *vanB*, and *bla*$_{KPC}$) using multiplex PCR. In this study, participants with positive blood cultures were randomized in the clinical laboratory to have blood culture characterization with either conventional testing methods including matrix-assisted laser desorption–ionization time of flight (MALDI-TOF), the BCID test, or the BCID test plus antimicrobial stewardship review. Participants in both BCID arms had faster time to antibiotic escalation, less treatment of contaminants, and less use of broad-spectrum antibiotics than had those receiving conventional testing. In addition, participants who received BCID paired with antimicrobial stewardship had more rapid antibiotic de-escalation than those who received BCID without stewardship. However, no differences were observed between the arms in mortality, length of hospital stay, infection with multidrug-resistant organisms or *Clostridium difficile*, or cost of care (20). Notably, the BCID test used in this study had greater impact on management of Gram-positive than Gram-negative infections, most likely because the diagnostic enabled detection of only a single resistance determinant (*bla*$_{KPC}$) from Gram-negative species, which was exceedingly rare at the time and did not provide rapid phenotypic susceptibility information for a full panel of antibiotics (20).

The recently completed RAPIDS GN trial evaluated the impact of a rapid phenotypic AST method for Gram-negative bacilli bloodstream infection and addresses some of the limitations of the BCID trial (19). This multicenter study evaluated the impact of the Accelerate PhenoTest™ BC Kit, performed on the original FDA-cleared Accelerate Pheno™ System, compared with standard of care (SOC) MALDI-TOF and BMD or agar dilution for AST. In this study, all blood cultures were reviewed by the stewardship clinicians. The arm with rapid testing had faster time to antibiotic change and optimal antibiotic

TABLE 3 | Randomized controlled trials evaluating clinical impact of rapid blood culture antimicrobial susceptibility testing methods.

Study, year, location	Study design	Rapid (intervention) method	SOC (control) method	Rapid test performance	Outcomes of rapid test compared with standard methods	Rapid testing paired with antimicrobial stewardship	Comment
Doern, 1994, USA (74)	Single-center prospective 2-arm RCT ($N = 573$)	Baxter MicroScan WalkAway-96 reported same day	Baxter MicroScan WalkAway-96 reported following day	Time to AST result 16 h faster than SOC	Decreased mortality, ancillary tests, cost Change in antibiotic therapy was 15 h faster in rapid AST arm No difference in LOS	No	Randomization scheme based on first letter of patient last name
Beuving, 2015, Netherlands (75)	Single-center prospective 2-arm RCT ($N = 250$)	Growth in presence of antibiotics assessed by 16S rRNA PCR	BD Phoenix	94% agreement with SOC AST Time to AST result 15 h faster than SOC	Decreased TOT No differences in mortality, LOS	No	Rapid AST was not implemented optimally and results were not used by clinicians Underpowered to detect differences in clinical outcomes
Banerjee, 2015, USA (20)	Single-center, prospective 3-arm RCT ($N = 617$)	BioFire BCID and BCID plus stewardship	MALD-TOF, agar dilution	97% agreement for on-panel organisms 19% of isolated organisms not on rapid test panel Time to AST result 49 h faster than SOC	Decreased TOT Faster time to escalation and de-escalation, less treatment of contaminants, less broad-spectrum antibiotic treatment No differences in mortality, LOS, adverse events, cost	Yes Audit and feedback by ID pharmacist or physician 24/7 in one intervention arm; treatment guidance comments included in microbiology result report for both intervention arms	More impact among Gram-positive than Gram-negative infections Population had low resistance rates Underpowered to detect differences in clinical outcomes
Kim, 2020, Korea (76)	Single-center, prospective 2-arm RCT of patients with hematologic malignancies ($N = 89$)	QMAC-dRAST (QuantaMatrix, Inc.)	MALDI-TOF, MicroScan, VITEK 2	Agreement with SOC not reported. Time to AST result 35 h faster than SOC	Decreased TOT Less broad-spectrum antibiotic use No differences in mortality, *Clostridium difficile*, multidrug-resistant infections	Yes ID team reviewed all patients	Excluded patients in both arms with off-panel organisms
Banerjee, 2020, USA (19)	Multi-center prospective 2-arm RCT of patients with Gram-negative bacteremia ($N = 448$)	Accelerate Pheno System	MALDI-TOF, broth microdilution or agar dilution	Time to AST 36 h faster than SOC	Decreased TOT No differences in mortality, LOS, adverse events, cost	Yes Audit and feedback by ID pharmacist or physician Mon–Friday during the day	Greater impact for more resistant isolates Population had low resistance rates Underpowered to detect differences in clinical outcomes

PCR, polymerase chain reaction; SOC, standard of care; MALDI-TOF, matrix-assisted laser desorption–ionization time of flight; TOT, time to optimal therapy; LOS, length of stay; AST, antibiotic susceptibility testing; ID, infectious diseases; BCID, Blood Culture Identification.

therapy but did not have any benefit in terms of mortality, length of stay, adverse events, or cost. Notably, the impact on antibiotic utilization varied by resistance profile of the blood isolate; compared with the SOC arm, in the rapid testing arm, time to any Gram-negative antibiotic change occurred 24 h faster for all patients, and antibiotic escalation occurred 43 h faster for patients with drug-resistant isolates (19). Lastly, a recent small study from Korea evaluated the impact of a rapid phenotypic AST method based on a microscopic imaging and microfluidic chip technology called dRAST (QuantaMatrix) (76). In this study, patients with hematologic malignancies and bacteremia were randomized to receive either the rapid testing method or conventional testing, which consisted of MALDI-TOF and MicroScan (Beckman Coulter Inc., Atlanta, GA) and VITEK 2 systems (bioMérieux, Inc.) for AST. Time to optimal therapy was significantly faster in the rapid testing arm than in the control arm, although there were no differences in mortality or other clinical outcomes (76).

Taken together, these studies demonstrate that a variety of rapid AST methods can shorten time to optimal therapy and improve antibiotic stewardship for patients with bloodstream infections. However, most RCTs do not demonstrate that rapid AST methods result in significant reductions in mortality, hospital length of stay, or adverse events, perhaps because larger studies are required to detect differences in these rare events. It is also notable that with the exception of the study by Kim et al., most of these trials were conducted in areas with low rates of antimicrobial-resistant bacteria, and it is possible that more clinical benefit would be observed in areas with higher rates of resistant infections (19, 20). Additionally, most of the studies demonstrate the importance of pairing rapid blood culture diagnostics with antibiotic stewardship team review, as has been emphasized by many others (20, 68, 69, 73, 77–80). A meta-analysis of primarily observational studies demonstrated mortality benefit when blood culture diagnostics were used with stewardship programs but not without stewardship programs (69). A decision analytic model demonstrated that rapid blood culture diagnostics were more cost-effective if implemented with stewardship than without stewardship (80).

CONCLUSION

In conclusion, technological advances that enable rapid AST from organisms growing in blood cultures have great potential to improve the care and outcomes for patients with sepsis. Numerous platforms are currently available or in development, all of which can provide rapid genotypic or phenotypic detection of resistance. However, the success of these technologies requires demonstration of clinical impact. Unlike trials that evaluate a direct action, RCTs conducted for rapid diagnostics are one step removed from the patient, requiring the physician to act on the result. This task has been shown to be best supported when ASPs are active participants in RCTs, showing significant reduction in time to therapy optimization through the use of rapid AST devices. However, the impact of more rapid therapeutic intervention remains largely theoretical. Shorter time to optimal antibiotic therapy should lead to reduced length of hospitalization and mortality, but studies conducted to date were not sufficiently powered to measure these endpoints. More subtle endpoints (e.g., impact to microbiome), alternative trial designs, and inclusion of patient preferences in endpoint determinations may all provide further insight into the value of these tests. Also worth noting is the fact that while significant improvement in hospitalization stays or mortality has not been demonstrated through rapid antimicrobial de-escalation, the opposite is also true—i.e., interventions conducted ∼1–2 days earlier in the course of sepsis do no harm. These data provide a valuable foundation to aid improved stewardship of antibiotics.

Another area of much-needed future research is implementation science. While most large academic hospitals have adopted rapid AST methods for blood cultures, their use is not universal (81). Similarly, extending these technologies to specimens other than positive blood cultures is challenging due to the high frequency of specimens that would need to be tested prior to a single positive result. For example, a recent survey of US hospital data for bronchoalveolar lavage specimens demonstrated only a third of specimens yielded a positive result, meaning expensive technology would be performed on two thirds of specimens with no results (82). Identifying patient populations likely to have the most benefit from these methods and determining how to encourage clinicians to act on rapid AST results are both critical for further development of rapid AST devices. Furthermore, determining the relative value of phenotypic vs. genotypic rapid AST methods is needed. To this end, an RCT is underway to evaluate patients with positive blood cultures tested by a genotypic (control) vs. rapid phenotypic (intervention) approach; the primary endpoint is duration of anti-pseudomonal beta-lactam therapy and anti-methicillin-resistant *Staphylococcus aureus* (anti-MRSA) therapy (clinicaltrials.gov, NCT03744728). Ongoing investment in infectious disease diagnostics and development of rapid AST technologies will be important for continued improvements in sepsis outcomes.

AUTHOR CONTRIBUTIONS

RB and RH reviewed the literature, drafted, and revised the manuscript. All authors agree to be accountable for the content of the work.

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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.

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Genetic Basis of Antimicrobial Resistant Gram-Negative Bacteria Isolated From Bloodstream in Brazil

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Multidrug-resistant microorganisms are a well-known global problem, and gram-negative bacilli are top-ranking. When these pathogens are associated with bloodstream infections (BSI), outcomes become even worse. Here we applied whole-genome sequencing to access information about clonal distribution, resistance mechanism diversity and other molecular aspects of gram-negative bacilli (GNB) isolated from bloodstream infections in Brazil. It was possible to highlight international high-risk clones circulating in the Brazilian territory, such as CC258 for *Klebsiella pneumoniae*, ST79 for *Acinetobacter baumannii* and ST233 for *Pseudomonas aeruginosa*. Important associations can be made such as a negative correlation between CRISPR-Cas and *K. pneumoniae* CC258, while the genes bla_{TEM}, bla_{KPC} and bla_{CTX-M} are highly associated with this clone. Specific relationships between *A. baumannii* clones and bla_{OXA-51} variants were also observed. All *P. aeruginosa* ST233 isolates showed the genes bla_{VIM} and bla_{OXA486}. In addition, some trends could be identified, where a new *P. aeruginosa* MDR clone (ST3079), a novel *A. baumannii* clonal profile circulating in Brazil (ST848), and important resistance associations in the form of bla_{VIM-2} and bla_{IMP-56} being found together in one ST233 strain, stand out. Such findings may help to develop approaches to deal with BSI and even other nosocomial infections caused by these important GNB.

Keywords: multidrug-resistance, bloodstream infections, gram-negative bacilli, whole-genome sequencing, Brazil, surveillance

INTRODUCTION

Multidrug-resistant (MDR) microorganisms are spread worldwide, and global efforts have been made on different fronts to reduce the incidence of MDR infections. Among them, strengthening antimicrobial resistance knowledge through surveillance and research is a major action (1).

Bloodstream infections (BSI) are usually associated with poor outcomes especially when adequate antimicrobial therapy and source control are delayed. Carbapenems have been used for the treatment of severe infections caused by gram-negative bacilli (GNB). However, carbapenem-resistant GNB, such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacterales*, are associated with decreased survival and have become a major challenge for therapeutics (2, 3). In this way, GNB bloodstream isolates are an important surveillance target for monitoring resistance and the World Health Organization (WHO) highlighted *A. baumannii*, *P. aeruginosa*, and *Klebsiella pneumoniae* as a critical priority to develop new antibiotics options (1).

Whole-genome sequencing (WGS) is a rapid molecular method for the search of molecular determinants of antimicrobial resistance (AMR). This tool has been proven to be fast and valuable to classify GNB in terms of resistance to the β-lactams and to guide antibiotic treatment decisions for BSI. WGS can detect not only important acquired genes but also chromosomal mutations, both of which contribute significantly to AMR (4). This approach improves our ability to analyze bacterial genomic content, and when these results are correlated with metadata and phylogenetic studies, we can understand with more precision the ways AMR bacteria multiply and spread (5).

Here, we conducted a surveillance about blood GNB isolates recovered from four of the five Brazilian regions using bioinformatic analysis of WGS data. The criteria and methods chosen are justified since as blood is a sterile site, these are more likely to represent true infections, and GNB is the group for which the highest resistance rates are currently found worldwide (2, 3). Phylogenetic analysis, clonal distribution, resistance mechanism diversity and other molecular aspects of nosocomial strains are described. Characteristics such as strain's national distribution, species diversity and the applied tools make this work the starting point for the construction of a new map of resistance in Brazil, contributing with global efforts to prevent the advance of AMR and assist in improving the therapeutic approach, especially for serious infections such as BSI.

MATERIALS AND METHODS

Bacterial Strains

The Laboratório de Pesquisa em Infecção Hospitalar (LAPIH-FIOCRUZ) takes part in a National Bacterial Resistance Surveillance Network headed by The General Coordination of Public Health Laboratories (Brazilian Health Ministry). Our laboratory routinely receives clinical GNB isolates from hospitals located in different Brazilian states to confirm the mechanisms of drug resistance. The aim of this study was to identify the genetic basis AMR of selected bloodstream and catheter tip bacterial strains recovered from Brazilian states from January 2019 to September 2020.

All gram-negative isolates received by LAPIH in this period were submitted to carbapenemase genes detection by PCR (6) and colistin MICs by the broth microdilution method (http://www.eucast.org). The specie identification was confirmed by biochemical tests and for Complex *A. baumannii* isolates were performed the detection of $bla_{\mathrm{OXA-51}}$ gene by PCR.

Only isolates of *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa*, the three serious cause of healthcare-associated infections and an emerging health threat worldwide, were selected to evaluate the antimicrobial resistance profile and to be submitted to WGS. The selection criterion per batch of isolates received over the period studied included representative carbapenemase-producing and/or polymyxin-resistant strains from different states.

Antimicrobial Susceptibility

Antimicrobial susceptibility profiles were determined by an automated Vitek 2 system (bioMérieux-Vitek, Hazelwood, Mo).

Genome Sequencing and Bioinformatic Analysis

WGS was performed using the Illumina Miseq (Illumina, San Diego, California, USA). A genomic library was constructed by transposon tagmentation with the Nextera XT DNA Sample Prep kit (Illumina, Inc., USA). Kraken2 was used to classify the reads taxonomically (7). The paired-end reads were *de novo* assembled using SPAdes v3.13.1 (8). Contigs shorter than 500 bp were discarded. CheckM was applied to assess the quality of genome sequencing and assembly, estimating their completeness, contamination, size and contigs parameters (9). The genome coverage was calculated using: coverage = (forward reads count * 2) * read length/total genome size. Species from *A. baumannii* were confirmed using Kmer-db (10) and reference strains (*A. baumannii* KL810966.1, *A. nosocomialis* GCF_000368085.1, *A. pittii* CP002177, and *A. calcoaceticus* NZ_LS999521).

Draft genomes were then annotated using Prokka (11). The prediction of the resistome and plasmid incompatibility (Inc) group were made using the ABRicate (T. Seemann, https://github.com/tseemann/abricate) against ResFinder (12), and PlasmidFinder (13), respectively. Only the results above 90% of coverage and identity using ABRicate were considered. Besides that, BLAST (blastx) alignment results were analyzed to identify mutations related to resistance (14). The proteins analyzed were PhoP and PhoQ (polymyxin resistance), besides RamR and AdrR (tigecycline resistance) for *K. pneumoniae*, using MGH78578 as reference; ColR, ColS, and PhoQ (polymyxin resistance), besides OprD (carbapenem resistance), AmpC (cephalosporin resistance) and MexT (different classes) for *P. aeruginosa* using PA01 as reference (AAG07497.1, the functional variant CAA07694 being used for MexT); and LpxA, LpxC, and LpxD (polymyxin resistance), besides AdeN and AdeR (tigecycline resistance) for *A. baumannii* using ATCC 19606 as reference. Furthermore, proteins PmrA and PmrB (polymyxin resistance), and GyrA, GyrB, and ParC (fluoroquinolones resistance) were analyzed for all these three species. Deleterious mutations were checked using PROVEAN web server (15). We only considered mutations classified as deleterious in Results and Discussion section. Presence of a CRISPR-Cas system was assessed by the CRISPRCasFinder (16). MLST was performed with the MLST software (T. Seemann, https://github.com/tseemann/mlst) and the PubMLST database (17).

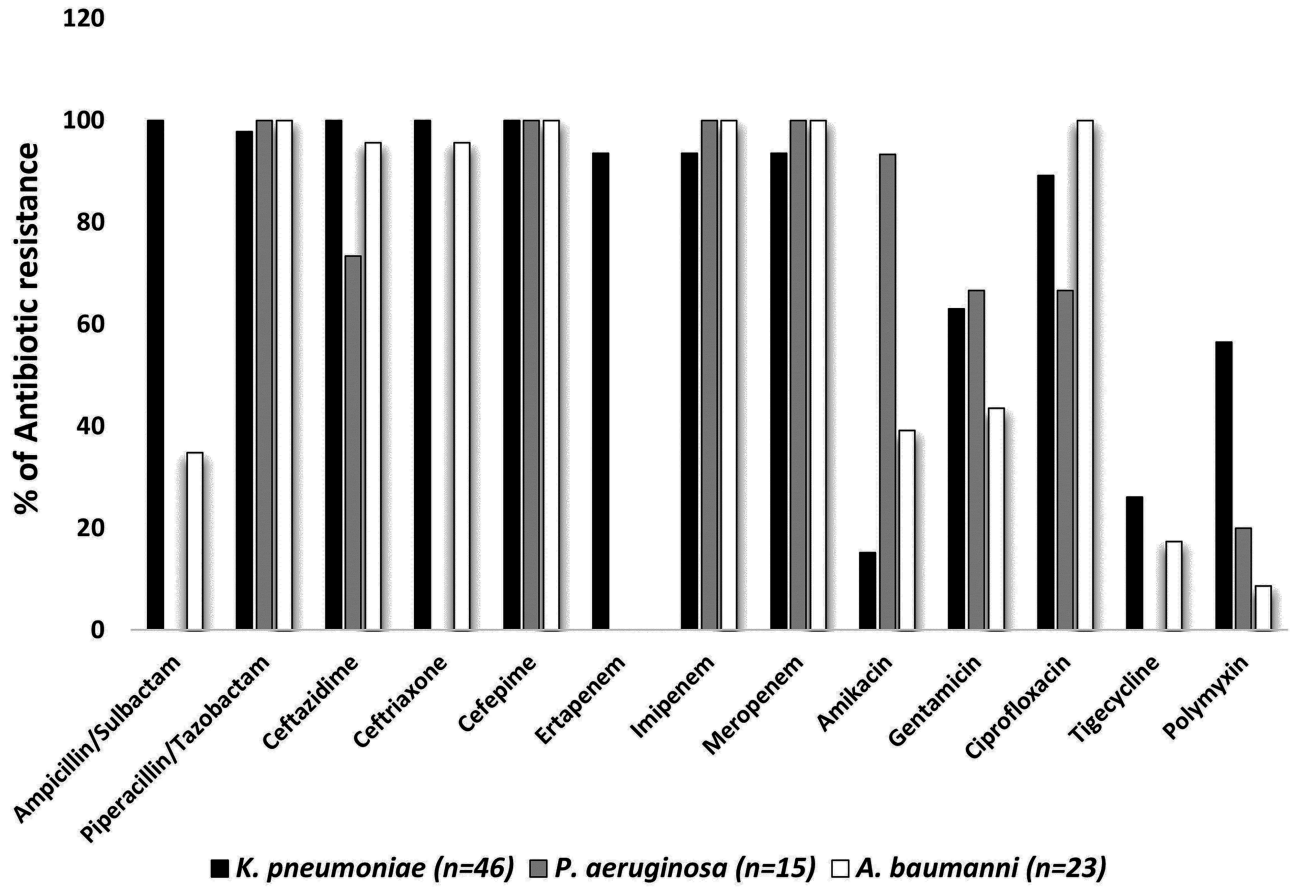


FIGURE 1 | Antibiotics resistance patterns in percentages from selected bloodstream GNB isolated from January 2019 to September 2020. Antimicrobial susceptibility tests were interpreted according to the European/Brazilian Committee on Antimicrobial Susceptibility Testing guidelines. The minimum inhibitory concentration for colistin was obtained using the broth microdilution test, according to the European/Brazilian Committee on Antimicrobial Susceptibility Testing guidelines.

Phylogenetic analysis were performed using kSNP3.0 software, without core genome option (18). The program Kchooser, which is part of the kSNP package, was used to identify the optimal kmer length. iTOL was applied for visualizing and annotating the phylogenetic trees (19).

Genome Accession Number

All draft genomes are available at GenBank BioProject accession PRJNA677881.

RESULTS

In the period of January 2019 to September 2020, 577 blood GNB isolates were received by LAPIH to characterize the resistance mechanisms (**Supplementary Table 1**). The isolates belonged to different species, the most frequent were *K. pneumoniae* (n = 200), *A. baumannii* (n = 192), and *P. aeruginosa* (n = 119). PCR analysis showed that the most prevalent carbapenemase gene among Enterobacterales isolates was bla_{KPC} (69,6%, n = 176) and among *Acinetobacter* species was $bla_{\mathrm{OXA-23like}}$ (89,6%, n = 172). Finally, *Pseudomonas* isolates were found to carry bla_{VIM} (n = 20), bla_{KPC} (n = 17), bla_{IMP} (n = 8), and bla_{SPM} (n = 4). However, 63.0% (n = 75) of *P. aeruginosa* isolates did not carry any of the investigated carbapenemases. Excluding species intrinsically resistant, polymyxin resistance was detected in 170 isolates (30.7%), belonging mainly to *K. pneumoniae* (n = 123/200, 61.5%). Furthermore, the plasmid-mediated *mcr-1* gene was found in 2 *Escherichia coli*.

Based on the results obtained, WGS was performed on 84 selected isolates and included the following species: *K. pneumoniae* (n = 46/200, 23.5%, being one *K. quasipneumoniae*), *A. baumannii* (n = 23/192, 12%), and *P. aeruginosa* (n = 15/119, 12.6%). These isolates were representative of carrying carbapenemase genes and/or polymyxin resistance recovered from the 7 Brazilian states included in the study belonging to 4 geographic regions: Northeast (51.2%), Southeast (35.7%), Midwest (6%), and North (7.1%) (**Supplementary Table 2**).

Antimicrobial resistance levels for the sequenced organisms are shown in **Figure 1**. All strains from the three species analyzed displayed resistance to cefepime. Most significantly, the vast majority of the *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa*

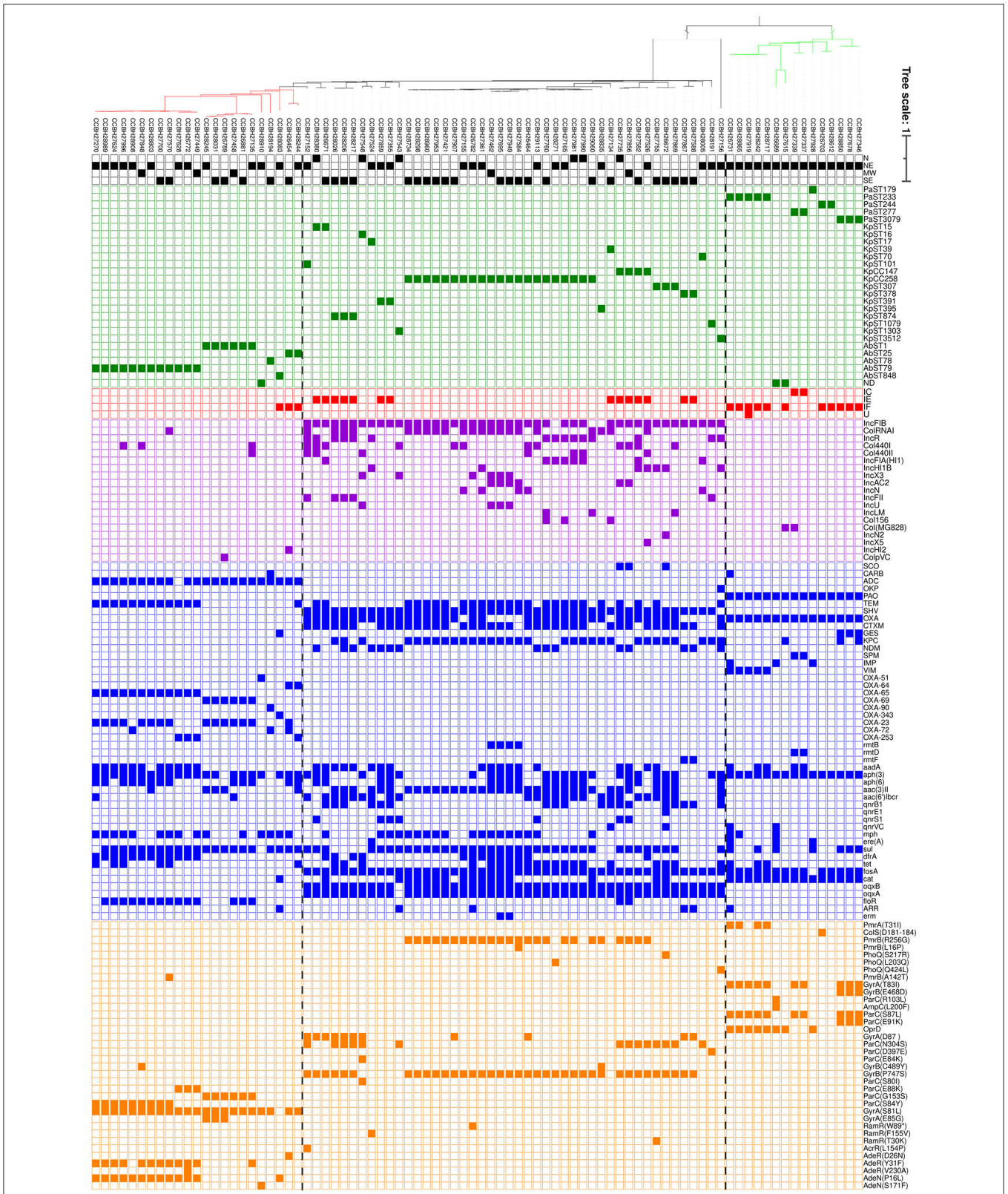


FIGURE 2 | Whole-genome SNP-based parsimony tree including isolates of *K. pneumoniae* (46 isolates, black clade), *A. baumannii* (23 isolates, red clade), and *P. aeruginosa* (15 isolates, green clade) generated by kSNP3.0. The branch lengths are expressed in terms of changes per number of SNPs. The tree was visualized using iTol (19). The panel shows the presence (filled squares) and absence (empty squares) of the characteristics analyzed. Each color corresponds to a characteristic: black (Brazilian regions), green (Sequence Types), red (Cas type), purple (Inc groups), blue (resistance acquired genes), and orange (deleterious mutations in proteins related to antimicrobial resistance). N, north; NE, northeast; MW, Midwest; SE, southeast; ND, not determined.

isolates showed resistance to piperacillin-tazobactam, imipenem, and meropenem (±98.33%). Ampicillin-sulbactam, ceftazidime, and ceftriaxone were not active against all *K. pneumoniae* strains and the resistance rates for ertapenem, ciprofloxacin, gentamicin, tigecycline, and amikacin were 93.43, 89.13, 63.04, 26.08, and 15.22%, respectively. The *P. aeruginosa* isolates showed high resistance to amikacin, ceftazidime, gentamicin, and ciprofloxacin (93.33, 73.33, 66.66, and 66.66%, respectively). Furthermore, the antibiotics resistance pattern for *A. baumannii* isolates was: ciprofloxacin (100%), ceftriaxone (95.65%), ceftazidime (95.65%), gentamicin (43.48%), amikacin (39.13%), ampicillin-sulbactam (34.48%), and tigecycline (17.39%). Finally, 27/46 (56.46%) *K. pneumoniae*, 3/15 (20%) *P. aeruginosa* and 2/23 (8.69%) *A. baumannii* isolates showed polymyxin resistance (MICs >2mg/L) by broth microdilution.

The bioinformatic analysis results are summarized in **Figure 2**. The acquired genes found are related to resistance to 11 different classes, including beta-lactams, aminoglycosides, sulfonamides, fluoroquinolone, macrolide, trimethoprim, tetracycline, fosfomycin, chloramphenicol, rifamycin, and macrolide/streptogramin/lincosamide (MLS phenotype). Most of the sequenced strains were positive for gene families *sul* (76.2%, sulfonamides resistance), *aph(3)* (73.8%, aminoglycosides resistance), and *fosA* (71.4%, fosfomycin resistance). Fifteen families of β-lactamase genes were found (bla_{KPC}, bla_{NDM}, bla_{SPM}, bla_{IMP}, bla_{VIM}, bla_{CTX-M}, bla_{GES}, bla_{TEM}, bla_{SHV}, bla_{SCO}, bla_{CARB}, bla_{ADC}, bla_{OKP}, bla_{PAO}, and bla_{OXA}).

The most prevalent resistance genes among *K. pneumoniae and K. quasipneumoniae* isolates were *fosA* (100%), bla_{SHV} (97.8%), and *oqxAB* (97.8%, fluoroquinolones resistance). Plasmids from the incompatibility group IncFIB were found in 93.5% of all isolates. The strains belonged to 20 STs. The CC258 (ST11, $n = 16$, ST258, $n = 4$, and ST437, $n = 1$) was the most prevalent clonal complex (45.7%) found in four Brazilian regions, followed by CC147 (ST147, $n = 1$, ST392, $n = 2$, and ST273, $n = 1$) (8.7%). All CC258 strains analyzed showed absence of CRISPR-Cas and 85.7% were found to carry the genes bla_{KPC}, bla_{CTX-M}, and bla_{TEM}. The *rmtB* gene (aminoglycosides resistance) was detected solely in ST258, in 4 strains. Of these, three are the only amikacin resistant strains from CC258. IncA/C2 was found only in ST258 strains. The CC258 showed a high Inc groups diversity, being IncFIB the most prevalent (90.5%), followed by ColRNAI (66.7%) and IncR (28.6%). In addition, deleterious mutations were identified in ParC (S80I) for all CC258 strains and in PmrB (R256G) for 90.5% of them. ParC could interfere in fluoroquinolones resistance, and indeed all CC258 strains were ciprofloxacin resistant. On the other hand, 5 *K. pneumoniae* strains from CC258 were polymyxin-susceptible despite the presence of mutation in PmrB (R256G). The CC258 strains with PhoQ (L203Q) or PmrB (L16P) deleterious mutations were resistant to polymyxin. All CC147 strains showed the adaptative immune system CRISPR-Cas type IE and were found to carry the resistance genes bla_{OXA-1}, bla_{SHV}, *oqxAB*, and *sul1*. IncFIB and deleterious mutations in PmrB (R256G), ParC (S80I), and ParC (N304S) were also found in all strains. Despite PmrB mutation, 75% of CC147 strains were polymyxin susceptible. In contrast, all of them were ciprofloxacin resistant and it could be related to ParC mutations. The ST3512 *K. quasipneumoniae* strain (CCBH27156), presented some unique characteristics, such as the simultaneous presence of the bla_{KPC} and bla_{NDM}, absence of the $bla_{SHV,}$ and mutation in PhoQ (Q424L), however, it was not associated with polymyxin resistance. Among all strains with PmrB R256G mutation, 33.3% were susceptible to polymyxin. The deleterious (T30K) and non-sense (W89*) mutations on RamR were only present in tigecycline resistant strains. Another 10 strains proved to be tigecycline resistant without any characteristic mutation identified. The *tet(x)* gene, related to tigecycline resistance, was not found.

The 23 *A. baumannii* strains analyzed belonged to 6 STs and were recovered from three Brazilian regions. ST79 was the most prevalent ST found (52.2%), followed by ST1 (26.1%) and ST25 (8.7%). The most common characteristics were the deleterious mutation S81L in GyrA (95.7%), and the resistance genes bla_{ADC} (95.7%), *aph(3)* (91.3%), and *sul* (91.3%). The resistance genes bla_{OXA-65} (bla_{OXA-51} allelic variant), $bla_{TEM-1A,}$ and *dfrA* (trimethoprim resistance) were only detected in all ST79 strains. The bla_{OXA-23} was the most prevalent carbapenemase gene among ST79 strains (66.7%). The carbapenemase gene $bla_{OXA-253}$ ($bla_{OXA-143}$ allelic variant), was also detected in three strains from this ST. The resistance gene bla_{OXA-69} (bla_{OXA-51} allelic variant), and the deleterious mutation G153S in ParC were only found in all ST1 strains. The carbapemase gene bla_{OXA-23} was also found in all ST1 strains. A few *A. baumannii* strains presented the Inc groups investigated (26.1%). A IncHI2 plasmid and the carbapenemase genes bla_{OXA-23} and bla_{OXA-72} (bla_{OXA-24} allelic variant), were found in one strain belonging to ST25. The CRISPR-Cas system type IF was detected in only three strains, assigned to ST25 and ST848. Interestingly, the only ST848 strain presented bla_{GES-11} and *arr* genes (rifamycin resistance) and the absence of S81L mutation in GyrA, differing from the other *A. baumannii* isolates. The only strain with a PmrB deleterious mutation showed polymyxin susceptibility (A142T in CCBH27570). We did not identify deleterious mutation associated to the two polymyxin resistant strains. The mutations AdeR(D26N), AdeR(V230A), and AdeN(S171F) were found in tigecycline-susceptible strains. Two mutations were majority related to ST79 (AdeR, Y31F and AdeN, P16L), present in both susceptible and resistant strains. The *tet(x)* gene was not found.

The 15 *P. aeruginosa* strains analyzed belonged to 5 STs and were from 1 Brazilian region. ST233 (33.3%) was the most prevalent ST found, followed by ST3079 (20%). All strains showed the resistance genes bla_{PAO}, *aph*(3), and *catB7* (chloramphenicol resistance). The CRISPR-Cas system was detected in 86.6% of the strains, being type I-F the most frequent. Deleterious mutations T83I and S87L in GyrA and ParC, respectively, were the most frequently found (66.7%), and were distributed in different clones. These two mutations were present in all ciprofloxacin resistant strains and absent in strains susceptible to this antibiotic. All 5 ST233 strains showed the genes bla_{VIM} and bla_{OXA486}. The PmrA deleterious mutation T31I was found in four strains belonging to this ST, and two of them are resistant to polymyxin according to MIC. Furthermore, one ST233 isolate carried the carbapenemase genes bla_{VIM-2} and

bla$_{IMP-56}$ together. The three ST3079 strains were the only ones to show the gene *bla*$_{GES-1}$ and the deleterious mutations in Parc (E91K) and GyrB (E468D). The genes *bla*$_{SPM}$, *bla*$_{OXA-56}$, and *rmtD* were found only in all ST277 isolates. A strain from ST244 with ColS amino acids deletion (D181–184) was polymyxin susceptible. The plasmid incompatibility group Col(MG828) was found in two *P. aeruginosa* isolates from different STs.

DISCUSSION

In the present study we analyzed gram-negative pathogens causing BSI and *K. pneumoniae* was the major species among MDR strains. A national study about nosocomial BSI in Brazil reveled that monomicrobial episodes of BSI were mostly caused by gram-negative bacteria, highlighting *K. pneumoniae* (20). Other Brazilian data about BSI, from a single hospital, showed that *K. pneumoniae* infections had a positive association with the MDR phenotype (21). Both studies corroborate the high prevalence of this specie among the isolates selected for the present work.

Antibiotic resistance within the group of gram-negative bacteria related to BSI is worrisome in Brazil, and the pathogens analyzed here are among the most outstanding (20, 21). According to our results, amikacin was the most effective antimicrobial against *K. pneumoniae*, and, *A. baumannii* and *P. aeruginosa* showed a great sensibility to polymyxin.

A predominance of worldwide distributed clones among *K. pneumoniae* (CC258), *A. baumannii* (ST79) and *P. aeruginosa* (ST233) strains was observed. Isolates from international high-risk CC258 have been found in all Brazilian states and are highly associated with the dissemination of carbapenemase KPC (6, 22). The ST1 is designed as the second major *A. baumannii* clone and is globally associated to OXA-23 (23), however in South America this carbapenemase has been most commonly related with ST79, the third major international distribution clone, as noted here (23, 24). The most prevalent ST in *P. aeruginosa*, ST233, is a worldwide disseminated MDR clone particularly linked to VIM-2, as was corroborated by our results (25).

The prevalence of gram-negative BSI has practical importance, especially when dealing with the issue of treatment, creating a vicious cycle of antimicrobial heavy use and resistance development (20). A great variety of resistance genes was found, highlighting *sul* and *fosA*. High prevalence rates of *sul* variants have been observed mainly in gram-negative bacteria isolated all over the world (26). Meanwhile, *fosA* is commonly found in *K. pneumoniae* and *P. aeruginosa* contributing to intrinsic fosfomycin resistance, but is largely absent in *A. baumannii* (27), as confirmed here. The resistance rates to carbapenems equal or higher than 93,48% can be associated to specific carbapenemases genes found more often. The carbapenemase *bla*$_{KPC-2}$ was very prevalent in *K. pneumoniae*, contrasting with *bla*$_{NDM-1}$. This difference is expected once *bla*$_{KPC-2}$ circulates in Brazil since 2009 (28), and *bla*$_{NDM-1}$ was detected only in 2013 in this country (29). Furthermore, most of the analyzed strains are CC258 *K. pneumoniae*, which is spread in Brazil mainly carrying *bla*$_{KPC}$ (22). The most prevalent carbapenemase in *A. baumannii* strains, OXA-23, was detected here in already associated clones as ST1, ST25 and ST79 (23, 30). Although this gene was not detected in some ST79 strains from this surveillance, all of them have OXA-65 carbapenemase, as previously reported in a Brazilian surveillance (31). The single strain from *A. baumannii* ST848 calls to attention since the occurrence of *bla*$_{GES-11}$, which mediates resistance to β-lactam and reduced susceptibility to carbapenem, has been reported in other continents and clones but neither in America nor in ST848 (32). Furthermore, this clone is not clearly associated with *A. baumannii* isolates from Brazil. The carbapenemases genes found in *P. aeruginosa* are highly associated with a specific clone. The association of *bla*$_{VIM-2}$ to ST233 (25) and *bla*$_{SPM-1}$ to ST277 (33) have been reported. On the other hand, the association of ESBL *bla*$_{GES-1}$ to ST3079 has not yet been described. Carbapenemase KPC-2 was also present in 2 of the 3 strains analyzed from this clone. It is important that the dispersion of *P. aeruginosa* ST3079 be monitored and the prevalence of these important genes confirmed. The coexistence of genes *bla*$_{VIM}$ and *bla*$_{IMP}$ in *P. aeruginosa* found here for a single strain was described before in India, associated with a MIC for meropenem higher than 32 μg/ml (34), so this association also demands attention.

Since resistance to carbapenems is usually associated with multidrug resistance, polymyxins became the last alternative (20). Here we detected different deleterious mutations in PhoQ for *K. pneumoniae*, PmrA for *P. aeruginosa* and PmrB for *K. pneumoniae* and *A. baumannii*. It is difficult to extrapolate whether some substitutions identified in proteins, mainly in *P. aeruginosa* and *A. baumannii*, leads to polymyxin resistance, and the levels of gene expression may vary and consequently influence the level of resistance (35). The deleterious mutation R256G in PmrB which seems common in *K. pneumoniae* CC258 and CC147, has been found in polymyxin-susceptible isolates (36), and the same was observed here. Other studies will be necessary to verify if PhoQ (L203Q) and PmrB (L16P) deleterious mutations are indeed associated to polymyxin resistant in *K. pneumoniae*. As far as we know, the PmrA deleterious mutation shown in *P. aeruginosa* (T31I) has not yet been linked to polymyxin resistance, but among the 4 strains that showed this mutation, 2 were resistant according to MIC. The PmrB A142T mutation in *A. baumannii* is present in a susceptible strain, and has not yet been linked to polymyxin resistance.

Although ParC deleterious mutations occurred in different *K. pneumoniae* clones and may cause ciprofloxacin resistance, we could not ignore the high frequency of *oqxAB* genes, which encode a efflux pump that confer resistance to multiple agents including fluoroquinolones. Some mutations observed for *P. aeruginosa* are relevant examples of gain-of-function and are associated with resistance to fluoroquinolones [GyrA (T83I), GyrB (E468D) and ParC (S87L)] (37). Indeed, we showed that the GyrA and ParC deleterious mutations were strict associated to ciprofloxacin resistance. For *A. baumannii*, the simultaneous presence of mutations in GyrA (S81L) and in ParC (S84L) have been associated to resistance to ciprofloxacin and nalidixic acid (38). All *A. baumannii* strains analyzed here are ciprofloxacin resistant and most of them have the GyrA S81L mutation (95.8%). For ST1, the deleterious mutations ParC (S84L e G153S)

could be noted simultaneously to GyrA(S81L), and for ST79 the concomitant mutation was ParC(S84Y). These associations could explain ciprofloxacin resistance for the major *A. baumannii* STs in this study.

Plasmid-mediated transfer of resistance has led to widespread dissemination, outbreaks, and untreatable infections. Molecular identification of plasmid and strain genotypes can distinguish whether the spread of AMR genes is driven by epidemic plasmids to different hosts or by clonal spread of bacterial organisms harboring these plasmids with AMR genes (39). Here, the diversity and abundance of Inc groups found in *K. pneumoniae* strains contrast with *P. aeruginosa* and *A. baumannii* results. The majority of *K. pneumoniae* strains analyzed have the IncFIB plasmid, a group known for their MDR characteristic (39, 40). In Brazil, the IncFIB plasmid has already been described as carrying bla_{KPC-2} and bla_{NDM-1} in *K. pneumoniae* (40, 41). A recent manuscript reported that plasmids with IncR, ColRNAI and IncF co-exists in KPC-2-producing *K. pneumoniae* strains from ST11 (42). Interestingly, we detected IncR in most ST11 isolates, but not in other CC258 clones. IncN was detected mainly in CC258, which is expected as in Brazil the spread of bla_{KPC-2} has already been related to dispersion of Tn4401 'b', carried by IncN plasmids mainly disseminated by this clonal complex (22). IncA/C2 was only detected in ST258 strains, the same strains which carry *rmtB* and *tet(G)* genes. This profile is very similar to plasmid pMTY16641, identified in the first KPC-producing *K. pneumoniae* ST258 isolated from a Japanese patient without a history of foreign travel (43). The incompatibility group IncHI2 was found in a single *A. baumannii* strain, the only one positive both for bla_{OXA-23} and bla_{OXA-72}, and as far as we know, this group has not yet been associated with such genes.

The only species for which almost all strains present CRISPR-Cas systems was *P. aeruginosa*. Types I-F and I-C identified here were already described in *P. aeruginosa* strains. Type I-F is the most widely distributed system among *P. aeruginosa*, while most type I-C-positive strains seem restricted to MDR clones, like ST277 (44), and a similar scenario was observed here. Only CRIPR-Cas type I-E is already described in the literature for *K. pneumoniae,* as was seen in this surveillance (45). It was also confirmed that this type is extremely rare in CC258 (45). However, other linages positive for bla_{KPC}, like CC147 and ST874, presented half or more positive strains for type I-E CRISPR-Cas system, so the negative association between CRISPR-Cas system and bla_{KPC} seems to be specific to CC258. In *A. baumannii*, only the CRISPR type I–F system has been found so far (46), and we detected it in 12.5% of the strains. One of these was also positive for IncHI2 contradicting the recently reported negative association between *A. baumannii* strains with CRISPR system and plasmid detection (46).

WGS is a powerful tool to provide reliable data for monitoring clonal dispersion, antimicrobial resistance and other related characteristics. In the presented study, it was possible to highlight high-risk clones circulating in Brazilian territory. Some important associations could be made between these clones, important resistance genes and specific deleterious mutations in genes also related to antibiotic resistance. In addition, some trends could be identified such as new MDR clones, and novel important resistance genes and clonal profiles that are circulating. Such findings may help to develop approaches to deal with BSI and even other nosocomial infections caused by these important GNB, mainly in Brazil but also in other countries, since most clones are globally dispersed.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA677881.

AUTHOR CONTRIBUTIONS

AC-A conceived the research. IO, LP, TO, CT-T, NC, NP, OC-N, BC, and DR were responsible for the execution of phenotypic and molecular tests to identify and detect resistance of Gram-negative bacteria. AC-A and CR participated in the selection of sequenced strains. MS, CR, and RL participated in method design, whole genome sequencing, and data handling. MS performed the data analysis. MS and CR made the figures. AC-A, MS, CR, RA, FS, EM, and RL wrote parts and edited the complete manuscript. All authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.635206/full#supplementary-material

Supplementary Table 1 | PCR and MIC results about the 577 blood GNB isolates received between January 2019 to September 2020.

Supplementary Table 2 | General information about 84 blood GNB isolates submitted to whole genome sequencing.

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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.

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Accuracy and Prognosis Value of the Sequential Organ Failure Assessment Score Combined With C-Reactive Protein in Patients With Complicated Infective Endocarditis

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This study aimed to evaluate the accuracy and prognostic value of the sequential organ failure assessment (SOFA) score combined with C-reactive protein (CRP) in patients with complicated infective endocarditis (IE). A total of 246 consecutive patients with complicated IE were included in the multicentric prospective observational study. These patients were divided into four groups depending on the SOFA score and CRP optimal cutoff values (≥5 points and ≥17.6 mg/L, respectively), which were determined using the receiver operating characteristic analysis: low SOFA and low CRP (n = 83), low SOFA and high CRP (n = 87), high SOFA and low CRP (n = 25), and high SOFA and high CRP (n = 51). The primary endpoint was in-hospital death, and the secondary endpoint was long-time mortality, defined as subsequent readmission and 3-years mortality in the follow-up period. High SOFA score and high CRP were associated with approximately 29.410% (15/51) of higher incidence of in-hospital death with an area under the curve of 0.872. Multivariate analyses showed that age [odds ratio (OR) = 2.242, 1.142–4.401], neurological failure (Glasgow Coma Scale ≤ 12) (OR = 2.513, 1.041–4.224), *Staphylococcus aureus* (OR = 2.151, 1.252–4.513), SOFA ≥ 5 (OR = 9.320, 3.621–16.847), and surgical treatment (OR = 0.121, 0.031–0.342) were clinical predictors for in-hospital death. On following up for 12–36 months, SOFA ≥ 5 (p = 0.000) showed higher mortality. A high SOFA score combined with increased CRP levels is associated with in-hospital mortality. Also, SOFA score, but not CRP, predicts long-term mortality in complicated IE.

Keywords: SOFA, C-reactive protein, complicated infective endocarditis, in-hospital death, long-time outcome 3

INTRODUCTION

Infective endocarditis (IE) causes nearly 20% of in-hospital mortality, 17% of 30-days mortality, 30% of 1-year mortality, and up to 40% mortality at 5-years follow-up, posing a diagnostic and therapeutic challenge to clinicians (1, 2). Therefore, early identification of patients at high risk of death or complications is essential to improve the outcome of this disease. Research works have shown the sequential organ failure assessment (SOFA) score and C-reactive protein (CRP) to be effective prognostic tools in the management of sepsis, infections as well as patients with IE (3–5). However, studies regarding the combined effect of SOFA and CRP on predicting adverse outcomes in patients with complicated IE remain unknown.

This is the first study documenting the combined effect of the SOFA score and CRP in predicting outcomes among patients with complicated IE.

MATERIALS AND METHODS

Patients Enrollment

A multicentric prospective observational study focusing on the impact of SOFA score and CRP level in evaluating the severity and the prognosis of complicated IE patients was conducted. The trial was conducted in six intensive care units in three big university-affiliated medical centers (Shenzhen People's Hospital, Longgang District People's Hospital of Shenzhen, and Guangdong General Hospital) in China. The minimal sample size

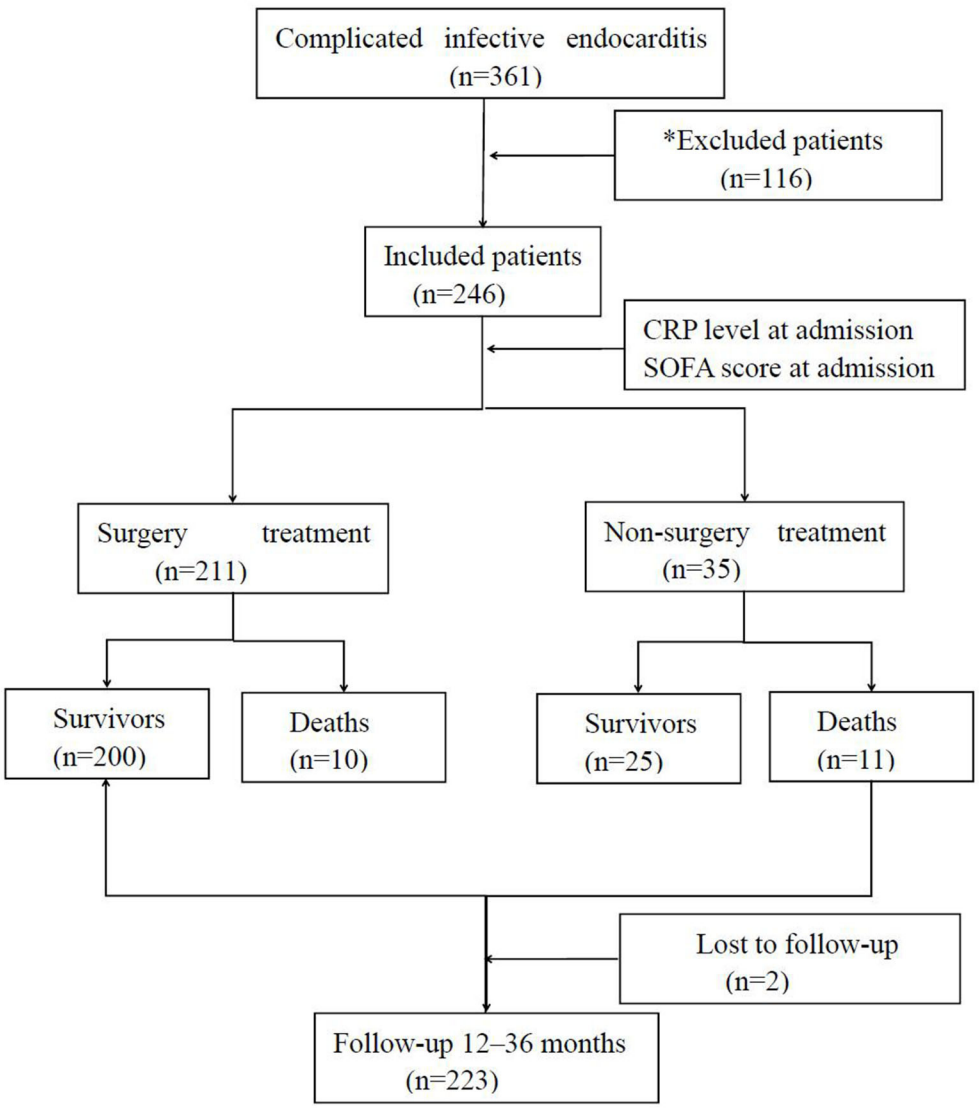


FIGURE 1 | Flowchart of the statistical analysis. *Patients were excluded on having non-complicated infective endocarditis ($n = 90$), prior infective endocarditis ($n = 10$), age <18 years ($n = 12$), and others ($n = 4$).

of each group was calculated by the chi-square test used by PASS 15 software (6). A total of 246 patients definitively diagnosed with complicated IE were consecutively screened between 2015 and 2019. Based on modified Duke criteria (7), patients were confirmed to have either IE or complicated IE if they met one or more of the following criteria: (i) presence of congenital heart disease (CHD) including any type of cyanotic CHD or any type of CHD repaired with a prosthetic material up to 6 months after the procedure (8); (ii) neurological complication including ischemic stroke, intracerebral or subarachnoidal hemorrhage, brain abscess, meningitis, and toxic encephalopathy (9); (iii) paravalvular abscess identified by echocardiography; (iv) embolic complications including pulmonary, cerebral, or systemic embolism (10); or (v) heart failure (11). The exclusion criteria included no complicated IE, prior IE, or age younger than 18 years (**Figure 1**). Finally, the patients were divided into four groups depending on the respective optimal cutoff value. All patients gave written informed consent before their enrollment. The institutional review board at the Shenzhen People's Hospital approved the study protocol.

Data Collection

Once the patients were enrolled, and serum was collected and sent for CRP analysis using an immunoturbidimetry assay with a range of 0–5 mg/L. Transthoracic echocardiography was performed, and SOFA scores were calculated within 24 h of diagnosis.

Study Endpoints

In-hospital mortality was considered as the primary endpoint. Long-time mortality with follow-up was the secondary endpoint. Long-term mortality was defined as subsequent readmission and 3-years mortality in the follow-up period.

TABLE 1 | Baseline clinical characteristics of patients according to SOFA and CRP.

Characteristics	Low SOFA, Low CRP ($n = 83$)	Low SOFA, High CRP ($n = 87$)	High SOFA, Low CRP ($n = 25$)	High SOFA, High CRP ($n = 51$)	*p-value*
Age (year)	41.86 ± 12.82	43.34 ± 13.93	47.88 ± 13.89	46.24 ± 13.52	0.126
Males, *n* (%)	64 (77.11)	56 (64.37)	18 (72.00)	40 (78.43)	0.781
AIE, *n* (%)	11 (13.25)	19 (21.84)	4 (16.00)	10 (19.61)	0.442
SIE, *n* (%)	72 (86.75)	68 (78.16)	21 (84.00)	41 (80.39)	0.512
Hypertension, *n* (%)	6 (7.22)	8 (9.20)	3 (12.0)	9 (17.65)	0.054
Diabetes mellitus, *n* (%)	0 (0)	3 (3.45)	0 (0)	5 (9.80)	*0.005*
Affected valve					
Aortic valve, *n* (%)	35 (42.17)	37 (42.53)	11 (44.0)	28 (54.90)	0.149
Mitral valve, *n* (%)	41 (49.40)	50 (57.47)	11 (44.0)	24 (47.06)	0.603
Triple vale, *n* (%)	7 (8.43)	13 (14.94)	3 (12.0)	6 (11.76)	0.612
Multiple valves, *n* (%)	8 (9.64)	9 (10.34)	2 (8.0)	4 (7.84)	0.745
Congenital heart disease, *n* (%)	14 (16.87)	8 (9.20)	3 (12.0)	6 (11.76)	0.416
Neurological failure (GCS≤12), *n* (%)	6 (7.22)	7 (8.05)	2 (8.0)	3 (5.88)	0.788
Paravalvular abscess, *n* (%)	4 (4.82)	10 (11.49)	1 (4.0)	4 (7.84)	0.707
Stroke, *n* (%)	1 (1.20)	7 (8.05)	4 (16.0)	14 (27.45)	*0.000*
Heart failure, *n* (%)	41 (49.40)	43 (49.43)	12 (48.0)	31 (60.78)	0.238
NYHA III–IV, *n* (%)	35 (42.17)	38 (43.68)	8 (32.00)	28 (54.90)	0.273
LVEF (%)	63.50 ± 8.74	62.58 ± 9.78	67.12 ± 8.67	60.64 ± 9.38	*0.044*
Temperature, °C	38.8 ± 1.17	38.75 ± 0.57	38.95 ± 0.76	39.08 ± 0.72	0.922
Pathogen, n (%)					
Staphylococcus aureus	2 (2.41)	8 (9.20)	3 (12.0)	14 (27.45)	*0.001*
Streptococci	10 (12.05)	11 (12.64)	4 (16.0)	9 (17.65)	0.052
Healthcare-associated infection	2 (2.47)	2 (2.31)	1 (4.0)	3 (5.88)	0.425
WBC, $\times 10^9$/L	7.3 ± 2.6	9.4 ± 3.7	8.4 ± 3.7	11.4 ± 2.7	*0.041*
CRP, mg/L	7.10 ± 5.23	39.89 ± 27.29	8.98 ± 6.88	37.95 ± 17.18	0.000
SOFA score	3.08 (3, 4)	3.21 (3, 4)	5.68 (5, 7)	6.51 (5, 8)	0.000
ESR, mm/h	20.22 ± 20.51	54.30 ± 36.61	28.32 ± 27.45	36.67 ± 31.88	0.000
Vegetation size ≥10 mm, *n* (%)	24 (29.63)	41 (47.13)	11 (44.0)	29 (56.86)	*0.014*
Surgery treatment, *n* (%)	77 (95.06)	77 (88.51)	22 (88.0)	35 (68.63)	*0.000*
In-hospital deaths	2 (2.47)	1 (1.15)	3 (12.0)	15 (29.41)	*<0.000*
Longtime mortality	0 (0)	6 (6.90)	2 (8.0)	8 (15.69)	*<0.000*

AIE, acute infective endocarditis; SIE, subacute infective endocarditis; NYHA, New York Heart Association; LVEF, left ventricular ejection fraction; WBC, white blood cell; CRP:C-reactive protein; ESR, erythrocyte sedimentation rate; SOFA, sequential organ failure assessment; GCS, Glasgow coma score. Italic values defined as reference value.

Statistical Analysis

Statistical Package for the Social Sciences 22.0 was used for all statistical analyses in this study. The receiver operating characteristic curve analysis was used in search of the optimal cutoff value of SOFA or CRP for in-hospital mortality. Included patients were divided into four groups depending on the respective optimal cutoff value. Values were reported as mean ± standard deviations, quartile ranges, or counts (percentages). The continuous data results were compared using a Student's *t*-test, analysis of variance, or the Kruskal–Wallis test, whereas the chi-squared or Fisher's exact test was used to compare the distribution of categorical data. Univariable and multivariable logistic regression analyses were used to evaluate the adjusted odds ratio (OR) for in-hospital death. The Kaplan–Meier method was used to analyze long-time survival with 12–36 (24, 36) months of follow-up. A ($p < 0.05$) was considered statistically significant.

RESULTS

Characteristics of the Patients

A total of 246 patients (178 males, mean age 44.00 ± 13.55 years) with complicated IE were included in this study. Patients were divided into four groups based on the SOFA score (≥5 points) and CRP level (≥17.6 mg/L) cutoff values: low SOFA and low CRP ($n = 83$), high SOFA and low CRP ($n = 25$), low SOFA and high CRP ($n = 87$), and high SOFA and high CRP ($n = 51$). Of 246 patients, 21 (8.54%) died during hospitalization. No statistically significant differences were found between the four groups with respect to demographic characteristics, risk factors, affected valve, echocardiographic findings, or clinical symptom. Patients with a high SOFA score and a high CRP level were associated with higher incidence of diabetes mellitus (9.8 vs. 3.45% vs. 0 vs. 0, $p = 0.005$), *Staphylococcus aureus* (27.45 vs. 9.20 vs. 12.0 vs. 2.41%, $p = 0.001$), stroke (27.45 vs. 16% vs. 8.05 vs. 1.2, $p = 0.000$), vegetation size ≥ 10 mm (56.86 vs. 47.13 vs. 44 vs. 29.63%, $p = 0.014$), in-hospital death (29.41 vs. 12 vs. 2.47 vs. 1.15%, $p < 0.000$), and long-time mortality (15.69 vs. 8 vs. 6.9 vs. 0%, $p < 0.000$). However, the rate of receiving surgery treatment was lower (68.63 vs. 88 vs. 88.51 vs. 95.06%, $p = 0.000$) (**Table 1**).

Predictive Value of the Sequential Organ Failure Assessment Score and the C-Reactive Protein Level for Adverse Outcomes

The receiver operating characteristic analysis revealed that SOFA score ≥ 5 was highly accurate in predicting the patient's in-hospital death [area under the curve (AUC) = 0.863, 95% confidence interval (CI), 0.814–0.904, $p < 0.001$] with a sensitivity of 85.71% and a specificity of 73.33%. CRP ≥ 17.6 mg/L was also accurate in predicting in-hospital death (AUC = 0.712, 95% CI, 0.651–0.768, $p < 0.001$) with a sensitivity of 85.71% and a specificity of 50.89%. The positive predictive values of SOFA score and CRP levels were 36.1 and 14.0%, respectively. The AUC of SOFA score combined with CRP in predicting patients' in-hospital death was 0.872 (95% CI, 0.825–0.912, $p < 0.001$) with a sensitivity of 80.95% and a specificity of 83.56% (**Figure 2**).

For in-hospital death, univariate analysis was used to identify predictive factors. Factors were related to a major risk for mortality including age (OR = 2.18, $p = 0.002$), hypertension (OR = 3.05, $p = 0.048$), neurological failure (Glasgow Coma Score ≤ 12; OR = 3.55, $p = 0.041$), *S. aureus* (OR = 2.45, $p = 0.011$), stroke (OR = 4.08, $p = 0.009$), heart failure (OR = 3.29, $p = 0.025$), CRP ≥ 17.6 mg/L (OR = 3.0, $p = 0.038$), and SOFA ≥ 5 (OR = 14.8, $p < 0.000$), whereas factor was related to survival was surgery treatment (OR = 0.11, $p < 0.000$). For in-hospital mortality, multivariate analysis revealed independent predictors such as age (OR = 2.242, 95% CI, 1.142–4.401, $p = 0.015$), Glasgow Coma Score ≤ 12 (OR = 2.513, 95% CI, 1.041–4.224, $p = 0.012$), *S. aureus* (OR = 2.151, 95% CI, 1.252–4.513, $p = 0.020$), surgery treatment (OR = 0.121, 95% CI, 0.031–0.342, $p < 0.00$), and SOFA ≥ 5 (OR = 9.320, 95% CI, 3.621–16.847, $p = 0.001$) (**Table 2**).

Long-Time Outcomes

Among the 225 patients after hospitalization, 2 (0.89%) patients were lost to follow-up. A total of 16 (7.17%) patients were dead within a follow-up time of 12–36 (24, 36) months. A lower cumulative rate of the long-term survivors with SOFA≥5 (log-rank test, $p = 0.000$) was demonstrated by the Kaplan–Meier analysis. However, we did not observe any significant difference in disease-free survival for CRP (log-rank test, $p = 0.654$) (**Figure 3**).

DISCUSSION

This study is the first clinical trial to evaluate the combinational effect of SOFA and CRP in predicting mortality in patients with

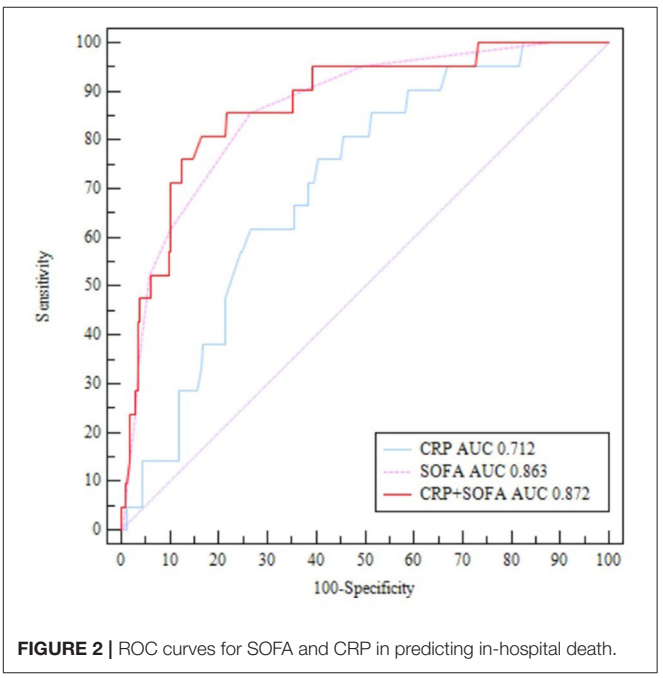


FIGURE 2 | ROC curves for SOFA and CRP in predicting in-hospital death.

TABLE 2 | Univariate and multivariate analyses of factors associated with in-hospital mortality.

Characteristics	Survivors ($n = 225$)	All-cause death ($n = 21$)	Univariate HR (95% CI)	*p-value*	Multivariate HR (95% CI)	*p-value*
*Age (year)	43.23 ± 13.19	54.77 ± 14.10	2.18 (0.66–4.52)	*0.002*	2.242 (1.142–4.401)	*0.015*
Males, *n* (%)	164 (72.89)	14 (66.67)	1.34 (0.52–3.49)	0.543		
AIE, *n* (%)	40 (17.78)	4 (19.05)	–	–		
SIE, *n* (%)	185 (82.22)	17 (80.95)	0.92 (0.29–2.88)	0.884		
Hypertension, *n* (%)	21 (9.33)	5 (23.81)	3.05 (1.01–9.12)	*0.048*		
Diabetes mellitus, *n* (%)	6 (2.67)	2 (9.52)	3.84 (0.73–20.36)	0.114		
Affected valve						
Aortic valve, *n* (%)	97 (43.11)	13 (61.90)	2.07 (0.83–5.20)	0.120		
Mitral valve, *n* (%)	117 (52.0)	9 (42.86)	0.91 (0.36–2.26)	0.831		
Triple vale, *n* (%)	28 (12.44)	1 (4.76)	0.34 (0.04–2.67)	0.308		
Multiple valves, *n* (%)	20 (8.89)	2 (9.52)	1.01 (0.4–2.56)	0.761		
Congenital heart disease, *n* (%)	28 (12.44)	3 (14.29)	1.16 (0.32–4.20)	0.820		
Neurological failure (GCS≤12), *n* (%)	14 (6.22)	4 (19.05)	3.55 (1.05–11.97)	*0.041*	2.513 (1.041–4.224)	*0.012*
Paravalvular abscess, *n* (%)	17 (7.56)	2 (9.52)	1.28 (0.28–5.97)	0.752		
Stroke, *n* (%)	20 (8.89)	6 (28.57)	4.08 (1.42–11.6)	*0.009*		
Heart failure, *n* (%)	105 (46.67)	16 (76.19)	3.29 (1.16–9.28)	*0.025*		
NYHA III–IV, *n* (%)	96 (42.)	13 (61.90)	2.18 (0.87–5.48)	0.100		
#LVEF (%)	63.10 ± 9.21	60.33 ± 24	0.39 (0.14–1.11)	0.78		
WBC	7.3 ± 3.1	8.8 ± 2.7	1.18 (0.28–2.97)	0.702		
Staphylococcus aureu	23 (10.22)	4 (19.05)	2.45 (1.25–5.57)	*0.011*	2.151 (1.252–4.513)	*0.020*
Streptococci	31 (13.78)	3 (14.29)	1.05 (0.5–2.97)	0.841		
CRP<17.6 mg/L, *n* (%)	109 (48.66)	5 (23.81)	–	–		
CRP≥17.6 mg/L, *n* (%)	116 (51.56)	16 (76.19)	3.00 (1.07–8.49)	*0.038*		
*#ESR mm/h	35.96 ± 27.41	38.38 ± 23.73	1.69 (0.62–4.60)	0.309		
Vegetation size ≥10 mm, *n* (%)	92 (40.89)	13 (61.90)	2.31 (0.92–5.81)	0.074		
Surgery treatment, *n* (%)	200 (89.29)	10 (47.62)	0.11 (0.04–0.28)	*<0.000*	0.121 (0.031–0.342)	*<0.000*
Emergency, *n* (%)	30 (13.39)	1 (4.76)	–	–		
Urgent, *n* (%)	39 (17.41)	1 (4.76)	–	–		
Select, *n* (%)	131 (58.48)	6 (28.57)	–	–		
SOFA at admission, *n* (%)						
SOFA 0–4 points	166 (73.78)	3 (14.29)	–	–		
SOFA ≥5 points	59 (1.78)	18 (14.29)	14.88 (4.80–39.38)	*<0.000*	9.320 (3.621–16.847)	*0.001*

**Age cutoff was 56 years. #LVEF cutoff was 58%. *#ESR cutoff was 56 mm. AIE, acute infective endocarditis; SIE, subacute infective endocarditis; NYHA, New York Heart Association; LVEF, left ventricular ejection fraction; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; SOFA, sequential organ failure assessment; GCS, Glasgow coma score. Italic values defined as reference value.*

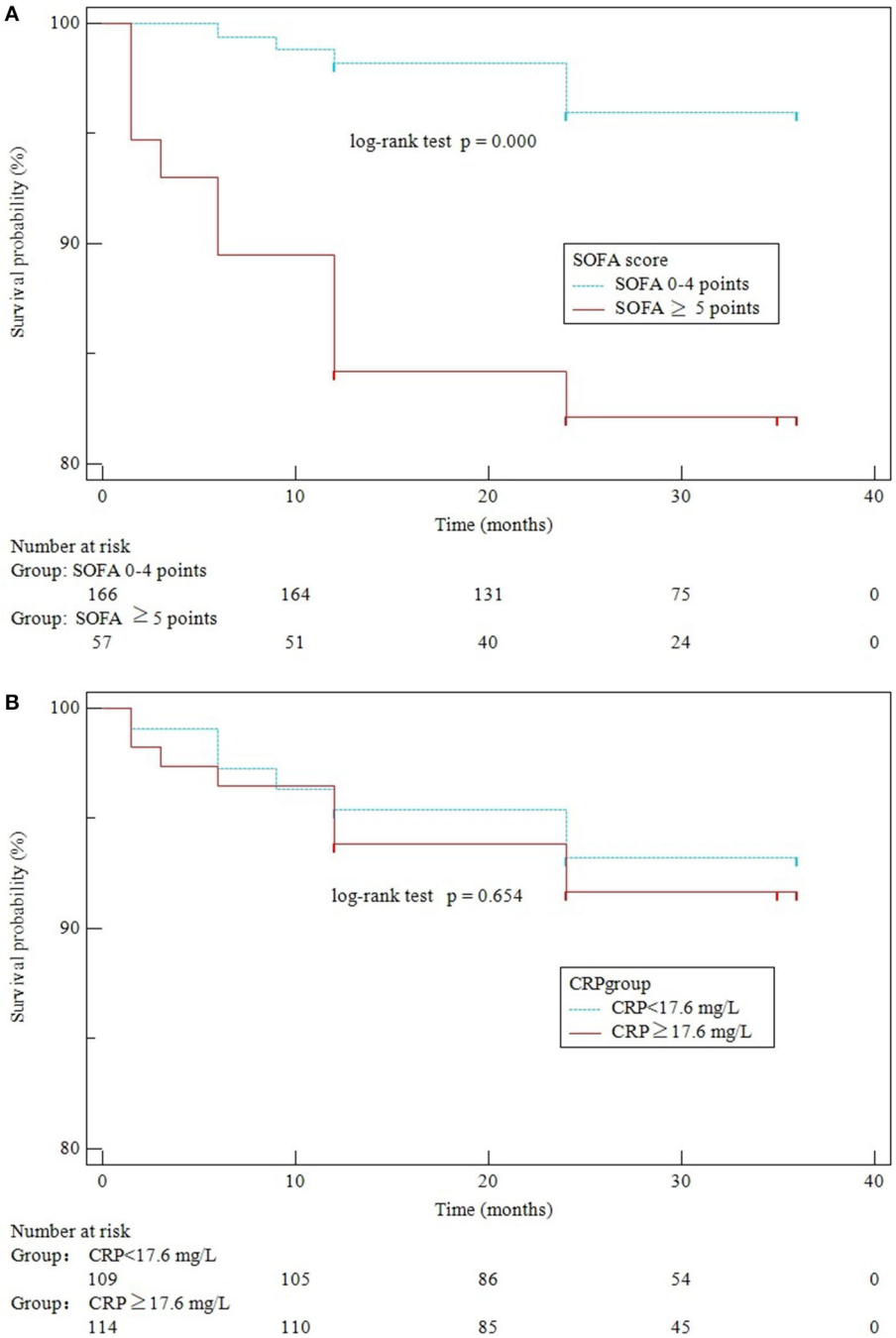


FIGURE 3 | Kaplan–Meier curves of SOFA scores **(A)** and CRP levels **(B)** for survival over 3 years of follow-up.

complicated IE. The major findings are as follows: (i) strong predictive value of SOFA ≥ 5 combined with CRP ≥ 17.6 mg/L for in-hospital mortality; (ii) high SOFA score, but not CRP, is independently associated with long-time mortality.

This is the first study documenting the combined effect of the SOFA score and CRP in predicting outcomes among patients with complicated IE. IE is a severe disease, causing 15–30% of in-hospital mortality (12, 13). However, early diagnosis of some prognostic factors may help in decreasing the mortality rates. A SOFA score of 2 or more was confirmed to be valid means of identifying sepsis with suspected infection and was demonstrated to be a significant predictor of intensive care unit mortality (14). CRP is an acute-phase inflammatory serum protein that responds rapidly to infection and is highly

accurate in predicting sepsis-suspected mortality in patients (5). Endocarditis with bacteremia leads to organ dysfunction and embolic complications in IE. Therefore, the SOFA score and the CRP level are determined to study the severity and prognosis of IE. The study by Kim et al. showed that higher SOFA [7(4–11) vs. 3(1–5), $p < 0.001$] was associated with poor outcomes in *S. aureus* bacteremia in IE (15). The data published by Asai et al. confirm that calculating the SOFA score could be a prognostic method for predicting in-hospital mortality in IE patients with a cutoff of 6 (AUC 0.915, sensitivity 76.9%, specificity 89.6%) (4). In our study, the SOFA score combined with the CRP level was considered a valuable factor in risk stratification (AUC = 0.872, sensitivity 80.95%, specificity 83.56%). The results of our study showed lower AUC and higher sensitivity when compared with those of Asai (that included coagulase-negative bacteremia patients without IE as a control group). A high SOFA score combined with increased CRP was significantly associated with in-hospital mortality with high sensitivity and specificity in this study.

The results from our study revealed that surgery treatment (OR = 0.121, $p < 0.000$) was a protective predictor, and *S. aureus* (OR = 2.151, $p = 0.020$) was an adverse predictor for the outcome during hospitalization in patients with complicated IE (16, 17). Patients with *S. aureus* infection are at the highest risk of death and need surgery in the active phase of the disease (18). Poor organ failure and more comorbidities among patients with high SOFA scores and high CRP levels might be associated with the low rate of receiving surgical procedures (68.63 vs. 88 vs. 88.51 vs. 95.06%, $p = 0.000$), likely attributing to the high death rate.

There is a high correlation of high CRP with a high SOFA score confirmed by the Spearman correlation test (analyze/correlate/bivariate) with r = 0.81 ($p = 0.004$). Therefore, the relation found of CRP with mortality was in fact indirect to the relation with SOFA score, and only SOFA $\geq$ 5 (OR = 9.320) was statistically significant in multivariate analysis.

As per the result of our study, high SOFA but not CRP is associated with high long-term mortality. CRP is an acute-phase inflammatory serum protein that is reactive to sepsis and can be suppressed through effective antimicrobial therapy or surgical treatment. However, cardiac or non-cardiac complications combined with patient characteristics are the main factors leading to a bad prognosis (19). The SOFA score was calculated for factors such as respiration, coagulation, liver function, circulatory systems, central nervous system, and renal function, which reflects the severity of organ failure and predicts underlying comorbidities (20).

However, there are a few limitations to this study. First, embolic complications are asymptomatic, therefore are not included in this study. Second, the SOFA score and CRP level should be calculated, recorded, and compared (i) before surgery, (ii) after surgery, and (iii) before discharge. Additionally, neurological complications after surgery were not included, which might contribute to postsurgical mortality. Finally, the sample size was small and only patients with complicated IE were included. However, this may not be applicable to all IE patients.

CONCLUSION

This study concluded that the SOFA score combined with the CRP level is a valuable prognostic tool to evaluate complicated IE. SOFA $\geq$ 5 combined with CRP $\geq$ 17.6 mg/L was significantly associated with in-hospital mortality. Also SOFA score, but not CRP, predicts long-term mortality in complicated IE.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Shenzhen People's Hospital. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

YL and HQ collected, analyzed, and wrote this manuscript. JY, DY, RC, and WB assisted in the conduct of the study. SD was the principal investigator. All authors contributed to the article and approved the submitted version.

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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.

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frontiers in Medicine

REVIEW
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Understanding and Managing Sepsis in Patients With Cancer in the Era of Antimicrobial Resistance

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Sepsis is a frequent complication in immunosuppressed cancer patients and hematopoietic stem cell transplant recipients that is associated with high morbidity and mortality rates. The worldwide emergence of antimicrobial resistance is of special concern in this population because any delay in starting adequate empirical antibiotic therapy can lead to poor outcomes. In this review, we aim to address: (1) the mechanisms involved in the development of sepsis and septic shock in these patients; (2) the risk factors associated with a worse prognosis; (3) the impact of adequate initial empirical antibiotic therapy given the current era of widespread antimicrobial resistance; and (4) the optimal management of sepsis, including adequate and early source control of infection, optimized antibiotic use based on the pharmacokinetic and pharmacodynamics changes in these patients, and the role of the new available antibiotics.

Keywords: sepsis, septic shock, cancer, bacteremia, bloodstream infection, neutropenia, antibiotic resistance

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INTRODUCTION

Sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection. It is associated with significant morbidity and mortality (1–3) that increase markedly when septic shock becomes established. Moreover, sepsis and septic shock are major healthcare problems, affecting millions of people worldwide each year, with the increasing cost of sepsis-associated medical care now estimated at $17 billion annually in the United States (4). Given the rapidly expanding elderly population with their associated immune senescence and frailty (5), the mortality rates associated with sepsis are expected to increase dramatically over the next 2 decades (6).

The epidemiology of sepsis in industrialized countries is mainly influenced by the age of the population and the increasing prevalence of comorbidities, such as chronic organ dysfunctions, non-cancer-related immunosuppressive diseases, or cancer itself. Patients with cancer are at more than ten times higher risk for sepsis than the general population, with some variability according to the cancer types (7, 8). Mortality due to sepsis has decreased over time in these patients, probably due to improvements in the general management of sepsis, advances in cancer therapies, and improvements in the intensive care unit (ICU) admission policies. Nevertheless, in recent decades, we are facing the alarming emergence of antimicrobial resistance among microorganisms that cause infection and sepsis, in both the general population and the immunosuppressed alike, which can negatively influence outcomes (9). Of special concern is the widespread emergence and dissemination of multidrug-resistant (MDR) Gram-negative bacilli (GNB), which are a common cause of infection and sepsis in patients with cancer. Several investigators have reported high rates

of bacteremia due to extended-spectrum β-lactamase (ESBL)-producing *Enterobacterales* (10–13), MDR *Pseudomonas aeruginosa* (MDR-PA) (12, 14–17), and carbapenem-resistant *Enterobacterales* (CRE) (18–22), among others, against which there are few treatment alternatives. This is of paramount importance because inadequate initial empirical antibiotic therapy can increase mortality when patients with cancer have infection due to MDR-GNB (11, 13–16, 18–23). Therefore, in the current era of widespread antibacterial resistance, there is an urgent need for the development of new agents with activity against MDR-GNB. In the meantime, novel ß-lactam/ß-lactamase inhibitors may be safe and effective options for treating infections due to some of these MDR-GNB (24, 25). In addition, specific strategies may help improve the overall prognosis of immunosuppressed patients with cancer, such as rapidly identifying sepsis (e.g., scores and biomarkers), optimizing ß-lactam antibiotic use (e.g., extended infusions), and optimizing source control and providing aggressive management in the ICU.

Finally, the pathophysiology of sepsis in the presence of cancer is especially complex because both entities share pathophysiological characteristics that result from the incapacity of the host's immune system to deal with an initial trigger. Thus, a dysregulated immune system seems common to both scenarios, raising the specter of their mutual impact on each other's course. Improving our knowledge about this bidirectional interaction between sepsis and cancer may lead to future research possibilities that could help modulate the dysfunctional immune system and the hyperinflammatory state, thereby improving sepsis control.

In this review, we aim to assess the prevalence, characteristics, etiology, and outcomes of sepsis in immunocompromised cancer patients and hematopoietic stem cell transplant (HSCT) recipients and to gain knowledge regarding the physiopathology of sepsis in these contexts. We also aim to review optimal management in the current era of widespread antimicrobial resistance. Finally, we will briefly comment on the current gaps in the literature and on directions for future research. Our focus is on sepsis due to bacterial infection. This review was not designed to provide evidence-qualified recommendations.

SEARCH STRATEGY AND SELECTION CRITERIA

We searched PubMed/MEDLINE for articles that were published from January 2000 to October 2020, using the following terms: "sepsis," "severe sepsis," "septic shock," "pathophysiology," "immunosuppression," "cancer," "solid tumor," "hematologic malignancy," "hematopoietic stem cell transplant," "neutropenia," "bacteremia," "bloodstream infection," "intensive care unit," "antibiotic resistance," "multidrug resistance," "mortality," "SOFA," "procalcitonin," "C-reactive protein," "adrenomedullin," "ceftolozane/tazobactam," "ceftazidime/avibactam," and "extended infusion." Articles resulting from these searches, together with any relevant references cited in those articles, were reviewed. We only included articles written in English and involving adult patients.

THE BURDEN OF SEPSIS IN CANCER PATIENTS

Some years ago, Angus *et al.* reported that one in 6 patients with sepsis presented a malignant underlying disease and that these patients suffered 30% excess mortality than other patients with sepsis (26). More recent ICU observational series have confirmed that about 15–20% of patients admitted to critical units have hematologic or solid malignancies (27–30), with sepsis being a leading cause of ICU admission in these patients (31, 32). Nevertheless, sepsis-associated mortality in cancer patients has decreased over recent decades (33–36), probably due to advances in sepsis diagnosis and management, cancer therapies, and ICU admission policies (37–39).

The current rates of in-hospital mortality of cancer patients presenting with sepsis and septic shock are ∼20 and 40%, respectively (36). Sepsis-related mortality relies on not only appropriate early management of multiple organ failure but also minimizing prolonged ICU stays and associated complications (40, 41). Moreover, the long-term outcome of cancer sepsis survivors after ICU admission is depended on the prognostic determinants of the underlying diseases as well as the possibility of continuing antineoplastic treatment, which may be hampered by loss of functional status and/or persistent organ dysfunction (42–45). Importantly, since early sepsis mortality has decreased over time, attention has recently been paid to late mortality after recovery from sepsis. Even though the exact causes of long-term sepsis mortality are still unclear, some investigations suggest that older age, comorbidities, and persistent organ injury are detrimental and lead to the immune system's dysfunction and suppression, with persistent inflammation and catabolism (46, 47).

The increased risk of sepsis in cancer patients is due to several factors: immunosuppression caused by the underlying disease, the specific onco-hematological treatments causing immunosuppression, and the invasive procedures used (e.g., long-term central venous catheters, urinary catheters, drainages, etc.). However, the cancer population is heterogeneous and there is great variation in the degree of immunosuppression. Hematological patients are at highest risk of infection and sepsis, particularly those with acute leukemia who often present prolonged and profound neutropenia (36, 48, 49), historically one of the most important risk factors for sepsis and mortality (50, 51).

Multiple myeloma and HSCT also place patients at higher risk of sepsis compared with other hematological malignancies (36, 40). HSCT recipients represent a unique population that is severely immunosuppressed due to the underlying disorder, the conditioning regimen, and the treatment of complications, such as graft-vs. host-disease (GVHD). In this setting, allogeneic transplant recipients presenting with GVHD seem to be at higher risk of sepsis and death compared to non-HSCT recipients and both autologous and allogeneic patients without GVHD, reaching mortality rates as high as 55% (52). Among patients with solid tumors, the most sepsis occurs with lung or gastrointestinal cancers, followed by other subtypes depending on the series.

In patients with solid malignancies, the site of primary or metastatic tumor often serves as the portal of entry (36, 48, 53, 54).

Even though febrile neutropenia (FN) is a frequent complication, occurring in 20–30% of patients with solid tumors and 80% of patients with hematological malignancies receiving chemotherapy, only 20–30% will develop bacteremia. Therefore, the rate of sepsis and septic shock globally is relatively low for FN. In line with this, a recent Brazilian study evaluating the frequency and epidemiology of early death and shock in 1,305 episodes of FN in 826 hematologic patients collected from 2003 to 2017 found that shock occurred in 42 (3.2%) on the first day of FN and early death occurred in 1.1% (55). In this study, predictors of septic shock were bacteremia due to *Escherichia coli* [odds ratio (OR), 8.47; 95% CI 4.08–17.55; $p < 0.001$), *Enterobacter sp.* (OR, 7.53; 95% CI 1.60–35.33; $p = 0.01$), and *Acinetobacter sp.* (OR, 6.95; 95% CI 1.49–32.36; $p = 0.01$).

PATHOPHYSIOLOGY OF SEPSIS IN CANCER PATIENTS

Sepsis-Related Immunosuppression

Sepsis is an extremely complicated process in which several situations may occur and lead to a persistent immunosuppression and hyperinflammation. On the one hand, it induces a severe state of immunosuppression that affects both cellular effectors of the innate and adaptative immune systems, changes that can persist even after recovery (56, 57). These comprise functionally essential cells, such as neutrophils, monocytes and macrophages, natural killer (NK) cells, dendritic cells, B lymphocytes, and T lymphocytes (including gamma delta T cells, T_H cell subpopulations and regulatory T cells). On the other hand, sepsis induces a state of complex immune dysfunction, including hyperinflammation (excessive release of inflammatory cytokines IL-1, TNF, and IL-7) (58), homeostatic dysfunction (59), complement activation, fibrinolytic and clotting system stimulation (60), redox imbalance (causing severe oxidative stress) (61), mitochondrial dysfunction (62), and molecular alterations (causing organ damage) (56).

Cancer-Related Immunosuppression

The immunosuppression in cancer that increases the risk of infection and sepsis is mainly associated with specific onco-hematological therapies that impair the immune system. Treatment with chemotherapy and radiotherapy alters the phagocytic activity of neutrophils and monocytes by depleting their circulating counts and impairing their capacity for chemotaxis and phagocytosis (63). As mentioned above, the risk of infection and sepsis is strongly associated with the depth and duration of neutropenia and monocytopenia (51, 52). Most cytostatics induce quantitative and/or functional modifications in lymphocytes and NK cells, while other anti-lymphoproliferative drugs and monoclonal antibodies (e.g., fludarabine, bendamustine, ibrutinib, rituximab, and alemtuzumab) can induce prolonged B- and/or T-cell lymphopenia (64). Corticosteroid use is also frequent in cancer patients, increasing the degree of immunosuppression. They cause both a pleiotropic dysregulation of innate and adaptive immune responses and a decrease in the activities of neutrophils, monocytes, macrophages, and lymphocytes (mainly $CD4^+$ T cells). At high doses, they also induce apoptosis, decrease IL-2 levels, and impair the Th2-cell response. Therapy with HSCT may delay immune reconstitution due to persistent lymphopenia, low cell diversity, and defective lymphocyte functions (65). In addition, chemotherapy and radiotherapy may impair other organ and tissue functions, limiting their capacity to deal with the initial aggression. In this regard, it has been suggested that the endothelial toxicity secondary to cytostatic agents may lead to microcirculatory alterations and an impaired vessel response to vasopressors (66).

There are specific cancer settings that may increase the risk of infection and sepsis, regardless of antineoplastic therapy. The involvement of the bone marrow and/or the presence of dysmyelopoiesis by certain hematological malignancies or by metastatic solid tumors may lead to important cytopenias and/or defective phagocytic activity of neutrophils and monocytes (67). Some lymphoproliferative disorders may also cause hypogammaglobulinemia, and the compression of anatomic structures and/or tissue infiltration by malignant cells can diminish local defense mechanisms.

Finally, tumor cells can escape cytotoxic cells by losing major histocompatibility class-1 molecules, leading to the inappropriate expression of checkpoint inhibitory molecules, and can exhibit functional defects that result in decreased antigen presentation, and altered dendritic, macrophage, NK, and CD8 T cell function (68). Whether tumor-related immune alterations increase the risk of infection still needs to be confirmed.

The Bidirectional Interaction Between Cancer and Sepsis

There are pathophysiological similarities between cancer and sepsis that favor the interaction between these two processes. Indeed, some malignancy-related conditions and adverse drug reactions can mimic sepsis (69) and may hinder differentiation between these entities. In particular, certain aggressive hematological diseases, such as acute leukemia and high-grade B-cell lymphoma may present multiple organ dysfunctions through several pathways, such as tissue infiltration by tumor cells, anatomical compression, intracellular metabolite release, altered coagulation, and hemophagocytic lymphohistiocytosis (70, 71). Currently available antineoplastic therapies, including T-cell based therapies (e.g., bispecific monoclonal antibodies and chimeric antigen receptor-T cells) or differentiating agents (e.g., all-trans retinoic arsenic or acid), may also produce acute systemic inflammatory syndromes that mimic sepsis (72, 73). Differentiating these entities is of paramount importance because managing these proinflammatory conditions varies significantly.

The similarities between cancer-related and sepsis-induced immune dysfunctions indicate that immune defects derived from infectious triggers may facilitate a favorable environment and promote tumor growth. Consistent epidemiological and experimental findings support a link between sepsis and further risk of cancer (74–77). Conversely, some historical reports and experimental studies have suggested that sepsis may instead have antitumoral activity (78, 79), with "cancer-then-sepsis" models suggesting that sepsis may induce tumor suppression (80–82).

Interestingly, recent data also suggest that antibiotic-induced dysbiosis (changes in the composition and diversity of gut microbiota) may alter the immune response to cancer (83, 84). Finally, it has been hypothesized that certain Toll-like receptors could have a role in modulating tumor growth in sepsis (85, 86).

CHARACTERISTICS, ETIOLOGY, AND OUTCOMES OF SEPSIS IN CANCER PATIENTS

There are several current studies evaluating the prevalence, clinical features, etiology, and outcomes of cancer patients with bacteremia, with reported rates of septic shock varying by series from 6 to 57% (11, 13, 15, 18–20). Nevertheless, very few investigations have focused on patients presenting with sepsis or septic shock (31, 36, 48, 49, 52, 55, 87, 88), and among these, some were retrospective, some used different sepsis definitions, and some did not provide comprehensive data. **Table 1** summarizes the most relevant data for cancer patients with sepsis or septic shock.

Overall, patients with hematological malignancies, mainly acute leukemia, are at greatest risk of sepsis (36, 48, 49). Patients with multiple myeloma (36, 48, 87) and non-Hodgkin lymphoma (31, 36, 55, 87) also seem to experience high rates of sepsis and septic shock. HSCT recipients have been included in only few series, but rates of sepsis range from 3 (87) to 12% (36), as reported in a recent large study of cancer with 2,062 patients admitted to seven European ICUs for sepsis or septic shock. In this study, data were extracted from the *Groupe de Recherche Respiratoire en Réanimation Onco-Hématologique* database, from 2006 to 2010. Patients more often had hematological (82.4%) than solid (17.6%) malignancies, and many (31%) had neutropenia at ICU admission. The 30-day mortality rate was 39.9% and decreased significantly over the study period [odds ratio (OR) 0.96; 95% CI, 0.93–0.98; $p = 0.001$].

Seeking to characterize the frequency and outcomes of sepsis in adult HSCT recipients and to compare them to non-transplant patients, Kumar et al. retrospectively analyzed a large nationwide administrative database from almost 20% of the community hospitals in the United States (52). Data were provided separately according to the reason for admission (engraftment admission or subsequent admission with and without GVHD). Of the 291,179 discharges with HSCT between 2000 and 2008, sepsis was identified in 21,898 (7.5%). The frequency of sepsis was 5 times higher in HSCT recipients than in non-transplant patients. Allogeneic transplant recipients and those with GVHD (10.4%) had significantly higher rates of sepsis than either autologous recipients (13.2 vs. 5.2%; $p < 0.001$) or those without GVHD (10.4 vs. 6.1%; $p < 0.001$). The unadjusted hospital-related mortality was significantly higher among allogeneic transplant recipients than non-HSCT recipients (55.1 vs. 32.9%, $p < 0.001$), but the mortality rates did not differ between autologous HSCT recipients and the non-transplant population. After adjustment, however, the odds of mortality were 3.81 times higher in allogeneic HSCT recipients (95% CI, 2.39–6.07) and 1.28 times higher in autologous recipients compared with non-transplant patients (95% CI, 1.06–1.53). **Table 2** displays more detailed information on the engraftment admissions and compares patients according to the type of transplant.

Among solid tumors, the rates of sepsis vary by the series, with gastrointestinal (31, 87) and lung cancers (48) being most susceptible. In a recent report of 1,009 patients with gynecological cancers receiving 10.239 cycles of chemotherapy, the incidence of septic shock during neutropenia was 3% and the mortality rate was 1.2% (88). The incidence of septic shock during neutropenia was also higher in patients older than 50 years (3.9 vs. 1.4%, $p = 0.034$), with a linear-by-linear association between the accumulated cycles of chemotherapy and the sepsis rate ($p = 0.004$). Also, patients who had received two or more courses of chemotherapy presented and increased incidence of neutropenic septic shock (NSS) compared with those receiving only one course (4.9 vs. 1.4%, $p = 0.002$). No significant differences were observed regarding the type of gynecological cancers and the status of the disease between patients with NSS and patients without NSS. The mortality rate of patients with NSS was 37.5%. In this study, the median age (64.0 vs. 56.5, $p = 0.017$) and the peak heart rate (149.5 vs. 123.5 min^{-1}, $p = 0.015$) were significantly higher in the group of patients who subsequently died of NSS than in those who survived.

In ∼30–50% of the sepsis and septic shock episodes occurring in patients with cancer, no microbiological diagnosis is achieved (31, 87). When a definitive microbiological diagnosis is made, blood cultures are the most useful tool because most episodes are secondary to bacteremia and/or high-inoculum infections, such as pneumonia, with frequent secondary dissemination to the bloodstream.

The site of infection is barely provided in the few series addressing sepsis in cancer patients. In a report addressing shock and early death in hematologic patients with FN, Rosolem et al. described the site of infection in 563 cases of sepsis (91% with septic shock) admitted in the ICU (31). The most frequent sites were the lung (44%), the abdomen (31%), and the urinary tract (8%), with 24 patients (4%) having more than one site. GNB were responsible for more than 50% of episodes, with *E. coli* (16%), *P. aeruginosa* (13%), and *Klebsiella pneumoniae* (13%) being most common. The overall ICU and hospital-related mortality were 51 and 65%, respectively; these were higher in patients with septic shock (62 and 74%) than in patients with either sepsis (36 and 55%) or sepsis (15 and 25%). Mortality rates were higher for patients with pneumonia and bacteremia than with gastrointestinal and urinary tract infections. End-of-life decisions were made for 29% of patients in their cohort.

In a subgroup analysis of a multicenter prospective cohort study in 28 Brazilian ICUs, 717 patients with cancer were analyzed (87). Among them, 37% had sepsis and 53% septic shock. The most frequent infection sites were the lungs (48%), abdomen (19%), bloodstream (primary) (19%), and urinary tract (17%). Half had a microbiologically confirmed infection, with GNB again being the most frequent cause (31%). ICU- and hospital-related mortality rates were 42 and 56%, respectively. End-of-life decisions were made in 17% of the patients.

TABLE 1 | Summary of the most relevant series of cancer patients presenting with sepsis and septic shock.

Study	Rosolem et al. (31)	Torres et al. (87)	Lemiale et al. (36)	[9]Kumar et al. (52)	
Study design	Secondary analysis of a prospective cohort study	Subgroup analysis of a multicenter prospective cohort study	Analysis of the GRRR-OH database which includes cancer patients from 1994 to 2015	Analysis of the healthcare cost and utilization project-nationwide inpatient sample (NIS) database	
Site	10-bed medical-surgical cancer ICU unit at Instituto Nacional de Cancer, Rio de Janeiro, Brazil	28 Brazilian ICUs	Seven European ICUs from France and Belgium	The database contains data from ∼1,000 (20%) community U.S. hospitals	
Study period	January 2003–July 2007	August 1–September 2007	2006-2010	2000–2008	
Number of patients	563 patients with sepsis	268 patients with sepsis	2.062 patients with sepsis or septic shock	6.168 engraftment admissions in HSCT recipients with sepsis among 79.287 discharges (7.7%)	
				2.750 admissions for sepsis in autologous HSCT recipients (5.2%)	3418 admissions for sepsis in allogeneic HSCT recipients (13.2%)
Age (y)	59.2 ± 17.8	63.1 ± 15.0	59 (48–67)	21.6% (≥65 y)	4.35% (≥65 y)
Male sex	301 (54%)	126 (47%)	1.275 (61.8%)	56.6%	6.34%
Hematologic disease	127 (23%)	35 (13%)	1.700 (82.4%)	100%	100%
- NHL	14%	4%	461 (22.4%)	7.8%	3.2%
- Leukemia	6%	4%	591 (28.7%	16.9%	62.5%
- Multiple myeloma	2%	3%	244 (11.8%)	37%	4.5%
- Hodgkin lymphoma	–	–	–	10.4%	1.8%
HSCT recipients	–	8 (3%)	250 (14%)[a]	100%	100%
- Solid tumor	436 (77%)	233 (87%)	362 (17.6%)	–	–
- Gastrointestinal	35%	25%	61 (16.9%)	–	–
- Head and neck	13%	7%	4 (0.1%)	–	–
- Lung cancer	6%	9%	48 (13.3%)	–	–
- Urogenital	6%	15%	43 (11.8%)	–	–
- Breast	5%	6%	55 (15.2%)	–	–
- Uterus	–	–	15 (4.1%)	–	–
Recurrence/progression	103 18%	35%	–	–	–
PS >2	294 52%	57%	–	–	–
Neutropenia (<500/mm^3)	71 13%	12%	640 (31%)	–	–
Comorbidities	349 (62%)	–	1.043 (50.6%)	–	–
- Diabetes mellitus	14%	–		6.8%	4.1%
- Hypertension	38%	–		–	–
- COPD	12%	–		11.8%	8.2%
Previous chemotherapy and/or radiotherapy	40%	54%	211 (58.3%)	–	–
Acquisition					
- Community-acquired	227 40%	32%	–	–	–
- Nosocomial	336 60%	67.5%	–	–	–
SOFA score (points) on the first day of ICU	8 (5–11)	9 (7–12)	6 (4–9)	–	–
Acute organ failures (n)	2 (1–3)	4 (3–4)	–	18% (≥3)	24.5% (≥3)
Mechanical ventilation	489 (87%)	51%	1.016 (49.3%)	30.9%	40.4%
Renal replacement therapy	110 (20%)	18%	420 (20.4%)	11.4%	19.3%
Vasopressor use	372 (64%)	59%	1.172 (56.8%)	–	–

(Continued)

TABLE 1 | Continued

Study	Rosolem et al. (31)	Torres et al. (87)	Lemiale et al. (36)		Kumar et al. (52)
Proof of infection					
- Clinically suspected	180 (32%)	133 (50%)	–	–	–
- Microbiologically proven	383 (68%)	135 (50.3%)			
Severity					
- Sepsis	48 (9%)	–	–	–	–
- Severe sepsis	143 (25%)	142 (53%)	–	–	–
- Septic shock	372 (66%)	126 (47%)	–	–	–
Pathogens					
Gram-positive	168 (30%)	34 (13%)	–	33%	38.3%
- Enterococci	92 (16%)	–	–	–	–
- *S. aureus*	61 (11%)	22 (8.2%)	–	–	–
- Group D streptococci	16 (3%)	–	–	–	–
- *S. pneumoniae*	13 (2%)	4 (1%)	–	–	–
- Other	12 (3%)	–	–	–	–
- CNS	297 53%)	13 (5%)	–	–	–
Gram-negative	91 16%	83 (31%)	–	16%	15.8%
- *Escherichia coli*	74 13%	27 (10%)	–	–	–
- *P. aeruginosa*	72 13%	29 (11%)	–	–	–
- *K. pneumoniae*	46 8%	27 (10%)	–	–	–
- *Enterobacter* spp	34 6%	–	–	–	–
- *Proteus* spp	18 4%	–	–	–	–
- *M. morganii*	14 3%	–	–	–	–
- *S. maltophilia*	92 16%	3 (1%)	–	–	–
- Other	43 8%	8 (2.9%)	–	–	–
Fungi			–	–	–
- *Candida* spp	35 6%	12 (4%)	–	3.3%	5.4%
- Other	8 1%	1 (1%)	–	2.2%[d]	5.8%[d]
Other infectious agents	19 (3%)	39 (15%)	–	12%[e]	13.4%[e]
Multidrug-resistant bacteria	81 14%	–	–	–	–
Site of infection					
Lung	246 44%	130 (48%)	–	–	–
Abdomen	172 31%	67 (25%)	–	–	–
Urinary tract	42 8%	45 (17%)	–	–	–
Skin/soft tissue	35 6%	26 (10%)	–	–	–
Primary bacteremia	24 4%	51 (19%)	–	–	–
Central nervous system	11 2%	3 (1%)	–	–	–
Surgical site infection	–	18 (7%)	–	–	–
Other/unknown	57 10%	18 (7%)	–	–	–
More than 1 site of infection	24 4%	71 (26%)	–	–	–
ICU LOS	9 (4–18)	7 (4–16)	–	–	–
Hospital LOS (days)	23 (11–43)	22 (13–38)	–	26 (21–37)	39 (30–58)
ICU mortality	289 51%	42%	–	–	–
In-hospital mortality	364 65%	56%	823 (39.9%)[b]	30.1%	55.1%

NHL, Non-Hodgkin lymphoma; HSCT, hematopoietic stem cell transplant; COPD, chronic obstructive pulmonary disease; SOFA, Sequential Organ Failure Assessment; ICU, Intensive care unit; CNS, Coagulase-negative staphylococci; LOS, Length of hospital stay; GRRR-OH, Groupe de Recherché Respiratoire en Réanimation Onco-Hématologique.
[a]Only data on allogeneic HSCT were provided; [b]Data on 30-day mortality were available in 943 patients (45.7%), approximated using hospital mortality in 879 patients (42.6%) and as last resort ICU mortality if the former were unavailable; [c]Only data regarding engraftment admission are provided in this table. Data on subsequent admissions in HSCT recipients with and without graft-vs. host diseases are provided in the complete version of the manuscript. [d]Infections due to Aspergillus spp.; [e]Clostridioides difficile colitis.

TABLE 2 | Risk factors associated with mortality in the most relevant series of sepsis and septic shock in cancer patients.

References	Patients studied	Outcome		OR (95% CI)	*P*
Rosolem et al. (31)	563 cancer patients with sepsis	In-hospital mortality	Admission to a medical ICU	2.19 (1.40–3.42)	0.001
			Active-newly diagnosed disease	1.76 (1.12–2.75)	0.013
			Active-recurrence/progression	2.42 (1.35–4.35)	0.003
			Performance status >2	3.57 (2.36–5.979)	<0.001
			Non urinary tract infection	3.28 (1.57–6.86)	0.002
			SIRS criteria ≥ 3	1.80 (1.20–2.72)	0.014
			Cardiovascular dysfunction	1.94 (1.27–2.94)	0.008
			Respiratory dysfunction	2.29 (1.24–4.23)	0.002
			Renal dysfunction	2.12 (1.34–3.35)	0.001
Torres et al. (87)	268 cancer patients with sepsis	In-hospital mortality	Organ dysfunction	1.48 (1.16–1.87)	0.001
			Hematological malignancy	2.57 (1.05–6.27)	0.038
			Performance status >2	2.53 (1.44–4.43)	0.001
			Polymicrobial infections	3.74 (1.52–9.21)	0.004
Lemiale et al. (36)	2.062 cancer patients with sepsis or septic shock	30-day mortality	Mechanical ventilation	3.25 (2.52–4.19)	<0.01
			Vasopressor use	1.42 (1.10–1.83)	<0.01
[a]Kumar et al. (52)	6.168 engraftment admissions in HSCT recipients with sepsis	Unadjusted in-hospital mortality	Allogeneic HSCT	2.12 (1.55–2.90)	NA
			Age 35–49 years	1.68 (1.08–2.60)	NA
			Age ≥65 years	2.08 (1.13–3.83)	NA
			Cirrhosis as comorbidity	4.49 (1.81–11.1)	NA
			Multiple myeloma	0.59 (0.40–0.89)	NA
			Respiratory failure	12.1 (8.64–16.8)	NA
			Cardiac failure	2.42 (1.59–3.66)	NA
			Renal failure	2.64 (1.95–3.56)	NA
			Metabolic failure	1.63 (1.07–2.49)	NA
			Hepatic failure	5.22 (2.29–11.8)	NA

[a]*Only data regarding engraftment admission are provided in this table. Data on subsequent admissions in HSCT recipients with and without graft-vs. host diseases are provided in the complete version of the manuscript.*

RISK FACTORS FOR MORTALITY IN CANCER PATIENTS WITH SEPSIS AND SEPTIC SHOCK

Table 2 shows the risk factors associated with mortality for the most relevant series of patients with cancer who developed sepsis or septic shock. The most commonly variables associated with mortality are those related to the underlying disease (mainly hematological malignancies) (52, 87) and its status (uncontrolled cancer and poor performance status) (31, 87), the presence of one or more organ dysfunctions (31, 52, 87), and the need for organ support (36). Some other variables identified as risk factors for mortality include older age (52, 88), comorbidities (52), infection site (particularly pneumonia) (31), polymicrobial infection (87). Of note, the year of ICU admission has also been shown to influence cancer patient's outcomes significantly, with decreased mortality rates observed over time (36, 52). The nutritional status of cancer patients may also play an important role in the development of sepsis and on its outcomes. In this regard, a study conducted in our institution involving head and neck cancer patients (who are particularly malnourished) with bacteremia identified hypoalbuminemia as independent risk factor for bacteremia and for early and overall mortality (89). Importantly, inadequate initial empirical antibiotic therapy (IEAT) is widely recognized as an important risk factor for mortality in all patients, including those with immunosuppression due to cancer (14, 18, 23, 90–92). Kadri et al. recently published a retrospective cohort analysis of electronic health record data from 131 hospitals in the US that included 21,608 patients with bacteremia who received empirical antibiotics between 2005 and 2014 (93). Among them, 4,165 (19%) received IEAT, which was independently associated with increased mortality (adjusted OR 1.46; 95% CI, 1.28–1.66); $p < 0.0001$), regardless of whether sepsis or septic shock was present. Infection due to antibiotic-resistant organisms was strongly associated with an increased risk of receiving IEAT (adjusted OR 9.09; 95% CI 7.68–10.76; $p < 0.0001$).

Several studies have shown that cancer patients with infections due to resistant pathogens are more likely to receive IEAT (90–92). In addition, many of these studies have shown that failure to cover resistant organisms, and particularly MDR-GNB, significantly and independently impairs their outcomes (10, 11, 14, 18, 20, 22, 23). The presence of septic shock and/or the need for ICU admission have also frequently been identified as risk factors for mortality in cancer patients with bacteremia (94). Therefore, it is reasonable to hypothesize that these factors (IEAT and sepsis/septic shock) synergize to affect the prognosis of these patients negatively. However, there remains no firm

evidence of how IEAT impairs outcomes in cancer patients with sepsis.

OPTIMAL MANAGEMENT OF SEPSIS IN CANCER PATIENTS

Early Recognition and Diagnosis

In 2017, Rhodes et al. published the "Surviving Sepsis Campaign," which are international guidelines for the management of adult patients with sepsis and septic shock. These guidelines provide the best evidence-based recommendations for the management of this life-threatening condition (95).

Several studies in the general population have demonstrated that the Sequential Organ Failure Assessment (SOFA) score better predicts hospital mortality for ICU patients with infection compared with the systemic inflammatory response syndrome. Therefore, a new definition of sepsis and septic shock was adopted in 2016 (1), and later studies have demonstrated that the new definitions are applicable to cancer with the same reliability as in the general population (94). In addition, the quickSOFA (qSOFA), constitutes a simple bedside clinical score that can be rapidly applied and allows the prompt identification of patients at greatest risk of need for admission to an intensive care unit. The qSOFA score is based in 3 simple variables, which include respiratory rate $\geq$22/min, alteration in mental status, and systolic blood pressure $\leq$100 mm Hg (96). The SOFA and qSOFA scores are therefore useful tools to identify and predict complications and mortality in these patients (97–99), and as such, should be applied to all cancer patients with suspected infection.

In recent decades, over 180 biomarkers have been evaluated as unsuitable for the diagnosis and prognosis of sepsis, being lactate one of the most frequently used (100). To date, none have demonstrated sufficient specificity or sensitivity for reasonable utility in clinical practice. Procalcitonin (PCT) and C-reactive protein (CRP) have perhaps been the most widely used, but they have limited ability to distinguish sepsis from other inflammatory conditions or to predict outcomes. More recently, elevated serum lactate has been used as a biomarker in the diagnosis of septic shock (1). There is also interest in the role of these biomarkers as diagnostic, prognostic, and theragnostic markers in febrile cancer patients, particularly those with FN. Despite the paucity of data about biomarkers in cancer patients, some reports have evaluated their role. In this regard, PCT has been shown to have better accuracy than CRP and IL-6 in differentiating infectious from non-infectious causes of fever in a meta-analysis of 27 studies in adult and pediatric cohorts (101). However, this meta-analysis included different types of underlying disease and evaluated different outcomes. Several other reports have shown that PCT can predict bacteremia in cancer patients with and without neutropenia, particularly those with infection by GNB, and that it may predict a need for organ support (102–106).

Adrenomedullin (ADM) is also elevated in sepsis, which results in pro-ADM that is present at higher levels in patients with localized infections and bacteremia than in healthy controls (107). Pro-ADM has shown to be more suggestive of sepsis than PCT in cancer patients (108), and its levels are more significantly elevated in patients with hematological cancers and localized infections than in those with no infections (108). Nevertheless, another study of critically ill cancer patient revealed that pro-ADM and PCT had similar areas under the roc curve for identifying bacteremia, both being superior to that of CRP (109). Other biomarkers, such as presepsin, IL-6, and IL-8, appear to be less useful (110). The role the currently available biomarkers in cancer patients with sepsis clearly needs to be elucidated further.

Antibiotic Therapy

In the current era of growing antimicrobial resistance, the following general considerations need to be assessed before deciding on empirical antibiotic therapy in cancer patients (particularly in FN): prior history of colonization/infection with resistant pathogens; the presence of other risk factors for antibiotic resistance; the local epidemiology and resistance patterns in that hospital, unit, and geographical area; and other patient-related factors that may predict a complicated clinical course (e.g., older age, comorbidities, localized infection, and shock).

After this evaluation, cancer patients with sepsis or septic shock need urgent therapy with a broad-spectrum anti-pseudomonal BLA with or without other agents that are active both against the suspected organisms and at the site of infection (111). It remains controversial whether adding a short-course aminoglycoside to a broad-spectrum BLA regimen can benefit severely ill patients. An important meta-analysis (112), as well as a recent prospective observational cohort study of 648 ICU patients (113), failed to show this association. In the study by Ong et al. there was no association with a faster reversal of shock or an increased 14-day survival; however, that study mainly included immunocompetent patients, and only 4% received IEAT, probably due to low local levels of antibiotic resistance (113).

Interestingly, in a previously published study we observed improved early (7- and 14-day) mortality rates in those who received initial combination therapy, who also presented more frequently with septic shock. In a prospective study of 510 hospitalized patients with bacteremia in the context of neutropenia due to hematological malignancy, we also observed better 30-day survival in those who received combination therapy (94). Similar findings have been reported in the general population by other investigators (114, 115). Therefore, while awaiting the results of well-designed randomized clinical trials, we advocate the inclusion of short-course aminoglycoside therapy with a BLA for IEAT when treating neutropenic cancer patients in centers with a high prevalence of multidrug resistance, especially if sepsis or septic shock are present.

The empirical use of the two novel antibiotics, ceftolozane/tazobactam and ceftazidime/avibactam, should be considered in cancer patients at risk of infection due to MDR-GNB (e.g., MDR-PA, CRE, or ESBL-*Enterobacterales*), particularly if they present with sepsis or septic shock. Targeted therapy with these agents should be also considered if other first-line antibiotics are not viable treatment options because of a lack of activity or a high-risk of toxicity. Although these novel antibiotics have not been specifically approved for neutropenic and/or cancer patients, they are being used in these setting due to the increasing problem of antibacterial resistance among

GNB. These drugs have been used in real-world settings, where they are reported to show clinical and microbiological success in high-risk hematological patients. However, there is a scarcity of data, and where it is present, it is based mainly on case series and case reports (116–122). **Table 3** summarizes the main clinical and microbiological data in these studies.

An alternative strategy for the treatment of infections due to resistant GNB organisms is to use existing BLAs by extended or continuous infusion to maximize their pharmacokinetic (PK) activity (123). Critically ill patients present certain physiopathologic changes, mainly due to an increased volume of distribution and an increased renal clearance, making them excellent targets for this strategy. In fact, two meta-analyses have shown an association between prolonged BLA infusions and lower mortality rates in critically ill patients (124, 125). Cancer patients with FN may be considered similar to critically ill patients in terms of the intra- and inter-individual variability of PK parameters (126–128).

Regarding the clinical impact of optimizing the use of BLAs, data are limited for neutropenic patients and restricted to certain antibiotics (129, 130). A recent single-center randomized clinical trial found that extended infusion with a BLA was associated with superior outcomes than intermittent infusion, the greatest benefit observable in patients with pneumonia (131). Nevertheless, that study had some methodological limitations, and there were no PK studies to support the clinical results (132). Currently, a multicenter, open label, randomized, superiority clinical trial is being performed (EudraCT 2018-001476-37, ClinicalTrials.gov: NCT04233996) to assess the clinical efficacy of extended BLA infusions in hematological patients with neutropenia. Other secondary outcomes include PK/pharmacodynamic target achievement, bacteremia clearance, CRP decrease, overall 30-day case-fatality rate, and adverse events. Finally, a population PK model of the BLA studied will be developed (133).

In the current era of emerging antimicrobial resistance, antimicrobial stewardship is of paramount importance in order to decrease the overall antimicrobial consumption and hinder resistance dissemination. In this regard, the last guidelines recommend applying antimicrobial stewardship strategies in patients with sepsis and septic shock, such as de-escalation and/or discontinuation of antibiotic within the first few days in response to clinical improvement and infection resolution and/or lack of evidence of infection (95). Cancer patients with sepsis and septic shock can be probably managed in the same way. In this line, we recently published a randomized clinical trial involving high-risk hematologic patients with febrile neutropenia without microbiologically documented infection, in which we demonstrated that empirical antibiotic therapy can be discontinued after 72 h of apyrexia and clinical recovery irrespective of the neutrophil count (134). This clinical approach showed to reduce unnecessary exposure to antimicrobials and to be safe.

ICU Management

The total number of patients with cancer who need ICU admission has increased dramatically over time, presently accounting for up to 15% of all admissions (8, 27–30). Overall mortality has also decreased and survivors achieve remission and quality of life after ICU admission, similar to non-ICU patients (37, 135). Some important changes in the management of critically ill cancer patients over recent decades have influenced these improved outcomes. These include the following:

a) Changes in ICU admission policies that may have favored faster admission of more candidates, leading to early treatment of organ dysfunction (37, 136).
b) Many so-called classic predictors of mortality (e.g., neutropenia, underlying disease, blood transfusion requirements, and second-line therapies) are no longer relevant or influence the therapeutic approach less (32, 137, 138).
c) Improved collaboration between hematologists/oncologists and intensive care providers (137–140).
d) Improved management of sepsis in neutropenic patients, including escalation and de-escalation strategies for antimicrobial therapy, source control (e.g., catheter removal), and/or conducting surgery if indicated regardless of the presence of cytopenias (141–143).
e) Using specific therapies for selected patients with hematological cancers in ICU has also proven to be feasible and associated with significant survival benefit (144–147).

CURRENT GAPS AND FUTURE RESEARCH

Although much progress has been made in the understanding and management of sepsis in cancer patients, much is still to be done.

The increasing number of cancer patients who potentially require ICU management will necessitate a comprehensive revision of ICU admission policies. Early recognition of sepsis based on routine clinical, biochemical, and radiological signs is still inaccurate in cancer. Future diagnostic strategies must therefore incorporate newer tests with improved diagnostic performances, easier non-invasive sampling, and shorter response time. None of the currently available biomarkers have demonstrated sufficient sensitivity and/or specificity for use in clinical practice. Identifying new biomarkers reflecting host response and/or pathogen invasion may allow better differentiation of infectious from non-infectious processes and the early and safe discontinuation of antimicrobial therapy.

In addition, gaining a better understanding of the mutual interaction between cancer and sepsis, as well as the alterations in innate and adaptive immune cell functions, could lead to the development of potential therapeutic interventions. Identifying biomarkers that can accurately detect and quantify immune suppression in cancer patients with sepsis will be key to the design of immunomodulatory therapeutic strategies.

The adequacy of IEAT should be improved in cancer patients with sepsis, and efforts should be made to ensure adherence to current guidelines, with adaptation to local epidemiology where necessary. Studies should also continue to clarify the role of new antibiotics, such as ceftolozane/tazobactam and ceftazidime/avibactam, in cancer patients with sepsis, particularly when used empirically. Other novel antibiotics

TABLE 3 | Summary of the main clinical and microbiological characteristics of the published case series and case reports on the use of ceftazidime/avibactam and ceftolozane/tazobactam in high-risk hematologic cancer patients.

References	Study design	Number of patients	Microorganisms mechanisms of resistance	Type of infections	Combination therapy	All-cause 30-day case-fatality rate	Recurrence/ resistance development	Comments
Ceftazidime/Avibactam								
Castón et al. (120)	Multicenter, retrospective. Patients treated with C/A were compared with those receiving other active antibiotics	8 C/A vs. 23 other active antibiotics	Carbapenemase-producing *Enterobacterales* (80.6% *K. pneumoniae*) 61.3% OXA-48, 38.7% KPC	Bacteremia	100% Combinations included: aminoglycosides (7), carbapenems (3), fosfomycin (2), tigecyclin (2), and/or colistin (2)	25% with C/A vs. 52% with other active agents	None/None	All treatment regiments were used as targeted therapy 77.4% were neutropenic The number of death events were too small to detect significant differences
Metafuni et al. (121)	Case series of patients presenting with persistent sepsis or septic shock	3	Carbapenemase-producing *K. pneumoniae* ($n = 2$) MDR-*P. aeruginosa* ($n = 1$)	Bacteremia	100% Meropenem (2), tigecyclin (3), colisitn (2)	33%	None/None	All treatment regiments were used as targeted therapy All patients were neutropenic
Hobson et al. (122)	Case report of a pediatric patient	1	NDM-1-producing *Morganella morganii*	Bacteremia	Aztreonam (ATM)	0	None/None	The patient was neutropenic MIC for the combination of ATM + C/A was 0.016 mg/L
Ceftolozane/Tazobactam								
Hakki and Lewis (116)	Retrospective case series	6 patients received 7 cycles of C/T	MDR-*P. aeruginosa*	Bacteremia (3), Pneumonia (3), SSTI (1)	None	0	1 case/1 case	Four patients (66.6%) were neutropenic and two (33.3%) were HSCT recipients In two cases C/T was initiated empirically
Fernández-Cruz et al. (117)	Retrospective, case-control. Patients treated with C/T were compared with those receiving other active antibiotics	19 C/T vs. 38 other active antibiotics	*P. aeruginosa* (51.2% were MDR)	Primary bacteremia (4) Pneumonia (5), perianal infection (3), UTI (2), SSTI (1)	42% Amikacin + levofloxacin (2), amikacin (4), colistin (1), and fosfomycin (1)	5.3% with C/T vs. 28.9% with other active agents	3 cases/None	C/T was used empirically in 3 cases, and as targeted 6 patients had secondary bacteremia >60% were neutropenic
Aitken et al. (118)	Case report of a pediatric patient	1	MDR-*P. aeruginosa*	Bacteremia	Tobramycin and ciprofloxacin	0	None/1	The patient was neutropenic The MIC to C/T increased from to 6 to 8 μg/mL
So et al. (119)	Case report	1	MDR-P. aeruginosa	Bacteremia	Tobramycin	0	None/1	The patient was neutropenic Bacteremia cleared with the combination of a pharmacodynamically driven dose of C/T and tobramycin with resultant synergy

MDR, multidrug-resistant; SSTI, skin and soft tissue infection; HSCT, hematopoietic stem cell transplant; UTI, urinary tract infection.

displaying activity against MDR-GNB are currently under clinical evaluation (e.g., imipenem/relabactam, plazomicin, cefiderocol, meropenem/vaborbactam, and eravacycline), and given time, these may improve the antibiotic armamentarium.

Finally, combining knowledge of more rigorous and thorough patient stratification and selection, strategic and careful long-term monitoring of immune function, and targeted immunomodulatory treatment could optimize clinical benefits for surviving initial sepsis.

AUTHOR CONTRIBUTIONS

CG and JC were responsible for the study conception and design. CG, AA-P, and GC performed the literature review. CG drafted the manuscript. All authors approved the final version of the manuscript.

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D-Dimer as Biomarker for Early Prediction of Clinical Outcomes in Patients With Severe Invasive Infections Due to *Streptococcus Pneumoniae* and *Neisseria Meningitidis*

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Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection; no current clinical measure adequately reflects the concept of dysregulated response. Coagulation plays a pivotal role in the normal response to pathogens (immunothrombosis), thus the evolution toward sepsis-induced coagulopathy could be individuate through coagulation/fibrinolysis-related biomarkers. We focused on the role of D-dimer assessed within 24 h after admission in predicting clinical outcomes in a cohort of 270 patients hospitalized in a 79 months period for meningitis and/or bloodstream infections due to *Streptococcus pneumoniae* ($n = 162$) or *Neisseria meningitidis* ($n = 108$). Comparisons were performed with unpaired *t*-test, Mann-Whitney-test or chi-squared-test with continuity correction, as appropriate, and multivariable logistic regression analysis was performed with Bayesian model averaging. In-hospital mortality was 14.8% for the overall population, significantly higher in *S. pneumoniae* than in *N. meningitidis* patients: 19.1 vs. 8.3%, respectively ($p = 0.014$). At univariable logistic regression analysis the following variables were significantly associated with in-hospital mortality: pneumococcal etiology, female sex, age, ICU admission, SOFA score, septic shock, MODS, and D-dimer levels. At multivariable analysis D-dimer showed an effect only in *N. meningitidis* subgroup: as 500 ng/mL of D-dimer increased, the probability of unfavorable outcome increased on average by 4%. Median D-dimer was significantly higher in *N. meningitidis* than in *S. pneumoniae* patients (1,314 vs. 1,055 ng/mL, $p = 0.009$). For *N. meningitidis* in-hospital mortality was 0% for D-dimer <500 ng/mL, very low (3.5%) for D-dimer <7,000 ng/mL, and increased to 26.1% for

D-dimer >7,000 ng/mL. Kaplan-Meier analysis of in-hospital mortality showed for *N. meningitidis* infections a statistically significant difference for D-dimer >7,000 ng/mL compared to values <500 ng/mL ($p = 0.021$) and 500–3,000 ng/mL ($p = 0.002$). For *S. pneumoniae* the mortality risk resulted always high, over 10%, irrespective by D-dimer values. In conclusion, D-dimer is rapid to be obtained, at low cost and available everywhere, and can help stratify the risk of in-hospital mortality and complications in patients with invasive infections due to *N. meningitidis*: D-dimer <500 ng/mL excludes any further complications, and a cut-off of 7,000 ng/mL seems able to predict a significantly increased mortality risk from much <10% to over 25%.

Keywords: D-dimer, biomarker, sepsis, meningitis, *Streptococcus pneumoniae*, *Neisseria meningitidis*, mortality

INTRODUCTION

Sepsis is a heterogeneous syndrome defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection, associated with an in-hospital mortality >10% (1, 2). The role of biomarkers [biological observations that substitute for and ideally predict a clinically relevant endpoint (3)] for diagnostic and prognostic assessment in case of sepsis has been extensively investigated in literature, but to date no current clinical measure seems able to reflect the concept of "dysregulated host response" (1); therefore, identifying a biomarker reflective of host-response interaction would be of great interest.

It has been known for several years that coagulation plays a pivotal role in the physiological host response to pathogens: immunothrombosis, mediated by immune cells and by specific thrombosis-related molecules, leads to the generation of a localized intravascular scaffold that facilitates the recognition, containment and killing of pathogens, limiting their diffusion through the circulatory system, thus protecting host integrity and limiting major organ damage (4): the loss of the physiologically localized activation of coagulation, the signature feature of disseminated intravascular coagulation (DIC), is a hallmark of sepsis and invasive infections (4). In general, during a systemic infection, both extrinsic and contact coagulation pathways are activated, the first mainly triggered by the intravascular tissue factor (TF) expressed on the monocytes and extracellular microparticles (MPs) surfaces, the second by the cell-free DNA associated with neutrophil extracellular traps (NETs) and by the phosphatidylserine residues present in various cell membranes (4). Sepsis-associated coagulopathy is characterized by concomitant activation of coagulation, down-regulation of physiological anticoagulants, and inhibition of fibrinolysis (5), finally leading to the generation of a variable amount of fibrin-related markers, such as the D-dimer.

Several biomarkers indicating a strong fibrinolytic shutdown, including low D-dimer levels, were found to be associated with a reduced survival in patients with sepsis (6); recently a very high mortality among the few sepsis patients having normal D-dimer levels (<500 ng/mL) was once again reported (7). On the contrary, other studies showed that the mortality is effectively predicted by high levels, with D-dimer ≥4,200 ng/mL effective in identifying the patients with infective endocarditis (IE) (8) and ≥4,000 ng/mL for those with bloodstream infections (BSI) (9); moreover, a linear relationship between high D-dimer levels and mortality in sepsis patients admitted to the emergency department has been reported (10). On-admission D-dimer levels ≥2,000 ng/mL were able to effectively predict in-hospital mortality also among Covid-19 patients (11).

D-dimer seems to be therefore an interesting biomarker in predicting prognosis in different models of infection, able to reflect the synthesis of the complex balance between the pro- and anti-thrombotic and the pro- and anti-fibrinolytic drive occurring during sepsis-related coagulopathy. However, the dysregulated host response occurring during an invasive infection depends on the specific pathogen involved (4, 12); it is plausible to hypothesize that the levels of a coagulative biomarker, and the related thresholds, should be assessed and differently interpreted for each pathogen. Infections due to *Streptococcus pneumoniae* and *Neisseria meningitidis* represent two excellent models of severe invasive infections due, respectively, to Gram-positive and Gram-negative cocci whose pathogenesis is usually characterized by an extensive coagulopathy and sometimes overt-DIC.

The aim of this study is to evaluate in a large cohort of patients with meningitis and/or BSI due to *S. pneumoniae* or *N. meningitidis* the main clinical and laboratory characteristics and variables associated with in-hospital mortality and complications, with a particular focus on the role of D-dimer as biomarker for early prediction of clinically relevant outcomes.

MATERIALS AND METHODS

Study Design, Setting, and Subjects

A retrospective study was conducted from November 2012 to May 2019 (79 months), enrolling all consecutive patients admitted for meningitis and/or BSI due to *S. pneumoniae* or *N. meningitidis* to the First Division of Infectious Diseases (FDID) of Cotugno Hospital, in Naples, Italy, a referral tertiary hospital covering an area (Campania region) with 5,800,000 inhabitants. This division has 25 beds dedicated to the management of invasive bacterial infections and other infectious diseases.

All patients enrolled in this study underwent blood cultures and, if not contraindicated, lumbar puncture.

In each patient, a complete blood analysis, including assessment of D-dimer, was performed within the first 24 h after admission. Patients for which the main laboratory and clinical data and primary clinical outcome were available were enrolled for the final analysis. No exclusion criteria were applied regarding the age of patients and the severity of clinical condition.

We assessed in each patient the presence of purpura with onset within 24 h after admission.

In each patient, within the first 24 h after admission, the ISTH overt-DIC score (13, 14), the SIC score (15), the Sequential Organ Failure Assessment (SOFA) score, were calculated according to current definitions, and multiple organ dysfunction syndrome (MODS) and septic shock (1) status was assessed.

Data Collection

The computerized database of the FDID was searched to identify all consecutive inpatients with meningitis and/or BSI due to *S. pneumoniae* or *N. meningitidis*, diagnosed between November 2012 and May 2019.

Data were obtained by electronic clinical chart review and interrogation of laboratory information systems and collected in a dedicated case record form.

Clinical information, including demographics, antimicrobial therapy, and laboratory data were retrieved from medical charts.

Clinical outcomes were retrieved from the electronic clinical chart related to the index hospitalization.

This study was exempt from institutional review board oversight because of its retrospective nature and the anonymity of pooled data.

Definitions

Sepsis and septic shock were defined according to the sepsis-3 definition (1).

Multiple organ dysfunction syndrome (MODS) was defined as the presence of altered function involving at least two organ systems in an acutely ill patient such that homeostasis cannot be maintained without intervention.

Purpura was defined as petechial rash rapidly spreading in extent and depth, evolving into skin necrosis.

Antibiotic therapy was defined as adequate if the isolated pathogen resulted susceptible to the tested antibiotic according to EUCAST clinical breakpoints effective during the study period.

Clinical Outcomes

The primary outcome was in-hospital mortality.

The secondary outcome was a composite of in-hospital mortality, amputations, hearing loss, neurological complications (stroke, transitory ischemic attack, brain abscess, epilepsy).

Coagulation Testing

Prothrombin time/INR, fibrinogen and D-dimer were measured using the ACL TOP analyzer (Instrumentation Laboratory-IL, Werfen Group).

Q.F.A. Thrombin (Bovine) kit was used for the quantitative determination of fibrinogen, based on the Clauss method, in human citrated plasma on the IL Coagulation Systems.

The quantitative determination of D-dimer in human citrated plasma was determined by the automated latex enhanced immunoassay HemosIL D-Dimer HS (0020007700).

D-dimer classes were divided as follows: <500, 500–3,000, 3,000–7,000, and >7,000 ng/mL. The cut-off values of 3,000 and 7,000 ng/mL were chosen according to the proposal of ISTH about the proper values for moderate (2 points) and severe (3 points) increase in the ISTH overt-DIC score (14).

Microbiology Laboratory Methods

Blood cultures were inoculated in BD BACTEC™ blood culture bottles (Becton, Dickinson and Company, NJ, U.S.) and monitored using BD BACTEC™ FX instrument for up to 5 days. Positive cultures were sub-cultured and identified to the species level by Vitek®MS or Vitek®2 (BioMérieux; Marcy-l'Étoile, France).

Cerebrospinal fluid (CSF) was first examined by a Gram-stained smear, and the appropriate culture media was inoculated. Blood agar and chocolate agar plates should be incubated at 35°C in an atmosphere enriched with carbon dioxide. CSF was cultured and identified to the species level by Vitek®MS or Vitek®2 (BioMérieux; Marcy-l'Étoile, France).

Susceptibility testing was performed using Vitek®2 system and interpreted according to EUCAST criteria effective during the study period.

Polymerase chain reaction (PCR) for the detection of meningococcal and pneumococcal DNA on blood specimens, antigen detection on CSF and Biofire® FilmArray™ meningitis/encephalitis (ME) panel (a multiplex PCR assay which can detect the most commonly identified pathogens in central nervous system infections) were performed when adequate and/or available during the study period.

Statistical Analysis

Descriptive analysis of the data was carried out using mean values and standard deviation (SD) or median values and interquartile range (IQR) for the quantitative variables, as appropriate, and percentage values for the qualitative ones. Normality of the variables was assessed with the Shapiro-Wilk-test. Comparisons between groups were performed with unpaired two-tailed *t*-test, Mann-Whitney-test or chi-squared-test with continuity correction, as appropriate.

The data related to in-hospital mortality was analyzed with Kaplan-Meier curves.

The association with the outcome of each considered predictor was investigated with univariable logistic regression. Predictors with a p-value <0.20 at the univariable logistic regression were considered for multivariable analysis.

The variable "Interaction" is "D-dimer-*N. meningitidis*," and represents the D-dimer in this specific subgroup; this explicitly entered the analysis because it expresses the main hypothesis of the work. For the D-dimer odds ratio (OR), an increase of 500 ng/mL was considered.

Multivariable logistic regression analysis was performed with Bayesian model averaging (BMA) (16) to address model uncertainty, producing a posterior probability for each possible model and predictor. As a result of multivariable analysis, in

addition to OR, the probability that the single variable has a non-zero effect in the final multivariable model [posterior probability, $p\ (b \neq 0)$] was reported. The variables with $p\ (b \neq 0) > 0.80$ were selected.

A p-value of <0.05 was considered statistically significant.

Analyses were performed using the *R* open-source statistical software and the SPSS statistical package (version 23 for Windows. SPSS, Inc. Chicago, Ill).

RESULTS

Patients' General Characteristics

During the 79 months study period, there have been 3,130 admissions in the FDID of Cotugno Hospital. We retrospectively included in the final analysis 162 patients with invasive infections due to *S. pneumoniae* (male: 48.8%; mean age: 45 years, SD 26; range: 0–89 years), and 108 patients with invasive infections due to *N. meningitidis* (male: 55.6%; mean age: 22 years, SD 22, range: 0–90 years), for a total of 270 cases. Only 3 patients out of 273 cases initially retrieved were excluded, 2 because missing data about D-dimer levels and 1 for incomplete data about several clinical characteristics.

Median age was significantly higher in *S. pneumoniae* than in *N. meningitidis* patients (53.5 years, IQR 21–67, vs. 15 years, IQR 3–39, $p < 0.001$).

Regarding 162 invasive infections due to *S. pneumoniae*, 132 patients had meningitis (86/132, 65.2%, with concomitant BSI), and 30 had isolated BSI. Regarding 108 invasive infections due to *N. meningitidis*, 90 patients had meningitis (66/90, 73.3%, with concomitant BSI), and 18 had isolated BSI.

Table 1 shows the results of the analysis of variables for different etiologies.

All patients (100%) received an adequate empiric antibiotic treatment before blood and CSF culture results and then an adequate targeted therapy; 96.7% were treated with corticosteroids.

The median in-hospital length of stay of overall population was 17 days (range 0–129 days).

TABLE 1 | Analysis of variables for different etiologies.

		***S. pneumoniae* (*n* = 162)**	***N. meningitidis* (*n* = 108)**	***p*-value**
Males, *n* (%)		79 (48.8%)	60 (55.6%)	0.274
Age (years), median (IQR)		53.5 (21.0–67.0)	15.0 (3.0–39.0)	**<0.001**
SOFA, median (IQR)		3.0 (2.2–6.0)	3.0 (2.0–5.0)	0.465
PLT ($\times 10^9$/mm^3), median (IQR)		195.5 (145.0–260.0)	167.0 (129.0–232.0)	**0.019**
Purpura, *n* (%)		14 (8.6%)	62 (57.4%)	**<0.001**
Splenectomy, *n* (%)		11 (6.8%)	1 (0.9%)	**0.031**
BSI isolated, *n* (%)		30 (18.5%)	18 (16.7%)	0.776
Meningitis isolated, *n* (%)		46 (28.6%)	24 (22.2%)	0.245
Meningitis + BSI, *n* (%)		86 (53.1%)	66 (61.1%)	0.122
D-dimer classes (ng/mL)	<500	35 (21.6%)	18 (16.7%)	**0.002**
	500–3,000	101 (62.3%)	52 (48.1%)	
	3,000–7,000	15 (9.3%)	15 (13.9%)	
	>7,000	11 (6.8%)	23 (21.3%)	
D-Dimer >3,000 ng/mL, *n* (%)		136 (84.0%)	70 (64.8%)	**<0.001**
D-Dimer >7,000 ng/mL, *n* (%)		11 (6.8%)	23 (21.3%)	**<0.001**
Fibrinogen (mg/dL), median (IQR)		619.5 (469.0–777.0)	574.5 (427.0–720.0)	0.114
D-dimer (ng/mL), median (IQR)		1055.0 (585.0–2239.0)	1314.0 (706.0–4223.0)	**0.009**
ISTH score positive, *n* (%)		4 (2.7%)	20 (20.6%)	**<0.001**
mISTH score positive, *n* (%)		13 (8.0%)	34 (32.1%)	**<0.001**
SIC score positive, *n* (%)		61 (37.9%)	63 (58.9%)	**0.001**
ISTH score, median (IQR)		1.0 (1.0–2.0)	2.0 (1.0–4.0)	**<0.001**
mISTH score, median (IQR)		1.0 (1.0–2.0)	2.0 (1.0–4.0)	**<0.001**
SIC score, median (IQR)		3.0 (2.0–4.0)	4.0 (3.0–4.0)	**0.003**
In-hospital mortality, *n* (%)		31 (19.1%)	9 (8.3%)	**0.014**
Composite of mortality or complications, *n* (%)		75 (46.3%)	20 (18.5%)	**<0.001**
Septic shock, *n* (%)		48 (29.6%)	40 (37.0%)	0.229
MODS, *n* (%)		57 (35.2%)	28 (25.9%)	0.101
ICU admission, *n* (%)		78 (48.1%)	65 (60.2%)	0.052

In all patients (pts), the ISTH overt-DIC score and the SIC score were calculated according to definitions (13–15) within the first 24 h after admission. The cut-off of ISTH overt-DIC score is 5 (13). The cut-off of modified ISTH (mISTH) overt-DIC score (without fibrinogen) is 4 (17). The cut-off of SIC score is 4 (15). Bold text indicates statistically significant difference ($p < 0.05$).

TABLE 2 | Univariable and multivariable logistic regression analysis (Bayesian model averaging).

	Univariable logistic regression		Bayesian model averaging	
	OR (95% CI)	***p*-value**	**OR (95% CI)**	***p* ($b \neq 0$)**
N. meningitidis-S. pneumoniae	0.250 (0.133–0.452)	<0.001	0.117 (0.111–0.124)	1.000
Fibrinogen	0.999 (0.998–1.000)	0.483	–	–
D-dimer (×500 ng/mL)	1.025 (1.007–1.047)	0.010	1.004 (1.003–1.006)	0.157
Male sex	0.533 (0.312–0.904)	0.020	0.886 (0.855–0.918)	0.206
Age	1.022 (1.011–1.033)	<0.001	1.001 (1.000–1.002)	0.071
ICU admission	2.587 (1.499–4.549)	<0.001	1.241 (1.175–1.310)	0.254
SOFA	1.221 (1.118–1.342)	<0.001	1.185 (1.174–1.956)	0.926
Platelet count	1.001 (0.998–1.003)	0.686	–	–
Splenectomy	0.968 (0.247–3.302)	0.959	–	–
Septic shock	2.808 (1.621–4.907)	<0.001	1.583 (1.486–1.687)	0.533
MODS	3.519 (2.012–6.234)	<0.001	1.003 (0.992–1.015)	0.030
Purpura	0.705 (0.385–1.261)	0.245	–	–
BSI isolated	0.628 (0.293–1.274)	0.211	–	–
Meningitis isolated	0.862 (0.458–1.587)	0.638	–	–
Meningitis + BSI	1.581 (0.927–2.725)	0.095	1.003 (0.996–1.010)	0.025
ISTH score	1.034 (0.882–1.208)	0.678	–	–
SIC score	1.224 (0.971–1.559)	0.092	1.010 (1.003–1.017)	0.054
Interaction (×500 D-dimer)	1.014 (0.996–1.027)	0.140	1.041 (1.038–1.045)	0.849

The variables that are selected in the final multivariable model are N. meningitidis–S. pneumoniae, SOFA and D-dimer-N. meningitidis. At multivariable analysis, the D-dimer has no effect on the whole population, but only on the subgroup of patients with N. meningitidis. As 500 ng/mL of D-dimer increase, the probability of a negative outcome increases on average by 4%.

TABLE 3 | *Streptococcus pneumoniae*.

	Survivors ($n = 131$)	**Deaths ($n = 31$)**	***p*-value**
Males, *n* (%)	64 (48.9%)	15 (48.4%)	0.963
Septic shock, *n* (%)	22 (16.8%)	26 (83.9%)	**<0.001**
MODS, *n* (%)	31 (23.7%)	26 (83.9%)	**<0.001**
Purpura, *n* (%)	7 (5.3%)	7 (24.1%)	**0.004**
BSI isolated, *n* (%)	29 (22.1%)	1 (3.3%)	**0.020**
Meningitis isolated, *n* (%)	43 (32.8%)	3 (10.0%)	**0.013**
Meningitis + BSI, *n* (%)	59 (45.0%)	27 (87.1%)	**<0.001**
Fibrinogen (mg/dL), median (IQR)	621.0 (458.0–777.0)	590.0 (500.0–780.0)	0.766
D-dimer (ng/mL), median (IQR)	974.0 (513.0–1652.0)	1578.0 (944.0–2872.0)	**0.009**
Age, years, median (IQR)	47.0 (13.0–65.0)	63.0 (49.0–48.0)	**0.002**
SOFA, median (IQR)	3.0 (2.0–5.0)	7 (4.0–8.0)	**<0.001**
PLT ($\times 10^9$), median (IQR)	200.0 (153.0–264.0)	169.0 (128.0–242.0)	0.085
ISTH score, median (IQR)	1.0 (1.0–2.0)	1.0 (1.0–3.0)	0.678
mISTH score, median [IQR]	1.0 [1.0 – 2.0]	1.0 [1.0 – 3.0]	0.550
SIC score, median (IQR)	3.0 (2.0–4.0)	3.5 (3.0–5.0)	**0.021**

Association between variables and in-hospital mortality. Bold text indicates statistically significant difference ($p < 0.05$).

143/270 (53%) patients needed admission in intensive care unit (ICU) during the hospital stay.

SOFA Score

48/270 patients (17.8%) had SOFA score<2 (absence of criteria for sepsis diagnosis): none of these died. No statistically significant difference ($p = 0.465$) was found for median SOFA scores between *S. pneumoniae* (3, IQR 2–6) and *N. meningitidis* (3, IQR 2–5) groups (**Table 1**).

For *S. pneumoniae* infections, the median SOFA was 7 (IQR 4–8) in non-survivors vs. 3 (IQR 2–5) in survivors ($p < 0.001$; **Table 3**), while for *N. meningitidis* infections it was 6.5 (IQR 4–9.5) vs. 3 (IQR 2–5) ($p = 0.010$; **Table 4**).

At the univariable and multivariable logistic regression analysis (**Table 2**), the SOFA score was significantly associated with in-hospital mortality in the overall population.

An acute change in total SOFA score ≥2 points, defining the sepsis-related organ dysfunction according to the sepsis-3

TABLE 4 | *Neisseria meningitidis*.

	Survivors ($n = 99$)	Deaths ($n = 9$)	p-value
Males, *n* (%)	55 (55.6%)	5 (55.6%)	1.000
Septic shock, *n* (%)	32 (32.3%)	8 (88.9%)	**0.001**
MODS, *n* (%)	21 (21.2%)	7 (77.8%)	**0.001**
Purpura, *n* (%)	57 (58.2%)	5 (62.5%)	1.000
BSI isolated, *n* (%)	16 (16.2%)	2 (22.2%)	0.643
Meningitis isolated, *n* (%)	23 (23.2%)	1 (11.1%)	0.680
Meningitis + BSI, *n* (%)	60 (60.6%)	6 (66.7%)	1.000
Fibrinogen (mg/dL), median (IQR)	576.0 (437.0–720.0)	233.0 (96.0–720.0)	0.233
D-dimer (ng/mL), median (IQR)	1140.0 (688.0–3543.0)	8051.0 (3859.0–40601.0)	0.001
Age, years, median (IQR)	15.0 (3.5–34.0)	5.0 (1.5–48.0)	0.739
SOFA, median (IQR)	3.0 (2.0–5.0)	6.5 (4.0–9.5)	**0.010**
PLT ($\times 10^9$), median (IQR)	170.0 (129.0–232.0)	155.0 (131.0–175.0)	0.405
ISTH score, median (IQR)	2.0 (1.0–4.0)	5.0 (3.0–5.0)	**0.035**
mISTH score, median (IQR)	2.0 (1.0–4.0)	5.0 (3.0–5.0)	**0.041**
SIC score, median (IQR)	4.0 (3.0–4.0)	4. 0 (3.5–5.0)	0.090

Association between variables and in-hospital mortality. Bold text indicates statistically significant difference ($p < 0.05$).

criteria, was present in 136 (84.0%) *S. pneumoniae* patients and in 86 (79.6%) *N. meningitidis* patients: the difference was not statistically significant.

Invasive Infection Type

At the univariable and multivariable logistic regression analysis (Bayesian model averaging), the type of invasive infection (meningitis with BSI, meningitis without BSI, isolated BSI) was not found to be a variable significantly associated with in-hospital mortality (**Table 2**).

Previous Splenectomy and Purpura at Admission

More patients with invasive infections due to *S. pneumoniae* than *N. meningitidis* previously underwent splenectomy (6.8 vs. 0.9%, $p = 0.031$), but at the univariable logistic regression analysis, a history of splenectomy was not found to be a variable significantly associated with in-hospital mortality (**Table 2**).

8.6% of *S. pneumoniae* patients had purpura at admission: 50% of these previously underwent splenectomy; out of the total of 11 splenectomized patients, 7 (63.6%) presented with purpura.

57.4% of *N. meningitidis* patients had purpura at admission: only 1 patient (1.6%) previously underwent splenectomy: in this patient D-dimer levels were <500 ng/mL.

Therefore, significantly more patients with invasive infections due to *N. meningitidis* compared to *S. pneumoniae* early presented purpura (57.4 vs. 8.6%, $p < 0.001$). Purpura was significantly more frequent in *N. meningitidis* than in *S. pneumoniae* patients regardless of D-dimer classes, and, independently by etiology, purpura prevalence was not significantly different between the different D-dimer classes. At the univariable logistic regression analysis (**Table 2**), the presence of purpura was not a variable significantly associated with in-hospital mortality.

D-Dimer

Data analysis for continuous variables (**Table 1**) showed that D-dimer levels were significantly higher in *N. meningitidis* than *S. pneumoniae* patients: median 1,314 ng/mL (IQR 706–4,223) vs. 1,055 ng/mL (IQR 585–2,239), $p = 0.009$.

35/162 (21.6%) *S. pneumoniae* patients and 18/108 (16.7%) *N. meningitidis* patients had D-dimer <500 mg/dL.

101/162 (62.3%) of *S. pneumoniae* patients and 52/108 (48.1%) of *N. meningitidis* patients had D-dimer 500–3,000 ng/mL.

15/162 (9.3%) of *S. pneumoniae* patients and 15/108 (13.9%) *N. meningitidis* patients had D-dimer 3,000–7,000 ng/mL.

11/162 (6.8%) of *S. pneumoniae* patients and 23/108 (21.3%) *N. meningitidis* patients had D-dimer >7,000 ng/mL.

Most patients thus had D-dimer levels belonging to the class 500–3,000 ng/mL.

Fibrinogen

Data analysis for continuous variables (**Table 1**) showed that fibrinogen levels were not significantly different between *S. pneumoniae* and *N. meningitidis* patients: median 619.5 mg/dL (IQR 469.0–777.0) vs. 574.5 mg/dL (IQR 427.0–720.0), $p = 0.114$.

At the logistic univariable regression analysis (**Table 2**) fibrinogen was not significantly associated with in-hospital mortality.

Only 4/249 (1.6%) patients, all presenting *N. meningitidis* BSI (1 isolated and 3 with meningitis), had fibrinogen levels <100 mg/dL; 3 of these 4 patients had shock and MODS, and 2 of these 3 died. Also elevating the cut-off to 200 mg/dL, we retrieved only 6 *N. meningitidis* and 3 *S. pneumoniae* patients with reduced levels: 7 of these 9 patients had shock and/or MODS and 4 out of these 7 patients ultimately died (mortality rate: 44.4%).

In definitive, only 9/249 patients (3.6%) had fibrinogen levels reduced under the normal value of 200 mg/dL.

MODS

MODS occurred in 35.2% of *S. pneumoniae* patients and in 25.9% of *N. meningitidis* patients, without statistically significant difference ($p = 0.101$; **Tables 1**, **5**). No significant differences were observed within each single D-dimer class.

Streptococcus pneumoniae patients with D-dimer levels <500 and 500–3,000 ng/mL showed similar rates of early presentation of MODS (31.4 and 29.7%, respectively), and the percentages were about the same also comparing the classes 3,000–7,000 ng/mL and over 7,000 ng/mL (60 and 63.6%, respectively, **Table 5**); for D-dimer levels >3,000 ng/mL a statistically significant ($p = 0.001$) two-fold increase of occurrence of MODS was observed.

In *N. meningitidis* patients, the percentage of MODS progressively and significantly increased as D-dimer increased (**Table 5**), from 11.1% for values <500 ng/mL to 52.2% for values >7,000 ng/mL ($p = 0.007$); when D-dimer exceeded 7,000 ng/mL the rate of MODS significantly increased compared to lower values ($p = 0.001$).

Of the *S. pneumoniae* patients who died, 83.9% had MODS, while this percentage was only 23.7% among patients who survived: this difference was significant ($p < 0.001$). For *N. meningitidis* patients the corresponding percentages were 77.8 and 21.2%, and the difference was still significant ($p = 0.001$; **Tables 3**, **4**). At the univariable logistic regression analysis, MODS was found to be a variable significantly ($p < 0.001$) associated with in-hospital mortality (**Table 2**).

Septic Shock

The occurrence of septic shock was not significantly different between *S. pneumoniae* and *N. meningitidis* patients (29.6 vs. 37%, $p = 0.229$) (**Table 1**), and was very high regardless of D-dimer class (**Table 5**).

Of the *S. pneumoniae* patients who died, 83.9% had septic shock, while this percentage was only 16.8% among patients who survived: this difference was significant ($p < 0.001$). For *N. meningitidis* patients the corresponding percentages were 88.9 and 32.3%, and the difference was still significant ($p = 0.001$; **Tables 3**, **4**). At the univariable logistic regression analysis, septic shock was found to be significantly ($p < 0.001$) associated with in-hospital mortality (**Table 2**).

About 33% of *N. meningitidis* patients with D-dimer levels <7,000 ng/mL and 52.2% of those with D-dimer levels >7,000 ng/mL early presented septic shock, without significant differences between every D-dimer classes ($p = 0.411$); also comparing only D-dimer classes under and over 7,000 ng/mL the difference was still not significant ($p = 0.090$).

In-hospital Mortality (Primary Outcome)

In-hospital mortality was 14.8% (40/270 patients) for the overall population, and was significantly higher in *S. pneumoniae* than in *N. meningitidis* patients: 19.1 vs. 8.3%, respectively ($p = 0.014$; **Table 1**).

At the univariable logistic regression analysis, the type of invasive infection (meningitis with BSI, meningitis without BSI,

TABLE 5 | Clinical status and outcomes in 270 patients with invasive bacterial infections by D-dimer class.

D-dimer class (ng/mL)	*S. pneumoniae* (*n* = 162)					*N. meningitidis* (*n* = 108)				
	In-hospital mortality	Mortality + complications	MODS	Septic shock	Purpura	In-hospital mortality	Mortality + complications	MODS	Septic shock	Purpura
<500	4/35 (11.4%)	12/35 (34.3%)*	11/35 (31.4%)	7/35 (20.0%)	2/35 (5.7%)*	0/18 (0%)	0/18 (0%)*	2/18 (11.1%)	6/18 (33.3%)	8/18 (44.4%)*
500–3,000	20/101 (19.8%)*	50/101 (49.5%)*	30/101 (29.7%)	29/101 (28.7%)	9/101 (9.0%)*	2/52 (3.8%)*	9/52 (17.3%)*	9/52 (17.3%)	17/52 (32.7%)	27/52 (51.9%)*
3,000–7,000	4/15 (26.7%)	6/15 (40.0%)	9/15 (60.0%)	7/15 (46.7%)	1/15 (7.1%)*	1/15 (6.7%)	3/15 (20.0%)	5/15 (33.3%)	5/15 (33.3%)	10/15 (66.7%)*
>7,000	3/11 (27.3%)	7/11 (63.6%)	7/11 (63.6%)	5/11 (45.5%)	2/11 (18.2%)*	6/23 (26.1%)	8/23 (34.8%)	12/23 (52.2%)	12/23 (52.2%)	17/23 (81.0%)*
Total	31/162 (19.1%)*	75/162 (46.3%)*	57/162 (35.2%)	48/162 (29.6%)	14/162 (8.7%)*	9/108 (8.3%)	20/108 (18.5%)	28/108 (25.9%)	40/108 (37.0%)	62/108 (57.4%)*

*Statistically significant differences ($p < 0.05$) comparing *S. pneumoniae* vs. *N. meningitidis*.

isolated BSI) was not significantly associated with in-hospital mortality, and this data is conformed to the Bayesian model averaging (**Table 2**).

At the univariable logistic regression analysis (**Table 2**), ICU admission was found to be significantly associated with in-hospital mortality.

The median survival time in patients who died was 1 day (range 0–18 days) in the *N. meningitidis* group and 21 days (range 0–129 days) in the *S. pneumoniae* group.

At the analysis of the association between continuous variables and outcomes (**Tables 3**, **4**), median D-dimer levels were significantly higher in non-survivors than in survivors, both for *S. pneumoniae* (1,578 vs. 974 ng/mL, $p = 0.009$) and *N. meningitidis* (8,051 vs. 1,140 ng/mL, $p < 0.001$).

In-hospital mortality gradually increased as D-dimer values increased, but the overall trend was significant for *N. meningitidis* group ($p = 0.010$) and non-significant for *S. pneumoniae* ($p = 0.420$).

At the univariable logistic regression analysis (**Table 2**), the following variables were significantly associated with in-hospital mortality: pneumococcal etiology ($p < 0.001$), female sex ($p = 0.020$), age ($p < 0.001$), ICU admission ($p < 0.001$), SOFA score ($p < 0.001$), septic shock ($p < 0.001$), MODS ($p < 0.001$), and D-dimer levels ($p = 0.010$).

For odds ratio (OR) of D-dimer, we considered an increase of 500 ng/mL. At multivariable analysis, the D-dimer did not show an effect on the whole population, but only on the group of *N. meningitidis* patients: in this group, as 500 ng/mL of D-dimer increased, the probability of unfavorable outcome increased on average by 4%.

For *N. meningitidis* the in-hospital mortality was 0% for D-dimer levels <500 ng/mL, very low (3.5%) for D-dimer levels <7,000 ng/mL, and increased to 26.1% for D-dimer exceeding the cut-off of 7,000 ng/mL (**Table 5**). Differences between classes were significant ($p = 0.010$), as well as between values under and above 7,000 ng/mL ($p = 0.003$).

Kaplan-Meier analysis of in-hospital mortality showed for *N. meningitidis* subjects a statistically significant difference for D-dimer levels >7,000 ng/mL compared to values <500 ng/mL ($p = 0.021$) and to values 500–3,000 ng/mL ($p = 0.002$; **Figure 1**).

For *S. pneumoniae* the trend of the in-hospital mortality increasing as D-dimer increased was not statistically significant ($p = 0.420$), and the mortality was very high (11.4%) also in case of normal D-dimer levels <500 ng/mL (**Table 5**).

The mortality related to *S. pneumoniae* infections was always higher than *N. meningitidis* (**Table 5**), and was statistically significant in the D-dimer class of 500–3,000 ng/mL, representative of most population (19.8 vs. 3.8%, $p = 0.008$); for classes <500 and 3,000–7,000 ng/mL the overall trend was confirmed but without reaching the statistical significance (11.4 vs. 0%, $p = 0.287$, and 26.7 vs. 6.7%, $p = 0.330$), and for D-dimer values over 7,000 ng/mL the mortality was not different (27.3 vs. 26.1%, $p = 1.000$).

Among patients with purpura the in-hospital mortality was 50% in case of *S. pneumoniae* infections and 8.1% for *N. meningitidis*. Purpura did not correlate with in-hospital mortality in *N. meningitidis* patients ($p = 1.000$), but was significantly more frequent in non-survivors from invasive infections due to *S. pneumoniae* (24.1 vs. 5.3%, $p = 0.004$) (**Tables 3**, **4**). At the univariable logistic regression analysis (**Table 2**), the

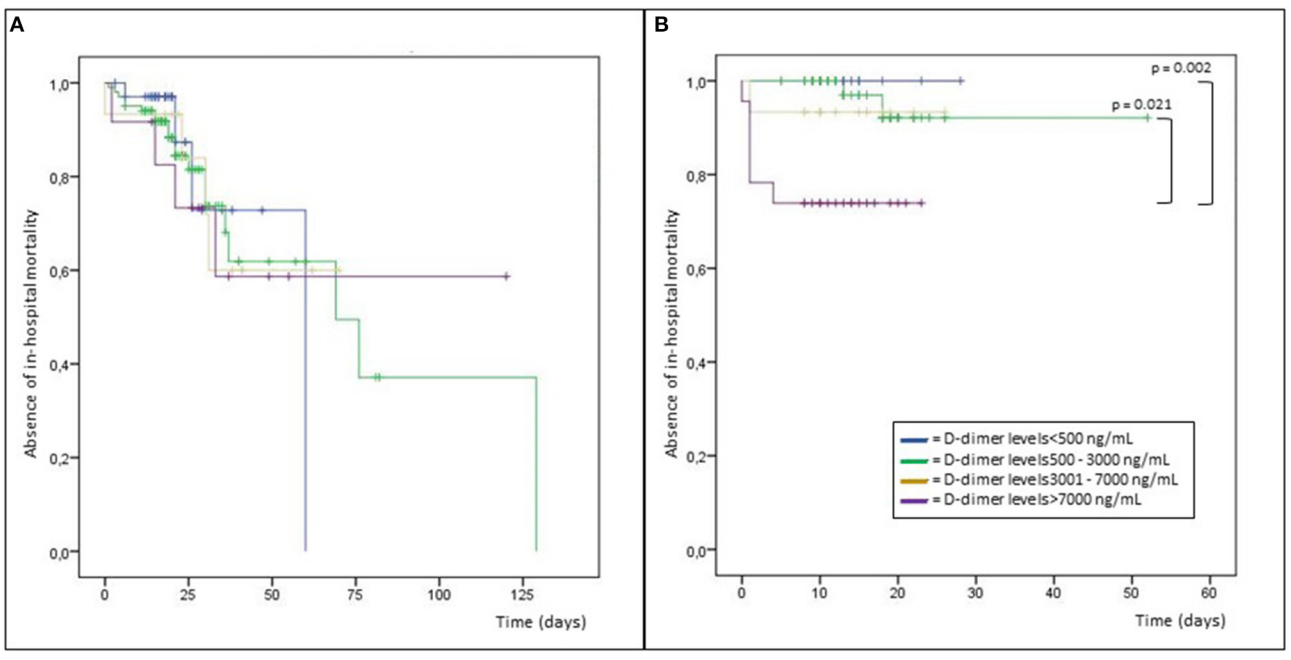


FIGURE 1 | Kaplan-Meier analysis of in-hospital mortality in patients with infections due to *Streptococcus pneumoniae* **(A)** and *Neisseria meningitidis* **(B)**.

presence of purpura was not significantly associated with in-hospital mortality.

Finally, we investigated the ability of D-dimer in predicting in-hospital mortality in the subgroup of patients early presenting with purpura. The rate of in-hospital mortality was 0% (0/45) in *N. meningitidis* patients with purpura and D-dimer levels <7,000 ng/mL and 29.4% (5/17) when D-dimer levels exceeded 7,000 ng/mL: this difference was statistically significant ($p = 0.001$).

The rate of in-hospital mortality was 0% (0/3) also in *S. pneumoniae* patients with purpura and D-dimer levels >3,000 ng/mL vs. 63.6% (7/11) among patients with D-dimer levels <3,000 ng/mL, but this difference was not significant ($p = 0.192$).

Composite Outcome of In-hospital Mortality or Complications

The composite outcome of in-hospital mortality or complications was 35.2% (95/270 patients) for the overall population, occurring more frequently in patients with invasive infections due to *S. pneumoniae* than in those with *N. meningitidis* (46.3 vs. 18.5%, $p < 0.001$) (**Table 1**).

For D-dimer levels <500 and 500–3,000 ng/mL, this outcome occurred significantly more frequently in *S. pneumoniae* patients than those with infections due to *N. meningitidis* (34.3 vs. 0%, $p = 0.004$, and 49.5 vs. 17.3%, $p < 0.001$, respectively), while the trend was still evident but not statistically significant for classes 3,000–7,000 and >7,000 ng/mL (40 vs. 20%, $p = 0.427$, and 63.6 vs. 34.8%, $p = 0.151$, respectively) (**Table 5**).

Among *N. meningitidis* patients there were no complications when D-dimer values were <500 mg/dL, but the risk significantly increased for higher values (17.3% for 500–3,000 ng/mL, 20% for 3,000–7,000 ng/mL and 34.8% for >7,000 ng/mL, $p = 0.029$). In *S. pneumoniae* patients, this composite outcome occurred instead very frequently independently by D-dimer classes (ranging from 34.3 to 63.6%, $p = 0.246$) (**Table 5**).

In case of purpura, among *S. pneumoniae* patients this composite outcome was met in 10/14 patients (71.4%), in *N. meningitidis* patients in 19.4% (12/62).

Finally, we conducted a sub-analysis about the predictive ability of D-dimer in the subgroup of patients with purpura. This composite outcome occurred in 11.1% (5/45) of *N. meningitidis* patients with D-dimer levels <7,000 ng/mL and in 41.2% (7/17) in those with D-dimer levels >7,000 ng/mL: the difference was statistically significant ($p = 0.013$). In *S. pneumoniae* patients with D-dimer levels >3,000 ng/mL this outcome occurred in 33.3% (1/3), compared to 81.8% (9/11) in patients with D-dimer levels <3,000 ng/mL: this difference was not significant ($p = 0.176$).

DISCUSSION

Coagulopathy is crucially involved in the pathogenesis of sepsis-related dysregulated host response to infections: the differences between Gram-negative and Gram-positive bacteria, due to profound differences in their cell wall composition and the specificity of bacterial exotoxins production (18, 19), and the consequent interaction with the innate immune system, affect both the induction of immunothrombosis and the evolution of coagulopathy. The Pathogen-Associated Molecular Patterns (PAMPs) are indeed specific for different microorganisms and are basically involved in determining the coagulopathy-related organ damage: the lipopolysaccharide (LPS), one of the most important cell wall component of Gram-negative bacteria, is precociously recognized by innate immune system *via* toll-like receptor (TLR) 4, while the lipoteichoic acid, a cell wall component of Gram-positive bacteria, is mainly recognized by TLR2 (12); as differences in activation of these receptors can result in different production of inflammatory cytokines by the host, we should also expect a peculiar pattern of coagulopathy. During BSI due to *N. meningitidis*, besides the presence of elevated levels of circulating LPS, monocytes have been shown to express high level of functional TF, correlating with disease severity, and TF-bearing circulant MPs able to initiate the extrinsic coagulation pathway have been isolated in large amount (20). Moreover, human monocytes exposed to LPS produce both TF and plasminogen activator inhibitor (PAI)-2, both favoring fibrin deposition, but evidence showed that cellular production of these molecules may be uncoupled, since exposition to alloantigens leads to high levels of TF with no concomitant increase in PAI-2 activity (21). Hence, the procoagulant and the fibrinolytic pathways can be differently involved. Therefore, a great heterogeneity in the hemostatic aspects of the immune response and distinct patterns of coagulopathy in response to different microorganisms are expected, causing distinctive imbalance of the main determinants, namely the activation of coagulation, the down-regulation of physiological anticoagulants, and the inhibition of fibrinolysis (5).

Coagulation biomarkers could be useful to characterize the extent and/or type of coagulopathy occurring during sepsis and/or severe invasive infections due to different pathogens. In effect, thrombin-antithrombin complex, PAI-1 and D-dimer have been already proposed as potential biomarkers for the identification of the clinical phenotypes of sepsis, being significantly associated with the δ phenotype, which is characterized by a distinctive pattern of organ dysfunction and higher mortality (2); thereafter, such biomarkers might be useful in identifying those patients most likely to benefit from anti-inflammatory, anti-coagulant or immunomodulatory strategies (17).

Several coagulation factors could be measured, but screening all patients with an extensive panel of biomarkers would be expensive and dispersive; moreover, the complex balance between the pro- and anti-thrombotic and the pro- and anti-fibrinolytic drive occurring in response to different pathogens could be more simply synthetized by a molecule produced at the end of these interconnected cascades.

D-dimer satisfies several ideal criteria that a biomarker should have (3), such as the plausibility (the credible mechanism connecting the marker with the pathogenesis of the disease) and the coherence (the consistency of the association between the marker and the natural history of the disease). A biomarker should ideally be consistent, that is the association should

persist in different individuals, in different places, in different circumstances, and at different times (3). Semeraro et al. (6, 7) reported a higher mortality in sepsis patients with low D-dimer, while other studies conducted on sepsis (10), but also on IE (8), BSI (9), and COVID-19 (11) conversely reported a worse prognosis for patients with high D-dimer values: in our opinion, these opposite findings should be interpreted not as an example of inconsistency of D-dimer as biomarker, but as the expression of its specificity to be differently associated with specific conditions and specific pathogens. This aspect has not been adequately taken into account in previous clinical studies: Schwameis et al. (9) enrolled in the same case series BSI due to *S. aureus, E.coli,* and *P. aeruginosa*, and Semeraro et al. (6) conducted their investigation using the data of the ALBIOS study (22), in which over 40% of patients did not have a microbiological diagnosis, and for the remaining no bacterial species was specifically reported or specifically studied for the interaction between coagulative biomarkers and clinical outcomes.

In our study we have clearly distinguished invasive infections due to *S. pneumoniae* from those due to *N. meningitidis*, and this was a strength of our study. Another strength is to have specifically investigated the association with relevant clinical outcomes depending by D-dimer levels early assessed at admission to the hospital. Median D-dimer levels were significantly higher in *N. meningitidis* than in *S. pneumoniae* patients.

Our results do not confirm the findings of Semeraro et al. (6, 7) about a higher mortality for low D-dimer levels, and agree with those of Turak et al. (8) and Schwameis et al. (9): we found for *N. meningitidis* an in-hospital mortality varying from 0% for D-dimer levels <500 ng/mL to 26.1% for values exceeding the cut-off of 7,000 ng/mL, progressively increasing on average by 4% as 500 ng/mL of D-dimer increase. For *S. pneumoniae* the mortality was always very high, regardless of D-dimer levels, being already 11.4% for normal values. Similarly, for *N. meningitidis* the composite outcome of mortality or complications increased as D-dimer increased. The strength of the association with the outcome (3), which is a central feature for a good biomarker, was thus more robust for invasive infections due to *N. meningitidis*. The only subgroup of our cohort in which lower D-dimer levels seemed associate with worse outcomes was that of *S. pneumoniae* patients with purpura, showing an in-hospital mortality of 63.6% when D-dimer levels were <3,000 ng/mL vs. 0% when D-dimer was >3,000 ng/mL: anyway, this difference was not significant. It is possible that the number of the sample size does not allow to highlight significant differences, so further studies are warranted to verify this finding.

It is noteworthy to observe that there have been no complications among *N. meningitidis* patients with normal D-dimer values <500 ng/mL, while for *S. pneumoniae* the composite of in-hospital mortality or complications occurred already in about one third of patients with normal values: this percentage was reached in *N. meningitidis* patients only for values >7,000 ng/mL. Therefore, D-dimer reflects only in part the complexity of the dysregulated host response finally leading to organ dysfunction and death. Since coagulopathy is a central element in the dysregulated host response leading to organ dysfunction, we assessed the association between D-dimer and MODS. In *N. meningitidis* infections the rate of MODS progressively and significantly increased as D-dimer increased; in *S. pneumoniae* invasive infections, a D-dimer cut-off of 3,000 ng/mL seems identify those patients with a significant two-fold increase of occurrence of MODS.

In our study, the mortality related to *S. pneumoniae* infections was higher than *N. meningitidis* in all D-dimer classes. A higher mortality in *purpura fulminans* due to *S. pneumoniae* compared to other pathogens (mainly *N. meningitidis*) has been recently reported by Contou et al. (23) in a multicenter French retrospective study including 306 cases admitted in ICUs from 2000 to 2016; the reason of a significantly higher mortality in that cohort compared to our data (41.2 vs. 14.8%) may be due to the different condition taken in account, specifically the enrollment only of the cases with evolution toward *purpura fulminans*. In our cohort only 8.6% of *S. pneumoniae* patients and 57.4% of *N. meningitidis* patients presented with purpura; like Contou et al. (23), also in our cohort the mortality was higher in this subgroup, 50 and 8.1% for *S. pneumoniae* and *N. meningitidis,* respectively, even if at the univariable logistic regression analysis purpura was not a variable significantly associated with in-hospital mortality.

The concepts of sepsis [according to the sepsis-3 definition (1)], coagulopathy (concerning pathophysiologic aspects and possibly reflected by D-dimer), overt-DIC [definable through clinical scores (13, 14)], even if interconnected, should be kept distinct, concerning different points of view of the same phenomenon, and it should be noted that these conditions often do not coexist, as evidenced by the discrepant percentages observed among our patients concerning presence of sepsis, elevated D-dimer levels, coagulopathy defined by SIC score (15), and overt-DIC defined by ISTH score (82.2, 80.4, 45.9, and 8.9%, respectively).

Finally, several studies have evaluated the role of prognostic biomarkers in case of bacterial meningitis: matrix metalloproteinases-8 assessed on CSF has been presented as an attractive prognostic biomarker in children (24), and longitudinal analysis of CSF lactate resulted to be an important predictor of prognosis (25). However, the advantage of measuring a biomarker directly from venous blood, in an easy and inexpensive way, with results rapidly available and everywhere, is evident.

D-dimer levels under a cut-off of 7,000 ng/mL, assessed within 24 h after admission, have shown to accurately predict a very low in-hospital mortality rate (3.5%) in case of infection due to *N. meningitidis*, and normal values <500 ng/mL excluded any further complications. For *S. pneumoniae*, D-dimer did not show the same ability to predict an increased risk of death, supporting the conclusion that D-dimer is a biomarker with a certain specificity in reflecting the dysregulated host coagulative response depending by specific pathogens.

Study Limitations

The main limitation of this study is represented by the retrospective design, with bias and confounding being known errors potentially affecting the validity of these studies. Nevertheless, we conducted data analysis performing

logistic regression analysis with Bayesian model averaging, to minimize confounders.

Another limitation is represented by a relatively small sample size that possibly did not allow to highlight significant differences in some cases, so further studies are warranted. Anyway, to the best of our knowledge, this cohort of patients with meningitis and/or BSI investigated for coagulation biomarkers early assessed within 24 h after admission represents one of the largest published so far.

Finally, our study has been conducted in a single center institution, thus composition of patient population, local resources, medical protocols, and staffing characteristics may limit the generalizability of results.

CONCLUSIONS

D-dimer is rapid to be obtained, at low cost and available everywhere, and seems to be an interesting biomarker able to reflect the concept of the dysregulated host response in case of invasive infections due to *N. meningitidis* and *S. pneumoniae*.

Assessment of D-dimer within 24 h after admission can help stratify the risk of in-hospital mortality and complications in patients with meningitis and/or BSI due to *N. meningitidis*: D-dimer values <500 ng/mL exclude any further complications, and a D-dimer cut-off of 7,000 ng/mL seems able to predict a significantly increased risk of in-hospital mortality from much <10% to over 25%. For *S. pneumoniae* invasive infections the mortality risk resulted always high, over 10%, irrespective by D-dimer values.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not provided by the participants' legal guardians/next of kin because for the retrospective nature of the study.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception of the work, acquisition, interpretation of data, in drafting the work, and approved the submitted version.

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MINI REVIEW
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Minireview on Novel Anti-infectious Treatment Options and Optimized Drug Regimens for Sepsis

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Sepsis, a life-threatening organ dysfunction caused by a dysregulated response to infection is a major public health concern, as it is a leading cause of mortality and critical illness worldwide. Antibiotics are one of the cornerstones of the treatment of sepsis; administering appropriate antibiotics in a rapid fashion to obtain adequate drug concentrations at the site of the infection can improve survival of patients. Nevertheless, it is a challenge for clinicians to do so. Indeed, clinicians today are regularly confronted with infections due to very resistant pathogens, and standard dosage regimens of antibiotics often do not provide adequate antibiotic concentrations at the site of the infection. We provide a narrative minireview of different anti-infectious treatments currently available and suggestions on how to deliver optimized dosage regimens to septic patients. Particular emphasis will be made on newly available anti-infectious therapies.

Keywords: antibiotics, multidrug resistant organisms, appropriate therapy, pharmacokinetics, gram-negative bacteria

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INTRODUCTION

Sepsis, a life-threatening organ dysfunction caused by a dysregulated response to infection (1), is a major public health concern, as it is a leading cause of critical illness and mortality worldwide. Indeed, data extrapolations from high-income countries suggest annual global estimates of sepsis cases and deaths in hospitals of over 50 million and over 5 million, respectively (2). Antibiotics are one of the cornerstones of the treatment of sepsis; administering appropriate antibiotics, defined as those that have *in-vitro* activity against the pathogen(s) responsible for the infection, in a rapid fashion to obtain adequate drug concentrations at the site of infection can improve patient survival (3–6).

Nevertheless, to give appropriate antibiotics to patients with sepsis is a challenge for the clinician because the risk of being confronted with an infection due to multidrug resistant (MDR) bacteria, particularly amongst Gram-negative bacteria, is increasing worldwide (7). To illustrate this point, we can look at the epidemiological situation in the European Union (EU) over the last 15 years concerning 2 important pathogens, *Escherichia coli (E.coli)* and *Klebsiella pneumoniae (K. pneumoniae)*. The estimated number of attributable deaths due to third generation cephalosporin (3GC) resistant *E. coli* was four times greater in 2015 than in 2007 (8). Resistance to 3GC is primarily caused by extended spectrum ß-lactamase (ESBL) enzymes; a matter of concern is that infections due to ESBL producing *E. coli* can either be community acquired or healthcare associated. The numbers of infections and deaths due to *K. pneumoniae* carbapenem-resistant strains also increased by over 6-fold from 2015 to 2018 (8). *K. pneumoniae* is currently the most frequent carbapenem-resistant Gram-negative bacteria causing infections (9–11). Indeed, *K. pneumonia* is

6–11 times more frequently responsible for carbapenem-resistant infections than *E. coli* (8, 12). The resistance to carbapenems, one of the last resort antibiotics, is often combined with resistance to other key antimicrobial groups, resulting in inappropriate initial therapy, but also suboptimal definitive therapy (8). Geographical distribution of resistance is highly heterogeneous, but the increasing rates of antimicrobial resistance is global (13).

To further complicate matters, it is also a challenge for clinicians to give adequate dosage regimens of antibiotics to patients with sepsis because standard dosage regimens often do not provide adequate antibiotic concentrations at the site of the infection (3). Standard drug regimens of antibiotics are determined from pharmacokinetic (PK) clinical studies in non-critically ill patients. However, when patients present severe infections, such as sepsis and septic shock, the PKs, or the concentration of antibiotics over time, particularly those that are hydrophilic (e.g., ß-lactams, aminoglycosides, and glycopeptides), are significantly altered, resulting in significant inter and intra-individual PK variability. These antibiotics have a distribution that is essentially extra-cellular, and their total body clearance (CL) is mainly dependent on renal mechanisms. Antibiotic concentrations may be lower than expected because of increased volume of distribution (Vd) due to fluid resuscitation, venous pooling, and hypoalbuminemia, but also due to increased drug CL because of high cardiac output and augmented renal clearance (ARC) (14, 15). Microvascular injury may also impair drug delivery to certain tissues (16). Low antibiotic concentrations may result in therapeutic failure, and/or facilitate emergence of resistance. Antibiotic concentrations may also be higher than expected, with risk of toxicity, because of decreased drug CL, particularly due to renal insufficiency. Toxic concentrations of glycopeptides and aminoglycosides are known to increase the risk of renal insufficiency (17), and accumulating data suggest that increased concentrations of ß-lactams can cause neurotoxicity (18). The PKs of antibiotics can be further altered by different renal replacement techniques and the use of extra-corporeal membrane oxygenation (ECMO).

It is in this light that clinicians have tried to optimize available antibiotic treatment regimens (by trying to attain the greatest efficacy, with the least toxicity) in the case of sepsis and septic shock, particularly in the case of infections due to MDR bacteria. Clinicians have also been anxiously waiting for new, effective antibiotics. The antibiotic pipeline has barely been trickling (19), yet several new antibiotics have finally made it to the market over the last couple of years, with indications to treat infections due to some of these problematic MDR Gram-negative pathogens. However, these antibiotics were not tested extensively in septic patients before getting European Medicinal Agency (EMA) and/or Federal Drug Administration (FDA) approval. In the light of these newly available therapeutic agents, we have performed a narrative minireview on their optimal antibiotic regimens in case of sepsis or septic shock. The antibiotics that will be reviewed here are ones that have received FDA and EMA marketing approval over the last 5 years for MDR Gram-negative infections: ceftolozane-tazobactam, cefiderocol, ceftazidime-avibactam, meropenem-vaborbactam, imipenem-relebactam, and eravacycline.

MATERIALS AND METHODS

This narrative review is based on a literature search performed in the Pubmed/MEDLINE database using combinations of pertinent keywords ("pharmacokinetics," "ICU," "sepsis," "septic shock," "MDR," "critically ill," "ceftolozone-tazobactam," "cefiderocol," "ceftazidime-avibactam," "meropenem-vaborbactam," "imipenem-relebactam," and "eravacycline"). Retrieved papers were reviewed, and further searches were conducted using the reference lists. The review was then organized into 4 chapters: (1) theoretical antibiotic Pharmacodynamic (PD) targets and optimized drug regimens, (2) PKs of novel antibiotics in septic patients and optimized drug regimens, (3) perspectives for the future, and (4) conclusions.

THEORETICAL ANTIBIOTIC PHARMACODYNAMIC TARGETS AND OPTIMIZED DRUG REGIMENS

In order to optimize an antibiotic dosage regimen, the desired target concentrations have to be known. In bacterial infections, the target concentration will depend on the minimal inhibitory concentration (MIC) of the infecting pathogen being treated, and on the PK/PD index (or the relationship between the dose and the effect obtained) that best describes the efficacy of the antibiotic. Antibiotics can indeed be divided into three different groups based on PK/PD indexes to schematically describe their efficacy:

- **Time dependent antibiotics (e.g., ß-lactam antibiotics):** efficacy is greatest when the serum concentration of the unbound fraction of the antibiotic remains above the MIC for a minimum period of time (fT > MIC). To increase efficacy, the time of the antibiotic infusion can be increased, even to the point of administering the antibiotics in continuous infusion (as long as the stability of the antibiotic permits).
- **Concentration-dependent antibiotics (e.g., aminoglycosides):** efficacy is greatest when a certain ratio of the peak concentration to the MIC of the pathogen during one dosing interval is attained (Cmax/MIC). To increase efficacy, the antibiotic dose can be increased to attain higher peak concentrations.
- **Concentration-dependent antibiotics with time dependency (e.g., glycopeptides, tetracyclines):** efficacy is greatest when a certain ratio of the area under the curve of the serum concentration of the free fraction of the antibiotic over time to the MIC of the pathogen is attained (AUC/MIC). To increase efficacy, the antibiotic dose and infusion time can be increased (20).

All of the novel antibiotics are ß-lactams, with the exception of eravacycline. We will therefore only discuss how to theoretically optimize dosage regimens for ß-lactams and tetracyclines in sepsis and/or septic shock.

ß-Lactam Antibiotics

Although there is no consensus concerning what specific PD target should be aimed for when administering ß-lactam

antibiotics, *in-vitro* and clinical data suggest that a value of at least 100% fT > 1xMIC, but possibly even 100% fT > 4xMIC, may be needed to be effective (21).

When standard dosage regimens of antibiotics are administered, a great proportion of patients have insufficient serum concentrations during the first 24 h of treatment (14). In order to obtain adequate serum concentrations as soon as possible after antibiotic treatment initiation, a loading dose based on PK model simulations has been suggested (22). However, these findings still need to be validated in a prospective study.

Maintenance doses are given after loading doses; these need to be adapted to the CL of the antibiotic. However, if an increased loading dose is not given to the patient, then data suggests that standard dosage regimens could be given during the first 48 h of treatment, regardless of the renal function, and then adapted to the creatinine clearance based on 8–24 h urine collects (23). Indeed, initial standard drug regimens allow for better early PD target attainment in patients undergoing continuous renal replacement therapy (CRRT) (24). Furthermore, acute kidney injury (AKI) is frequent in patients admitted for severe infections, but can resolve rapidly after admission, as shown in a retrospective study evaluating data from a clinical database on 18,500 patients with predominantly pneumonia, intra-abdominal and skin-infections, where almost 1 in 5 patients presented AKI upon admission but was resolved within 48 h in over 50% of them (25).

To maintain serum concentrations above the MIC of the pathogen for the longest period of time, ß-lactam antibiotics can be administered as extended or continuous infusions. Although this improves PK/PD target attainment, it still remains to be proven that this mode of administration improves clinical outcomes (26). Indeed, extended or continuous infusions may require different PK/PD targets than intermittent infusions to get the same level of bacterial cell kill (27).

As clinicians aim to attain PD targets rapidly, the risk of reaching toxic concentrations increases. Nevertheless, there is currently no consensus concerning thresholds for ß-lactam toxicity; concentrations >10 times the MIC of the pathogen is the most frequently one currently used (22, 28).

Finally, to guide ß-lactam treatment, therapeutic drug monitoring (TDM) should be used in a routine fashion, as recommended in the recent position paper on antimicrobial TDM in critically ill patients, on behalf of different international scientific societies (28). Nevertheless, one of the challenges is that few centers have access to TDM of ß-lactams (29).

Tetracyclines

Tetracyclines are not first choices to treat patients in sepsis or septic shock because only tigecycline is formulated for intravenous formulation, and early on during its' market-life, the FDA emitted a black box warning due to concerns about increased mortality associated with this antibiotic. The increased risk of death has since been attributed to therapeutic failure due to underdosing. Because the efficacy of this drug is best described by the PK/PD parameter, AUC_{0-24}/MIC, to optimize PD target attainment, doses and frequency of administration can be increased. By increasing dosage regimens (i.e., 200 mg loading dose followed by 100 mg q12h, instead of 100 mg loading dose followed by 50 mg q12h), increased PD target attainment with better clinical outcomes than with smaller dosage regimens has been observed (30).

NOVEL ANTIBIOTICS

Ceftolozane-Tazobactam

This is a new antibiotic composed of a new cephalosporin associated with tazobactam, a well-known ß-lactamase inhibitor. It is active against MDR *Pseudomonas aeruginosa,* even those that are carbapenem-resistant, and ESBL producing Enterobacteriaceae. However, it is not active against carbapenem-resistant Enterobacteriaceae (CRE) (31). The drug is approved by the FDA and EMA for the treatment of intra-abdominal infections, complicated urinary tract infections, hospital acquired and ventilator-associated bacterial pneumonia (VAP). The antibiotic is also used by clinicians for off-label indications, particularly when faced with infections due to resistant pathogens with few or no other treatment options. The standard drug regimen is 1.5 g q8h, and 3 g q8h for VAP (**Table 1**) (37).

In a multicentric (22 hospitals), retrospective study in Italy from 2016 to 2018, 101 patients with serious and diverse infections due to *P. aeruginosa* (over 50% of strains were extensively resistant) were treated with this antibiotic with standard drug regimens. Clinical success (i.e., complete resolution of clinical signs/symptoms related to the infection and no microbiological evidence of infection) was observed in 83.5% of cases. However, lower success rates were observed in patients with sepsis or those receiving CRRT (37), possibly due to lack of PD target attainment in patients with sepsis. Indeed, in a population PK study of unbound ceftolozane and tazobactam in 12 critically ill patients without renal dysfunction, the probability of PD target attainment was ≥90% in patients receiving 1.5g q8h, but only for strains of *P. aeruginosa* with a MIC ≤2 mg/L. The 3g q8h regimen was needed to obtain a similar probability of PD target attainment for strains of *P. aeruginosa* with MICs of 4 mg/L. Finally, a loading dose followed by continuous infusion (1.5 g, then 4.5 g/24 h or 3 g, then 9 g/24 h) was needed to obtain optimal PD target attainment if MICs of *P. aeruginosa* were 8 and 16 mg/L, respectively (37). Another population PK model-guided evaluation of dosing in 6 patients undergoing continuous veno-venous hemodiafiltration (CVVHDF) showed that CL of ceftolozane-tazobactam is decreased in this situation. Nevertheless, in order to attain the PD target rapidly, a loading dose is necessary, followed by a reduced dosage regimen (**Table 1**). The optimal dosage regimen will depend on several factors, some being the specific CVVHDF settings, and the patient's residual renal function (38).

Finally, septic patients also sometimes benefit from ECMO. An *ex-vivo* model and then an *in-vivo* porcine model showed that ECMO has little influence on the PKs of ceftolozane/tazobactam, suggesting that no dose adaptation should be performed for these patients (39).

All of the proposed dosage regimens, particularly in the critically ill patients with sepsis still need to be validated in prospective clinical studies. No data is currently available

TABLE 1 | PK/PD indexes and drug dosage regimens in adults of these novel antibiotics.

Antibiotic	PK/PD index for efficacy	Standard drug dosage regimen	Drug regimens in septic or critically ill patients
Ceftolozane-tazobactam (32)	%fT > MIC	Always to be administered in 1 h Creatinine clearance*: >50 mL/min: 1.5 g q8h 30–50 mL/min: 750 mg q8h. 15–29 mL/min: 375 mg q8h. End stage renal disease on hemodialysis: 750 mg, followed by 150 mg q8h	Always to be administered in 1 h Creatinine clearance*: >50 mL/min: 3 g q8h or. 1.5 g followed by 4.5 g CI 30–50 mL/min: 1.5 g q8h 15–29 mL/min: 750 mg q8h End stage renal disease on hemodialysis: 2.25 g followed by 450 mg q8h
Ceftazidime-avibactam (33)	%fT>MIC	Always to be administered in 2 h Creatinine clearance*: ≥ 50 mL/min: 2 g q8h 31–50 mL/min: 1.25 g q8h 16–30 mL/min: 0.75/0.1875 g q24h End stage renal disease or renal dialysis: 0.75/0.1875 g q24h	Always to be administered in 2 h Creatinine clearance: ≥ 50 mL/min: 2 g q8h 31–50 mL/min: 1.25 g q8h 16–30 mL/min: 0.75/0.1875 g q24h End stage renal disease or renal dialysis: 0.75/0.1875 g q24h
Cefiderocol (34)	%fT > MIC	Always to be administered in 3 h Creatinine clearance*: ≥ 120 mL/min (or ARC): 2 g q6h ≥ 60 ML/min: 2 g q8h ≥ 30 to < 60 mL/min: 1.5 g q8h ≥ 15 to < 30 mL/min: 1 g q8h End stage renal disease: 0.75 g q12h	Always to be administered in 3 h Creatinine clearance*: ≥ 120 mL/min (or ARC): 2 g q6h**
Meropenem-vaborbactam (35)	%fT > MIC	Always to be administered in 3 h Creatinine clearance*: ≥ 40 mL/min: 4 g q8h 20–39 mL/min: 2 g q8h 10–19 mL/min: 2 g q12h <10 mL/min: 1 g q12h	CVVH: Low efferent: 500 g q8h** High efferent: 1 g q8h**
Imipenem-relebactam (36)	%fT > MIC	Infusion time: 30 min. Creatinine clearance*: ≥ 90–149 mL/min: 1,250 mg q6h ≥ 60–89 mL/min: 1,000 mg q6h ≥ 30–59 mL/min: 750 mg q6h ≥ 15–29 mL/min: 500 mg q6h End stage renal disease: 500 mg q6h	No data
Eravacycline (57)	AUC/MIC_{0-24}	1 mg/kg q12h (even for obese patients) If taking CYP3A4 inducers: 1.5 mg/kg q 12h	No data

*PK/PD, Pharmacokinetic/Pharmacodynamic; %fT > MIC, percent of the time that the free fraction of the antibiotic remains above the minimal inhibitory concentration of the pathogen; AUC/MIC, area under the curve of the concentration of the free fraction of the antibiotic/MIC; CI, continuous infusion; ARC, augmented creatinine clearance; CVVH, continuous veno-venous hemofiltration. *Based on the Cockcroft-Gault equation. **Based on Monte Carlo simulations; dosage regimens not yet clinically validated.*

concerning need for dosage adjustment in special patient populations such as the obese, the elderly, or patients with severe hepatic impairment.

Ceftzidime-Avibactam

This is a combination of an existing cephalosporin, ceftazidime, and a new non-ß-lactam ß-lactamase inhibitor, avibactam which restores *in-vitro* activity of ß-lactams against Ambler class A [ESBLs and *Kl. pneumoniae* carbapenemases (KPC)], class C (AmpC), and certain enzymes from the class D (Oxa-48 carbapenemases). There is no activity against the Ambler class B enzymes (metallo-ß-lactamases), *Acinetobacter spp*, *Stenotrophomonas maltophilia*, or anaerobes. The antibiotic is registered by the FDA and the EMA to treat complicated intra-abdominal infections, complicated urinary tract infections, HAP, and VAP. The EMA has also approved its use for the treatment of infections due to Gram-negative bacteria for which few therapeutic options are available. The PK/PD index that best describes its efficacy is the %T> MIC. The standard dosage regimen is 2.5 g q8h for a patient with normal or augmented renal clearance, administered in 2 h. The doses must be decreased in patients with renal impairment (**Table 1**).

A population PK study on data from the five phase III trials was performed to validate these standard dosage regimens. The PK dataset for ceftazidime was made up of 9,155 observations from 1,975 subjects, and for avibactam, 13,735 observations from 2,249 subjects. A very wide range of clinical characteristics were captured, such as estimated creatinine clearances varying from 11 to 610 mL/min, and clinical presentations varying from the healthy volunteer to the patient with VAP. The covariates tested (that could have a possible significant effect

on the PKs of ceftazidime and/or avibactam) were numerous, including disease status/indication of treatment, ARC, markers of systemic illness, severity of illness identified by the Acute Physiology and Chronic Health Evaluation version II (APACHE II), sex, age, obesity, body weight, race, and creatinine clearance. Creatinine clearance was the key covariate that predicted CL of both ceftazidime and avibactam. The covariates that affected the VD of both ceftazidime and avibactam were body weight, and mechanical ventilated patients when treated for nosocomial pneumonia, and treatment indication (40). Another PK study was carried out in 10 critically ill patients receiving ceftazidime-avibactam to once again evaluate adequacy of proposed standard dosage regimens (41). Both studies showed that standard dosage regimens resulted in a >90% probability of attaining the PD of 8 mg/L during >50% of the time for a relatively heterogenous patient population. However, this PD target is significantly lower than the suggested ß-Lactam targets of 100% fT> MIC or 100% fT> MICx4 in critically ill patients. Nevertheless, this standard dosage regimen of ceftazidime-avibactam has resulted in superior clinical success and survival than other treatment regimens to treat life-threatening infections by carbapenem-resistant pathogens (42, 43).

However, data on optimal dosage regimens in patients with septic shock, and those receiving CRRT are scarce. There are currently only 2 case reports with PK data in critically ill patients with infections due to MDR *Pseudomonas aeruginosa* (MIC: 8 mg/L). Both regimens (2.5 g q8h and 1.25 g q8h) attained the PD target of 100%fT > 4xMIC (44, 45).

Cefiderocol

Cefiderocol is a catechol-type siderophore cephalosporin with very potent *in-vitro* activity against CRE and drug resistant non-fermenting Gram-negative bacilli. It uses the siderophore-iron complex pathway to penetrate Gram-negative membranes, like a Trojan horse. Once inside the bacteria, cefiderocol separates from the iron, and binds to penicillin-binding proteins to inhibit peptidoglycan synthesis. This antibiotic seems to have enhanced stability against hydrolysis by many ß-lactamases and carbapenemases. The antibiotic has been approved by the FDA and the EMA for Gram-Negative bacterial infections when other treatments might not work. The standard dose regimen is 2 g q8h in a 3 h infusion, but it needs to be reduced in case of renal insufficiency and increased in case of ARC (**Table 1**).

No PK data on cefiderocol is available in patients with sepsis, or patients in the ICU. However, in a phase III, double-blind, randomized trial for HAP, VAP, or health care associated pneumonia caused by Gram-negative pathogens, cefiderocol was non-inferior to meropenem concerning all-cause mortality at days 14 and 28 (46). On the other hand, in another phase III randomized (CREDIBLE-CR: NCT02714595), open-label study comparing cefiderocol to best available treatment (chosen by the investigator, with a combination of up to 3 drugs) for the treatment of severe infections caused by carbapenem-resistant Gram-negative pathogens, clinical cure rates were similar in both groups, however all-cause mortality was numerically higher in the cefiderocol arm on days 14, 29, and 49. The greatest imbalance with death was on day 49 in patients with APACHE II scores ≥ 16 and those with infections due to *Acinetobacter baumanni*, *Pseudomonas aerugi*nosa or *Stenotrophomonas maltophilia* (47).

Nevertheless, population-PK models were developed based on PK data obtained from phase I studies: plasma and urine concentrations from healthy individuals ($n = 54$) and plasma concentrations from individuals with varying renal function ($n = 37$). Monte Carlo simulations were then performed to determine optimal dosage regimens in patients with varying renal functions. Simulations showed that patients with ARC would benefit from an increased dosage regimen (i.e., 2 g q6h) (48). This proposed dosage regimen still needs to be validated in the clinical setting.

Meropenem-Vaborbactam

Vaborbactam is a boron-based ß-lactamase inhibitor with activity against serine-ß-lactamases. It is a particularly potent inhibitor of KPC. When combined to meropenem, an existing carbapenem, it restores activity against KPC-producing carbapenem-resistant Enterobacteriaceae, both *in-vitro* and in preclinical models. The drug is approved for complicated urinary tract infections by the FDA, in addition to complicated intra-abdominal infections, and HAP, and VAP by the EMA. The standard drug regimen is 4 g q8 in 3 h infusions.

TANGO II is a randomized trial to compare the efficacy and safety of meropenem-vaborbactam to the best available therapy in adults with serious infections due to CRE. Seventy-seven patients were randomized, and 47 patients had confirmed CRE infections, but exclusion criteria were an APACHE score > 30, and an immediately life-threatening disease. Therefore, no patients with sepsis or septic shock were included in the study. Treatment with meropenem-vaborbactam for CRE infections was associated with improved outcome to best available treatment: increased cure, decreased mortality, and reduced nephrotoxicity (49). Nevertheless, no PK data is yet available in patients with severe infections.

An *ex-vivo* study has been performed to characterize the effects of continuous veno-venous hemofiltration therapy on the PKs of meropenem-vaborbactam. The study showed that there was little adsorption (<10%) of meropenem and vaborbactam by the extra-corporeal circuits. Furthermore, clearance of vaborbactam was 20–40% lower than the clearance of meropenem for an array of different settings and filters tested. Dosing according to currently optimized regimens for meropenem probably allows for adequate PD target attainment while ensuring adequate ß-lactamase inhibition during the entire dosing intervals: 500 mg/500 mg q8h for low effluent flow rates or 1 g/1 g q8h for higher effluent rates (50). Once again, these dosage regimens still need to be clinically validated.

Imipenem-Relebactam

This is a combination of an existing carbapenem (imipenem-cilastatin) and a new non-ß-lactam ß-lactamase inhibitor, relabactam. It inhibits activities of certain ß-lactamases (Ambler Class A (e.g., KPC) and C (e.g., AmpC) cephalosporinases) but does not have activity against class B metallo-ß-lactamases, and class D carbapenemases. The addition of relebactam greatly improves the activity of imipenem against *Enterobacteriaceae*

TABLE 2 | Intravenous antibiotics active *in-vitro* against gram-negative bacteria in phase I-III studies (19).

Antibiotic	Compound	Clinical phases completed or on-going
Benapenem	Carbapenem	Phase II trial completed in cUTIs: NCT04505683
SPR741 + ß-lactam	Polymyxin	Phase I trials completed: NCT03022175, NCT03376529
SPR206	Polymyxin	Phase I trial completed: NCT03792308
Apramycin	Aminoglycoside	Phase I trial completed: NCT04105205
Enmetazobactam (AAA 001) + cefepime	Clavulanic acid + 4th generation cephalosporin	Phase III trial on complicated urinary tract infections: completed: NCT03687255
Durlobactam (ETX2514) + sulbactam	Diazadicyclooctane-type ß-lactamase inhibitor + clavulanic acid type BLA inhibitor	Phase II completed for pyelonephritis and complicated urinary tract infections: NCT03445195 Phase III trial on-going: NCT03894046
Taniborbactam (VNRX-5133) + cefepime	Boronate + 4th generation cephalosporin	Phase III trial recruiting on complicated urinary tract infections: NCT03840148
Nacubactam (OP0595) + meropenem	Diazadicyclooctane-type ß-lactamase inhibitor + carbapenem	Phase I trials completed: NCT02134834, NCT02972255, NCT02975388, NCT03174795
Zidebactam + cefepime	Diazadicyclooctane-type ß-lactamase inhibitor + PBP2 + 4th generation cephalosporin	Phase I trials completed: NCT02532140, NCT02674347, NCT02707107, NCT02942810, NCT03630094
TP-6076	Tetracycline	Phase I active, not recruiting: NCT03691584

spp. (particularly those that are ESBL, KPC, and AmpC producing) and *Pseudomonas spp*. in function of the presence or absence of ß-lactamase enzymes. However, the activity of imipenem is not improved against *Acinetobacter baumannii*, and *Stenotrophomonas maltophilia* (51). The antibiotic is currently only approved for complicated urinary tract infections, including pyelonephritis. The dosage regimen is 500/250 mg q6h.

Only one trial has evaluated this antibiotic in ICU patients. In this phase III trial RESTORE-IMI 1, imipenem/relebactam was compared to imipenem and colistin in infections (HAP, VAP, complicated intra-abdominal infections, or complicated urinary tract infections) due to imipenem resistant bacteria. A favorable overall response was observed in 70% of patients; results were similar in both arms. However, over 50% of patients in the microbiological intention-to-treat arm presented a complicated urinary tract infection, and over two-thirds of patients had an APACHE score < 15 (52). Clinical and PK data from patients with sepsis or septic shock is clearly lacking.

Eravacycline

This is the first fluorocycline antibiotic in the tetracycline class. It disrupts bacterial protein synthesis by binding to the 30S ribosomal unit. It has excellent *in-vitro* activity against Gram-negative bacteria (e.g., CRE, *A. baumannii* and *S. maltophilia*), but no activity against *Pseudomonas sp*. The PK/PD parameter that best describes its' efficacy is the AUC/MIC. The proposed current standard dosage regimen is 1 mg/kg q12h, to be increased to 1.5 mg/kg if patients are taking strong CYP3A4 inducers, with no drug dosage adjustment for renal or hepatic failure. Furthermore, this dosage regimen based on actual body weight appears to obtain high rates of clinical cure, without excess toxicity issues in obese patients. Indeed, cure rates were similar to those observed in obese patients treated with carbapenem containing regimens in a *post-hoc* analysis of pooled data from the Investigating Gram-negative Infections Treated with Eravacycline (IGNITE) 1 and IGNITE 4 phase 3 clinical trials comparing eravacycline to ertapenem and then to meropenem for complicated intra-abdominal infections. Drug discontinuation rates were no different between these two groups either (53).

This antibiotic has been approved both by the FDA and the EMA for complicated intra-abdominal infections, after showing non-inferiority to levofloxacin and ertapenem in the IGNITE 1 and 4 trials, where 80% of patients had APACHE II scores < 10. However, the antibiotic failed to show non-inferiority when used to treat complicated urinary tract infections when compared to levofloxacin in IGNITE 2, and ertapenem in IGNITE 3 because of poor urinary tract PKs (54). No other data is currently available concerning use of the antibiotic in bacteremia, in sepsis or septic shock.

PERSPECTIVES FOR THE FUTURE

Clinicians need to remain vigilant concerning possible future therapeutic options because other antibiotics and anti-infectious agents, than those presented in this paper, are in the development pipeline. **Table 2** provides a list of intravenous antibiotics active *in-vitro* against MDR Gram-negative bacteria currently in phase 1 to III trials (19). Because this paper reviews treatment options for patients with sepsis, only antibiotics with intravenous formulations have been presented.

CONCLUSIONS

New antibiotics active against MDR Gram-negative bacteria have finally made it to the market, providing the clinician with more arms to fight difficult-to-treat infections. Nevertheless, further evaluation of these novel antibiotics in the real-life setting is warranted. As a general rule, there is little data concerning the use of these novel antibiotics in patients with sepsis, particularly to treat infections due to MDR Gram-negative bacteria. Few post-marketing trials, including PK trials in special patient populations have been performed. Furthermore, case reports of rapid emergence of resistance to some of

these antibiotics (e.g., ceftazidime-avibactam) have already been reported (55). Finally, these new antibiotics are significantly more costly than older antibiotics; they should only be administered to patients when no other effective therapeutic alternatives are available.

In order to use these new antibiotics in the most optimal fashion, more data (including PK data) on the efficacy of these antibiotics in critically ill patients, and those with sepsis or septic shock to treat MDR infections need to be obtained.

AUTHOR CONTRIBUTIONS

MH wrote the article on her own.

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Conflict of Interest: MH has received service fees from Pfizer and Gilead for participating in scientific and advisory board meetings in the domain of anti-fungal therapy.

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PERSPECTIVE
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Related Factors to Streptococcus pneumoniae Invasive Infection and Clinical Manifestations: The Potential Role of Nasopharyngeal Microbiome

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Infections of the lower respiratory tract, such as pneumonia, are one of the leading causes of death worldwide. *Streptococcus pneumoniae* might colonize the upper respiratory tract and is the main aetiological agent of community-acquired pneumonia (CAP). In the last decades, several factors related to the host, the microorganism and the antibiotic therapy have been investigated to identify risk factors associated with the development of invasive pneumococcal disease (IPD). Nevertheless, these factors themselves do not explain the risk of developing disease or its severity. Recently, some studies have focused on the importance of nasopharyngeal (NP) microbiome and its relation to respiratory health. This review presents existing evidence of the potential role of NP microbiome in the development of IPD.

Keywords: microbiota, nasopharynx, *Streptococcus pneumoniae*, pneumococcal infections, invasive pneumococcal disease

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INTRODUCTION

Lower respiratory infections, considering also pneumonia, represent the third cause of death worldwide, with a median of 4.2 million deaths (7.1% of total deaths) per year (1). Combination of pneumonia and Influenza ranked 8th among leading causes of mortality in developed countries. Also, they are the main cause of death due to infection (2). *Streptococcus pneumoniae (S.pneumoniae)* is the most common pathogen implicated in community-acquired pneumonia (CAP) and accounts for two-thirds of the mortality in hospitalized patients (3).

Invasive pneumococcal disease (IPD) refers to an infection confirmed by the isolation of *S.pneumoniae* from a normally sterile site (pneumonia, parapneumonic empyema, meningitis, bacteraemia/sepsis, peritonitis and arthritis) (4). Its incidence, despite its remarkable reduction since the introduction of pneumococcal conjugated vaccine (PCV), is still 10–20/100,000/year in developed countries and could be even higher in developing countries (5–11).

To date, the risk of developing pneumococcal pneumonia and also IPD has been associated with the interplay between host susceptibility and pathogen virulence.

Many factors have been described regarding host susceptibility for the development and prognosis of pneumococcal pneumonia, as age, comorbidities and previous vaccination status (12–15). Severity and mortality in pneumococcal pneumonia are also related to strain characteristics, coinfection and antibiotic treatment (16, 17).

However, these elements do not fully explain by themselves the potential risk for infection and its severity. Despite recent advances in this field, it is still not known why some individuals present asymptomatic NP colonization; others develop localized infection and others, IPD.

The aim of this review was to present the current evidence for the role of different factors –including the potential role of NP microbiome- involved in the dissemination of *S.pneumoniae* from the nasopharynx to other human body sites and its subsequent clinical manifestations.

METHODS

A literature search of PubMed and WOS databases conducted between January 2000 and February 2021 using the parameters [microbiome (MeSH Terms)] OR [infection, streptococcus pneumoniae (MeSH Terms)] OR [pneumonia (all fields)] generated >180,000 abstracts.

Titles and abstracts were assessed, as a rule of thumb, and clinically relevant articles were reviewed. References of the selected articles were also reviewed and those which could fit the topic were read and evaluated as well.

HOST CHARACTERISTICS

Baseline Characteristics and Clinical Features

S.pneumoniae is a large contributor to mortality worldwide (12, 13, 18). It is the most common documented etiology of severe bacterial respiratory tract infection in children, but also in the adulthood. The spectrum of disease ranges from colonization (especially among children under 5 years of age) to mucosal disease (otitis media, sinusitis, pneumonia), and to invasive infections (bacteremia, meningitis, endocarditis, others) (18).

Increasing age, male gender, toxic habits (as smoking and alcohol abuse), liver or renal disease, solid organ tumors, immunosuppression (HIV infection, asplenia) and higher Charlson Comorbidity Index have been identified as independent risk factors for high mortality among patients with community-acquired, bacteraemic pneumococcal pneumonia.

Among all the host factors previously described, age has been identified as the strongest predictor of death, even in patients without significant comorbidity (13, 19, 20).

Vaccination

S.pneumoniae infection is one of the most vaccine-preventable diseases. In the 1980s and 1990s, the 23-valent-polysaccharide vaccine (PPSV23) was the only direct prevention measure available (12). Polysaccharide antigens produce the activation of mature B cells. However, protein-polysaccharide conjugate pneumococcal vaccines (PCV) have a T-cell-immune response and are effective in immunosuppressed patients (21). In 2000 the 7-valent vaccine (PVC7) -against seven serotypes- was licensed for children in the USA. After that, the increment in relevance of non-vaccine serotypes, conducted to the development of new vaccines: PCV10 (PCV7 plus 1, 5 and 7F), and PCV13 (PCV10 plus 3, 6A and 19A) (22). At present, there are two new conjugate vaccines in development (PCV15 and 20vPnC) (23).

Many investigators have focused on the subsequent impact of young children vaccination in the incidence of IPD in other age groups. Although there are some reports of this incidence being increased (24, 25), there is solid evidence of a sustained decrease in IPD incidence in vaccinated children and adults, -including the immunosuppressed population- supporting the use of PVCs (12, 22, 26–29).

Genetic Polymorphisms

The innate immune system represents the first non-specific step in host defense. The recognition of pathogens by the host immune system is a necessary requisite for the initiation of a response (15). A bunch of different receptors are placed on the cell surface of the epithelial barrier and on hematopoietic cells. These receptors recognize diverse pathogen antigens. After these pattern recognition, a very complex net of intracellular signaling pathways is triggered in order to develop a response of the host against the pathogen (30).

In the 1980s, genetics was found to be a major determinant of susceptibility to infectious diseases. Extreme-phenotype studies in patients with recurrent IPD were successful in the identification of factors associated with increased susceptibility (31). Different genetic variants in the proteins involved in the signaling pathways have been identified in the last 20 years as elements that either increase the risk or confer protection against pneumococcal pneumonia. A recent meta-analysis showed that variants in *CD14* and *MBL2* genes were associated with susceptibility to pneumococcal disease. Several other host genetic polymorphisms have been identified that potentially influence susceptibility and outcome of pneumococcal disease, although most of these findings have not been confirmed in independent studies (32).

Thus, all these findings support the hypothesis that genetic variants may explain, at least partially, the host susceptibility to pneumococcal pneumonia and IPD.

BACTERIAL AND TREATMENT FACTORS

The outcome of IPD can be affected by host factors, such as age (the very young and the very old), underlying conditions, low socioeconomic status and quality of life; but also by bacterial factors such as the serotype (16).

Serotype, Invasiveness, and Severity

At least 100 serotypes of *S.pneumoniae* has been identified, based on antigenic differences in their capsular polysaccharides (18).

Main serotypes found in NP carriage and invasive disease are quite similar worldwide (33, 34). Likewise, the frequency by which a certain serotype causes invasive disease per carriage episode is a stable property along the time. There is an inverse relationship between frequency of carriage and invasiveness: serotypes which are less commonly carried cause more frequently invasive disease whilst the serotypes most prevalent in carriage are less invasive (35). Also, it has been described that serotypes which have less risk of causing invasive disease are associated

with more severe disease and mortality, even in healthy individuals (36–38).

According to the studies of Brueggemann et al. serotypes 1, 3, 4, 5, 7F, 8, 9A, 9V, 12F, 14, 18C, and 19A are considered highly-invasive serotypes while the remaining serotypes were considered non-highly-invasive or opportunistic (39).

A meta-analysis of IPD outcome by serotype in 2011 was consistent with these findings: there is a relationship between IPD and pneumococcal serotype. The risk for IPD has a direct relation with the serotype prevalence, and is inversely correlated with invasiveness. Besides, the findings of this study suggested a potential mechanism for the epidemiologic relationships between serotypes. The most prevalent among carriage isolates are the more heavily encapsulated, which rarely cause bacteraemic invasive disease, but can cause more severe disease when they do invade (16).

Impact of Initial Antimicrobial Treatment

The effect of *S.pneumoniae* resistance to antimicrobials and subsequent discordant antimicrobial therapy (DAT) has been largely investigated with controversial results. While some studies observed that patients who received DAT did not have a higher mortality rate (40), others concluded that discordant antibiotic prescribed at admission was strongly related with higher mortality (41).

More recently, in a study conducted in Spain to assess the relationship between empirical antibiotic treatment and mortality in CAP due to *S.pneumoniae,* the authors evaluated some clinically relevant situations. Among the subgroup of bacteraemic patients, the choice of empirical antibiotic combination other than monotherapy with β-lactam or macrolide combination of β-lactam and macrolide or levofloxacin alone or in combination was associated with higher mortality (17).

Regarding beta-lactam therapy, pharmacokinetic and pharmacodynamic studies suggest that there is no association between mortality and penicillin MIC $\geq$2 mg/mL in patients that receive penicillin monotherapy at a dosis of $\geq$10 MU per day (usually $\geq$12 MU per day) (42).

NASOPHARYNGEAL MICROBIOME AND RESPIRATORY DISEASE

The human body is considered to be a super-organism which is composed of more microbial cells than body cells (43). The term *microbiome* was suggested by Joshua Lederberg and refers to the collective genomes of our endogenous microbes or microbiota. This human microbiota plays essential roles in human body functions, as the maintenance of the integrity of the epithelium (44), the modulation of the immune response (45, 46), and the "colonization resistance" to avoid the invasion of pathobionts, microorganisms which can live as commensal symbionts but, under not yet completely characterized circumstances (related to host genetics, microbial context, etc.) could act as pathogens and cause invasive disease (47–52). Research on this topic has largely focused on the gut microbiota; however, recent studies provide growing evidence of the importance of respiratory ecosystems on human health.

Classically, the lung has been considered sterile. More recently, the use of molecular typing [especially quantitative PCR for 16S ribosomal RNA (rRNA)] has allowed identification of pathobionts in culture-negative respiratory specimens (53, 54). These techniques have permitted the identification of five phyla in the lungs of healthy individuals: Actinobacteria, Proteobacteria, Bacteroidetes and Firmicutes (55), including low levels of oral bacteria, like those of the genera *Prevotella* and *Veionella* (56, 57). The composition of the lower respiratory tract (LRT) microbiota seems to depend on the upper respiratory tract (URT) microbiota composition, due to aspiration of oropharyngeal secretions, micro aspiration (i.e., while sleeping) or direct contact by continuous mucosa (48, 58, 59). Some studies report that there are indistinguishable microbiota patterns along the respiratory tract in healthy individuals, with decreasing biomass content from upper to lower respiratory tract (56, 60). One study found high viral and bacterial microbiota concordance between nasopharyngeal and endotracheal samples during the course of childhood LRTIs, suggesting that upper respiratory tract (URT) samples could be a valid proxy for lung microbiota during disease states (61). These findings have now changed the assumption that the lower airways are normally sterile (62).

Lung colonization by microbiota starts after birth and therefore remains unaltered during the whole lifetime (63–65). Gut and lung microbiota are actually connected in the gut-lung immunity axis. The metabolites produced by the gut microbiome (short-chain fatty acids) can reach other organs and have some influence in the respiratory diseases (66, 67). Recently, it has been shown the role of the gut-lung immunity axis in the pneumococcal pneumonia, by whom the integrity of the gut microbiota plays a main role in the host defense (68).

The colonization of the URT is the essential first step for a respiratory infection, either of the URT, LRT or a disseminated infection (33). Between the different species of the microbiota, there can exist positive interactions (as mutualism or commensalism) or negative interactions (antagonism) (48). Thus, some members of the microbiome have potential advantageous effects on ecosystem equilibrium, health and functionality (69). Examples of these members in the URT microbiota are *Dolosigranulum* spp. and *Corynebacterium* spp., as they have been related to respiratory health and negatively associated to disease caused by pathobionts, such as *S.pneumoniae* (48, 70, 71). The impediment for the pathogens to find necessary resources because the available nutrients might be used by a diverse local microbiome is one of the mechanisms described behind colonization resistance (48). Certain microorganisms have clearly been identified as having the ability to exclude pathogens from the NP ecosystem (72). In the case of *S.pneumoniae*, for instance, the release of free fatty acids -which come from the skin surface tiacylglycerols- by the *Corynebacterium* species *C.accolens,* has been described as one of the mechanisms which could inhibit pneumococcal growth (71). Furthermore, colonization resistance may be enhanced by interactions with the host immune system (48).

The URT is considered to be a major reservoir for potential pathogens, including *S. pneumoniae*, from where it could expand and arrive to the lungs and potentially cause infection (33). Thus, the study of the microbiome in the respiratory tract will shed light in the understanding of susceptibility to pulmonary infections, pneumococcal pneumonia or even IPD.

Main advances on this field have been achieved on pediatric population and adult population with chronic respiratory diseases, as chronic obstructive pulmonary disease (COPD). A relation of recent studies addressing the relationship between respiratory microbiome and infection is showed in **Table 1**. In samples from the respiratory tract of children, it has been described a high inter-individual variability in bacterial composition (81). Despite this variability, the bacterial composition has been proposed to be within discrete categories where some bacterial taxa dominate the community. In infants between 1 and 3 months of age, the hypopharynx was found to have five "pneumotypes" which follow concrete trajectories during the child's development (82). Distinct microbiota profiles have been detected in young children (under 2 years old). Early-life profiles are associated with microbiota stability and change patterns of change during the two first years of life. During the first 2 years, stable microbiota profiles were characterized by an early colonization of *Moraxella*, and *Dolosigranulum* combined with *Corynebacterium*, whereas instability was associated with profiles dominated by *Haemophilus* and *Streptococcus*. Moreover, infant feeding and frequency of respiratory infections in the parents were associated with the patterns of change. A history of breastfeeding and a reduced number of consecutive respiratory infections were associated with stable microbiota profiles (63).

Studies of the early URT microbiota in children have connected the early microbiota to the development of disease later in life and to the impact of important drivers. Accelerated microbiota maturation (enriched of *Neisseria* spp. and (facultative) anaerobes, mainly oral species including *Prevotella*) is associated with microbiota instability and increasing number of respiratory tract infections (RTIs) over the first year of life. These changed dynamics could be observed within the first month of age and, as stated previously, might be connected to important drivers: a healthy microbiota development and stability might be linked to vaginal delivery and breastfeeding, although this connection is controversial (83, 84).

In 2020 Chapman et al. conducted a large, retrospective study in children in order to address the reasons why some infants are more susceptible to respiratory infections than others (79). In this study, the cohort was divided into two groups: "infections and allergy prone group (IAP)" and "non-infection and allergy prone group (NIAP)." Males and daycare attendance were independently identified as risk factors for IAP. In terms of microbiome characteristics, colonization of NP in an early-age (between 6 and 36 months) with any of the three pathobionts most frequently associated with respiratory infections (*S.pneumoniae, H.influenzae* and *M.catarrhalis*) was associated with the IAP group.

More concretely, regarding the potential relationship between pneumococcal infection and microbiome, a case-control study was conducted in Spain to compare the nasopharyngeal microbiota of children with IPD (cases) and healthy children (controls) -representative of a healthy nasopharyngeal niche. In this study, bacterial richness and diversity were significantly higher in the groups of children who developed IPD. Three clusters corresponding to three different nasopharyngeal-types (nasopharyngeal types A, B or C, respectively) were observed. These patterns were significantly associated with the classification of the patients into cases and controls. The most frequently detected pattern in children with IPD (observed in 50.0% of the cases) was nasopharyngeal-type B, mostly represented by the genera *Streptococcus* (36.9%), *Staphylococcus* (21.3%), *Veillonella* (9.8%) along with a diversity of anaerobic genera (*Prevotella* and *Porphyromonas*). The other two nasopharyngeal types [type C, composed of *Haemophilus* (52.1%), *Moraxella* (31.4%) and *Streptococcus* (11.4%)] and type A [composed mainly of the genera *Dolosigranulum* (44.3%), *Moraxella* (29.3%) and *Haemophilus* (10.5%)] were detected in 32.1 and 17.9% of children with IPD, respectively. Conversely, the nasopharyngeal-type A was the most frequently related with healthy controls, leading to the hypothesis—and according to previous studies (63)—that *Dolosigranulum* sp. could confer resistance against pneumococcal infection (70). A clear imbalance was observed with a high frequency of *Veillonella* and other oral microorganisms which were be relatively infrequent in controls. These results were surprising given that higher bacterial diversity has been associated with health, and lower bacterial diversity has been associated with disease (85).

In adults, a recent study was designed to evaluate the characteristics of the NP microbiota in the pneumococcal acquisition (86). At baseline, samples from the NP of the healthy volunteers enrolled, showed mainly species from four genera: *Corynebacterium, Dolosigranulum, Staphylococcus* and *Streptococcus.* From these frequently found bacteria, the NP microbiome could be divided into five different profiles (A-E). Profiles B-E were dominated by *Staphylococcus* sp., *Streptococcus* and *Corynebacterium* sp. or *Corynebacterium* sp. and *Dolosigranulum* sp. combined, whilst profile A showed more diversity. The latter profile was more frequently associated with *S.pneumoniae* carriage (86). In other study, where bronchoalveolar lavage samples were analyzed, two pneumotypes in asymptomatic adults were found: one of them, enriched with supraglottic-characteristic bacteria *Prevotella* and *Veillonella* was associated with higher levels of inflammatory markers (57).

A large revision on the role of microbiome in the innate immune response related to chronic lung diseases (CLD) was published in 2020 (87). COPD is a progressive inflammatory disorder characterized by persistent airflow limitation, obstructive bronchiolitis and parenchymal destruction (88) with a high disease burden and related mortality rates. An specific pattern of microbiota has been identified in COPD patients, in comparison with healthy controls (87). *Proteobacteria* were more frequent in COPD than *Bacteroidetes* (and *Prevotella* spp. was specially reduced) (89). COPD mortality is higher during periods of acute exacerbations (AECOPD). During AECOPD, patients show a temporally dynamic sputum microbiome

TABLE 1 | Studies of respiratory microbiota related to lower respiratory tract infections.

Reference	Population, number of patients	Methods	Main findings
Zhou et al. (73)	Adults, 101	Observational, prospective	• *Streptococcus, Staphylococcus, Pseudomonas*, and *Acinetobacter* were identified as the most important species in hospital-acquired pneumonia
Bousbia et al. (74)	Adults, 210	Observational, prospective, case-control	• *Pseudomonas aeruginosa* and *Streptococcus* sp. were as common in pneumonia patients as in controls • Different microbiota patterns were presented in different forms of pneumonia
Iwai et al. (75)	Adults (HIV-infected), 15	Observational, prospective, case-control	• HIV-patients had increased abundance of species including several pathogenic microorganisms compared to controls
Chaban et al. (76)	Adults, 65	Observational, cross-section	• Respiratory microbiota composition of H1N1 patients relied on 11 different patterns dominated by one or two microorganisms
Leung et al. (77)	Adults, 22	Observational, prospective, longitudinal	• H1N1 infection could provide with previously unrecognized pathogens which could travel to the LRT and cause infection in weakened patients
Biesbroek et al. (63)	Children, 60	Observational, prospective, longitudinal	• Distinct patterns of NP microbiota in healthy children from infancy were associated with stable composition and less susceptibility for further respiratory infections
de Steenhuijsen Piters et al. (78)	Adults (elderly), 191	Observational, prospective, longitudinal with two cohorts	• Pneumonia in either young and elder adults is related with URT microbiome dysbiosis • Dysbiosis was characterized by bacterial overgrowth of *S.pneumoniae*, *Rothia* ad *Lactobacillus* and absence of anaerobic bacteria
Man et al. (61)	Children, 29	Matched case-control study	• Presence and severity of LRTI was associated with the URT microbiota composition • *S.pneumoniae* and *Haemophilus* were associated with disease
Camelo-Castillo et al. (70)	Children, 56	Observational, prospective, longitudinal	• Three nasopharyngeal microbiota patterns significantly associated with case and healthy controls • The dominated by *Streptococcus* sp. type was more frequent in cases; the type mainly composed by *Dolosigranulum* sp. was more frequent in controls.
Chapman et al. (79)	Children, 358	Retrospective	• NP colonization of *Moraxella* at early infancy was associated with less microbiota diversity and co-colonization with *S.pneumoniae* and *H.influenzae* • Colonization with *Moraxella* supposed an increased risk for respiratory illnesses
Rueca et al.(80)	Adults, 31	Observational, cross-section, case-control	• Patients with SARS-CoV-2 infection had a complete depletion of *Bifidobacterium* and *Clostridium* • In the case group, *Salmonella*, *Scardovia, Serratia* and *Pectobacteriaceae* were present

Abbreviations: LRT: lower respiratory tract, NP: nasopharyngeal.
URT: upper respiratory tract, LRTI: lower respiratory tract infections.

(90). In addition, the sputum microbiome profile at first day of exacerbation is related to 1-year mortality. The absence of *Veillonella* increases mortality risk by 13-fold whereas the presence of *Staphylococcus* increases the risk by 7-fold. When these two factors were combined in the same individual, 1-year mortality risk in COPD increased by 85-fold (91). Thus, reduced diversity of microbiome in sputum of AECOPD patients confers a poor prognosis, which is consistent with the previous association of higher bacterial diversity and health (90, 91).

Asthma has largely been studied on its relationship with microbiome. Several studies have identified, on one hand, the respiratory microbiota pattern related to asthma (high abundance of *Haemophilus influenzae, Streptococcus pneumoniae, Staphylococcus aureus,* and *Moraxella catarrhalis*, which can potentially play a pathogenic role) (87, 92) and on the other, that these species have a role on the inflammatory response which could determine the outcomes in asthma (93).

Apart from CLD, a limited number of studies have investigated the possible influence of the URT microbiome on the development of LRTIs in adult patients. In general, published studies t include moderate sample sizes without a healthy control group (73, 74), or specific risk groups like

HIV (human immunodeficiency virus)-infected patients (75), or patients infected with the pandemic H1N1 influenza virus (76, 77).

In the last year, the spreading of SARS-CoV-2 a novel *beta-coronavirus*, has provoked a world pandemic. COVID-19, the disease caused by this new virus, ranges from an asymptomatic estate to a severe pneumonia associated to a potentially lethal adult respiratory distress syndrome (SDRA) (94). In the search of answer to the question of why this respiratory virus could affect humans in such a different way, the role of respiratory microbiota has been put on the spot. A very recent review about the role of respiratory microbiota in COVID-19 patients (95) concluded, based on previous evidence, that the dysbiosis in the microbiota in COVID-19 patients would potentially lead to infection or progression of the disease. However, studies on the characteristics of microbiome in COVID-19 patients are still scarce and conducted in a small number of patients to make them suitable for extrapolating conclusions (80, 96).

Although it seems reasonable that the respiratory microbiome composition may play a role in the development of IPD, there is, to our knowledge, one single study that has investigated this association in elderly and young adults (78). In this study by de Steenhuijsen et al. the differences in microbiota profiles between patients with pneumonia and their healthy controls were identified as an independent factor. In the elderly, pneumonia was associated with *Rothia, Lactobacillus* and *Streptococcus (pseudo) pneumoniae* whilst healthy adults showed greater diversity and higher richness of especially three different patterns of microbiome (*Prevotella melaninogenica, Veillonella* and *Leptotrichia*) in the oropharynx samples (78).

THERAPEUTIC OPTIONS IN RESPIRATORY INFECTIONS

One of the main applications of the knowledge of microbiota composition are the potential therapeutic options. The modification of microbiota profiles to a protective pattern which could lead to a less degree of tissue inflammation, damage and therefore disease, is an attractive approach for this novel research (97).

A previous systematic review evaluated 23 trials in children to evaluate the efficacy of probiotics for prevention and treatment of recurrent respiratory infections (RRI) (98). After meta-analysis and considering the available evidence, probiotics were postulated as a possible alternative therapeutic option for RRI in children. However, the probiotic strain, dosage and administration forms were very heterogeneous among the different studies analyzed.

Human-associated microorganisms are able to produce secondary or specialized metabolites (natural products) which could mediate in the interactions between host and microbes and between microbes themselves (99). These natural products released by non-pathogenic species could be a novel source of antimicrobials, due to their antimicrobial activity against pathogen species (99, 100). More recently, Manti et al. conducted a prospective study in order to prove the *a priori* protective role of *Streptococcus oralis* and *Streptococcus salivarius* using them as a therapeutic option (101). Ninety-one children between 1 and 12 years old were prospectively included in a single-open study, in which a nasal spray composed of *Streptococcus oralis* 89a and *Streptococcus salivarius* 24SMBc was administered. After probiotic treatment, clinical improvement was reported, even in children with previous history of atopy or allergies. However, results were only applicable for the initial 3-months, due to a lack of follow-up period in the study protocol.

Phage therapy has arisen as a promising therapeutic tool in the era of antimicrobial resistance (102). Phage lysins, which are encoded by phages, are cell wall hydrolases that selectively act against different peptidoglycan bonds. These proteins can attack especially Gram-positive bacteria by splitting the bacterial cell wall (103). Some lysins have demonstrated activity against *S.pneumoniae* (104). In a recent research, a new phage lysine, 23TH_48 has been postulated as a potential therapeutic weapon for pneumococcal infections, combined with other lysins or antibiotics. This sinergyc combinations could be used to broaden the spectrum of action and improving their antimicrobial activity (103).

DISCUSSION

Recent advances in molecular typing techniques have identified not only that the lungs are not sterile –as was classically believed- but also that the respiratory tract is colonized by microbial species, which change between healthy individuals and those affected with respiratory diseases (53–57). Microbial populations play an important role in health. Along the human airways, structures above the vocal cords are exposed to high bacterial burden producing contamination of lower airway secretions from the URT (57).

We have reviewed the most important factors known to be associated with pneumococcal disease and we have focused on the available evidence of the role of respiratory microbiome in the development of respiratory infections in children and adults. It has not been established how these previously identified factors might impact in respiratory microbiota development and thereby in susceptibility to LRTIs. Despite the many comorbidities and conditions that have been identified as risk factors for the development of IPD, only the extremes of age (<5 years and >60 years old) have been strongly consistently found to be a major risk for IPD (13, 19, 20). Lung colonization is believed to start early after birth, with different profiles related with different factors, such as infant feeding. Stable patterns were associated with less risk of respiratory infections whilst changing patterns were associated with increased incidence of respiratory infections (63). The presence of these changing microbiota patterns–with *Haemophilus* and *Streptococcus* dominant profiles- in children under 2 years of age could explain the higher incidence of IPD in this population. On the other hand, changes on respiratory microbiota through age in the adults were also associated with a higher risk for LRTI. The absence of anaerobic species in the very old –a phenomenon linked to increasing age- could

be associated with a high susceptibility for pneumonia at the extreme of life (78).

Vaccination against pneumococcal has changed the epidemiology of the pneumococcal infections and, despite controversial results of previous studies, there has been a decrease in IPD incidence in the vaccinated population, even in the immunosuppressed (12, 22, 26–29). Pneumococcal vaccination has had also an impact in NP microbiota characteristics. In spite of a lack of evidence for a different composition between vaccinated and non-vaccinated children, a higher abundance was identified in patients after PCV 10 vaccine. This complexity could explain that, after vaccination, individuals are less prone to suffer acute respiratory tract infections (105).

Several host genetic polymorphisms which control the pathways of the immune system to combat bacterial infection have been identified as a risk factors for protection or susceptibility to pneumococcal pneumonia (32). Considering that some factors -as, for example, CXCL16- are regulated by microbiota through modulation of the quantity of iNKT cells in the gut and lung, leading to a higher tissue inflammatory response (46), these genetic variations could lead to a different expression of signaling proteins which could be, in turn, modified by the different microbiota patterns conferring more risk or protection against pneumococcal pneumonia.

Studies in recent years have focused on characterization of the respiratory microbiota, and concluded that the LRT microbiota composition comes from the URT (48, 53–59). Due to its role in the regulation of the immune response and inflammation (45, 46), the respiratory microbiome has been associated to the development and exacerbations of chronic lung diseases, as asthma and COPD (87–93). However, the most relevant findings in this review have been the implication of respiratory microbiome in pneumonia. Thus, colonization of the NP with either *S.pneumoniae*, *H.influenzae* or *M.catarrhalis* in children was associated with a tendency for respiratory infections and allergy in the pediatric period. Moreover and related to the scope of this review, in the study conducted by Camelo-Castillo et al. different microbiota patterns on the NP of children, were associated to IPD or asymptomatic colonization in this population (70).

Conversely, in the adult less is known on the impact of microbiome development of LRT infections, including pneumonia and more specifically, pneumococcal pneumonia and IPD. De Sreenhuijsen et al. found -in contrast with the previous studies in children- that anaerobic species were highly represented in old patients with pneumonia, who also had a less diverse and rich oropharyngeal microbiota profile (78).

The identification of microbiota profiles associated to IPD or asymptomatic colonization may be of clinical value as disease biomarkers. According to recent encouraging data about the potential role of probiotics in the treatment and prevention of respiratory infections (mainly in pediatric population) (97, 98, 101), the characterization of beneficial bacteria in adults -preventing or protecting against pneumococcal infection- would allow integrating those microorganisms in a probiotic preparation for the treatment or prophylaxis of pneumococcal infections and IPD.

The main limitation of our study is that we have tried to conduct an unbiased, accurate review of the most relevant literature regarding the evidence on the role of the respiratory microbiota in the development of pneumococcal pneumonia and especially, IPD. However, some relevant publications could have gone unnoticed by our research system and therefore not included in the current literature review.

Finally, considering that the microbial ecosystem of adults is relatively stable in the absence of gross perturbations, the role of microbiome in IPD in children might be likely reproducible in adults.

Data regarding the potential relationship between NP microbiome and the risk of developing IPD in adults, viral coinfection and severity of disease are scarce, and specific research in this area is needed. Although NP microbiome in patients with IPD has not been properly characterized yet, there seem to be discordant results between pediatric and adult populations. New, longitudinal studies, with larger number of participants and a homogeneous system to collect samples should help to elucidate the potential role of the previously observed microbial species in adults and their relationship with increasing or reducing risk for the development of respiratory infections, especially IPD.

DATA AVAILABILITY STATEMENT

The original contributions generated for the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

DH, EC, LB-P, and CM-A contributed to the conception and the design of the review. DH, EC, LB-P, and BD contributed to the literature research and selection. JG contributed to the coordination of the review. BD and EC were the major contributors in writing the manuscript. All the authors read and approved the final manuscript.

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Novel Cephalosporins in Septic Subjects and Severe Infections: Present Findings and Future Perspective

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In past decade, cephalosporins have developed significantly, and data regarding novel cephalosporins (i.e., ceftobiprole, ceftaroline, ceftolozane/tazobactam, ceftazidime/avibactam, and cefiderocol) within septic and bacteremic subjects are rising. These compounds generally offer very promising *in vitro* microbiological susceptibility, although the variability among gram-negative and -positive strains of different cohorts is noticed in the literature. We require further pharmacological data to measure the best dose in order to prevent sub-therapeutic drug levels in critically ill patients. These new compounds in theory are the sparing solution in the *Enterobacteriales* infection group for different antimicrobial classes such as aminoglycosides notably within endovascular and GNB-bacteremias, as well as colistin and carbapenem-sparing strategies, favoring good safety profile molecules. Moreover, new cephalosporins are the basis for the actual indications to open up new and exciting prospects for serious infections in the future. In future, patients will be addressed with the desirable approach to sepsis and serious infections in terms of their clinical situation, inherent features of the host, the sensitivity profile, and local epidemiology, for which evidence of the use of new cephalosporin in the treatment of severe infections will fill the remaining gaps.

Keywords: cephalosporin, sepsis, severe infections, blood-stream infections, multi-drug resistant bacteria

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INTRODUCTION

Sepsis has been defined as "one of the oldest and most elusive syndromes in medicine" (1). In 2017 Rudd et al. estimated, worldwide, 48.9 million cases of sepsis and 11.0 million deaths due to sepsis (2). Sepsis complexity resides in a dysregulated host response to an infection and in jeopardy to develop acute organ dysfunction with a high risk of in-hospital mortality rates estimated between 25 and 30% (3). This syndrome requires urgent treatment; thus, an awareness of the presenting characteristics of sepsis is highly important (4). In medicine, we must always look to the past with an eye to the future. From this perspective, this paper evaluates and discusses the available data on novel cephalosporins (i.e., ceftobiprole, ceftaroline, ceftolozane/tazobactam [C/T], ceftazidime/avibactam [C/A], and cefiderocol) (5) in the antimicrobial management of sepsis and severe infections to assess whether these new molecules can provide innovative answers to ancient questions (6).

CEPHALOSPORINS WITH A MAIN ANTI-MRSA ACTIVITY

Ceftobiprole

Ceftobiprole medocaril is currently approved in Europe as an extended-spectrum cephalosporin for adult community acquired and nosocomial, non-ventilator-associated pneumonia (i.e., CAP and HAP) (7, 8), and skin and soft tissue infection (SSTIs), including diabetic foot infections (9, 10).

Clinical application and actual experiences with ceftobiprole are limited but nevertheless promising for sepsis and bloodstream infections (BSIs) (11, 12). A discreet proportion of gram-positive sepsis was included in phase III trials for CAP, HAP and SSTIs (7–10). Rello et al. (13) developed two interesting grouped analyses—a test of cure (TOC) and mortality—for ceftobiprole, vs. comparators (e.g., vancomycin, linezolid, and ceftazidime) for *Staphylococcus* spp. BSIs through the extrapolation of the clinical data directly from phase III studies (7–10). In the TOC analysis, the clinical cure rate of the ceftobiprole group (48.9 percent, 22/45 patients) was similar to that of the comparators (44.0 percent, 22/50), specifically the subgroups of coagulase-negative staphylococci (CoNS; 45.5 vs. 45.5%), methicillin-sensitive *Staphylococcus aureus* (MSSA; 44.4 vs. 46.7%), and methicillin-resistant *S. aureus* (MRSA; 55.6 vs. 22.2%) (12, 13). Furthermore, the 30-day all-cause mortality in the ceftobiprole group was 8.9% (4/45) vs. 16.0% (8/50) in the comparator group (12, 13). In the ceftobiprole group, death rates were zero for MRSA bacteraemic patients compared with 22.2 per cent in the comparator cohorts (12, 13). Despite the interesting results, the conclusions drawn by Rello et al. had been obtained from a sample of just 18 patients, without a complete analysis of all data in the entire subgroup (13).

Ceftobiprole (alone or in combination) could therefore play an important role in treating endovascular infections due to its high bactericidal activity, favorable resistance profile, and potential synergism with other antigram-positive molecules (11–13). In a rat model of endocarditis, a subtherapeutic dose of ceftobiprole plus vancomycin was as effective against MRSA and vancomycin intermediate-resistant *S. aureus* strains as ceftobiprole standard dose monotherapy (14, 15). Theoretically, ceftobiprole used to treat endocarditis could permit a nephrotoxic-sparing strategy, thus avoiding the aminoglycoside and glycopeptide side effects and the need for therapeutic drug monitoring. Ceftobiprole also plays a vital role in penicillin-allergic subjects with severe gram-positive infections (11–13). A daptomycin-based scheme, complemented with an adjunct of ceftobiprole, also seems promising in clinical applications within endocarditis therapeutic management (16, 17). An ongoing non-inferiority trial (double-blinded and randomized, https://clinicaltrials.gov/ct2/show/NCT03138733) comparing standard dose ceftobiprole and daptomycin (6 mg/kg/24 h) in adult patients with *S. aureus* bacteraemia (including right-sided infective endocarditis) will provide more information (18). One potential concern surrounding ceftobiprole is determining the adequate dosage for bacteraemia and endocarditis. The prospect of accomplishing this target for MRSA strains with the ongoing approved dose is >90% with MIC of 4 mg/L (19–21).

A higher exposure (100% T > MIC) is however associated with strong bactericidal action and therefore is the preferred goal for severe and high inoculum infections (19–21). With the current dose the probability of receiving 100% > T > MIC for MRSA strains will be lower, but this can be improved significantly through the prolonged infusion (over 4 h) or ongoing infusion of patients at higher doses (500 mg/6 h or 1 g/8–12 h) (19–21).

Little is known about CSF penetration for ceftobiprole, which may still be a valuable option in primary meningitis caused by *Streptococcus pneumoniae* (including penicillin- and ceftriaxone-resistant strains) and secondary, post-surgical meningitis requiring both gram-positive and susceptible gram-negative coverage (11–13, 22). The antibacterial activity was comparable to cefepime with β-lactamase-negative strains of *Escherichia coli*, *Klebsiella pneumoniae,* and *Haemofilus influenzae* in the animal model proposed by Stucki et al. (22). Moreover, the same authors (22) discovered that ceftobiprole reached about 16% of serum levels through inflamed meninges compared to about 2% of serum levels through uninflamed meninges in a rabbit meningitis model, similarly to cefepime pharmacokinetic in CSF.

This fifth-generation cephalosporin has been approved for official indications of CAP and HAP adults in 12 European countries, Canada, and Switzerland (11). Moreover, ceftobiprole blends excellent spectrum with beta-lactam safety for low to moderate MDR HAP pathogenes in frail patients who could be at great risk from adverse effects caused by MRSA or anti-MRSA coverage, including oxazolidinones or glycopeptides (6, 11, 12).

There are promising activities of this novel cephalosporins on MRSA isolates, including Panton-Valentine Leukocidine positive strains, whether or not it is a slight change depending on the type of SCCmec, as shown in recent findings concerning ceftobiprole isolates in Phase III SSTI and pneumonia studies (23, 24). In severe adult CAP, coinfection, or superinfection over viral pneumonia, ceftobiprole can be a suitable option when the risk for MRSA or susceptible *P.aeruginosa* coinfection is high (6, 11, 12).

VAP still represents an area of uncertainty according to clinical trial results (8) due to the inadequate sample size and substantial baseline sample heterogeneous features (6). Ceftobiprole data for BSIs in real life are limited, but promising, despite the need for further pharmacokinetic data in special populations such as patients with critical illness or elevated creatinine clearance (25).

Ceftaroline

Ceftaroline is a fifth-generation cephalosporin with a peculiar affinity to the penicillin binding protein (PBPs) 2a, an MRSA-specific protein, with an excellent spectrum of activity on common bacterial causes of CAP (26, 27) and SSTIs (28). Ceftaroline has activity against a wide spectrum of gram-positive bacteria including MSSA and MRSA, also including

some resistant *S. aureus* strains (vancomycin intermediate, heterogeneous vancomycin intermediate, vancomycin-resistant, or daptomycin non-susceptible) and MDR *S. pneumoniae* (29–31). Moreover, ceftaroline also exhibits activity against a broad group of gram-negative, ESBL-negative, or AmpC-producing *Enterobacteriaceae* (29–31). The US Food and Drug Administration (FDA) approved a label expansion for the treatment of *S. aureus* bacteraemia associated with SSTIs in adults in 2015 and in the pediatric population in 2016 (32). The literature regarding the bacteraemic cohorts of patients treated with ceftaroline (alone or in combination) is growing and has been mostly comprised of subjects with a diagnosis of either persistent bacteraemia or MRSA bacteraemia that is not susceptible to vancomycin or daptomycin (32–47). Persistent bacteraemia (with or without a known focus of infections) is not an uncommon finding, which may be associated with increased mortality and morbidity, and refers to blood cultures that were positive within the same infectious episode and on different days (33, 34). Interesting observations on time to eradication of MRSA in BSIs were extrapolated from a retrospective matched case-control study by Paladino et al. (33): the ceftaroline cohort reported a median time to eradication that was about half the median time of the control group treated with vancomycin (4 vs. 8 days; interquartile range [IQR]: 3.0–7.5 days vs. 5.8–19.5 days; $P = 0.02$). The time to eradication was further reduced to 2 days (IQR: 1–4 days) in Arshad et al.'s (34) multicenter observational study of 211 patients (33). Clinical success in the most representative studies has ranged from 60 to 88% (34–39). Interestingly, Arshad et al. (34) reported a clinical cure rate of 69.7% when ceftaroline was used as a monotherapy and 64.9% when it was used in combination. Furthermore, even a microbiological cure reported good results, between 70 and 100%, but these were variable depending on the time of consideration (34). Mootz et al.'s (45) retrospective comparative effectiveness study included adults hospitalized with sepsis who received ceftaroline or daptomycin within 14 days of hospital admission. Patients treated with ceftaroline were less likely to experience readmission at 30 days (25 vs. 37%, $P = 0.06$), 60 days (27 vs. 44%, $P = 0.008$) and 90 days (28 vs. 46%, $P = 0.01$) compared to those treated with daptomycin (45). Moreover, the ceftaroline group showed a lower in-hospital mortality rate (7 vs. 12%, $P = 0.4$) at 30 days (3 vs. 9%, $P = 0.1$), 60 days (6 vs. 12%, $P = 0.2$), and 90 days (7 vs. 15%, $P = 0.1$) vs. the comparator (45).

Ceftaroline, a rapid BSI clearance beta-lactam, also serves as an interesting solution to complicated endocarditis (46). This is illustrated by the retrospective CAPTURE analysis involving 55 patients receiving ceftaroline treatment with gram-positive endocarditis (47). The overall success rate in this study was 70.9% (47). In particular, ceftaroline therapies were extremely effective as a first-line therapy (75.0%) as well as in patients suffering from right-sided endocarditis (80.8%) and MRSA (77.3%) (47).

Ceftaroline is currently used in the management of persistent gram-positive bacteraemia (36) and, also due to the wide spectrum of microbiological activity comprising gram-negative organisms, may be a feasible choice for catheter-related and -associated BSIs (12, 32). The bactericidal and time-dependent activity of ceftaroline, as well as ceftobiprole, could potentially be implemented in therapeutic schemes for bacteraemia and endovascular infection to improve the safety of kidney function, limiting glycopeptides and the need for therapeutic monitoring, with consistent advantages in centers with scarce laboratory resources (11, 12, 32, 33). Ceftaroline could also be used in the treatment of severe infections, including both primary (e.g., post-traumatic) and secondary (e.g., post-surgical) bacterial meningitis, although few data are available for CSF drug concentrations (48, 49).

Stucki et al. (48) estimated that 15% of the serum level of CSF penetrated inflammatory meninges and about 3% of non-inflamed meninges. Mermer et al. (49) found that in an experimental meningitis model both ceftaroline and vancomycin have similar antibacterial efficacy in treating MRSA. Pani et al. (32) confirmed the use of ceftaroline as the fifth off-label indication for meningitis in their latest systematic research.

CEPHALOSPORINS WITH MAIN ANTI-GRAM-NEGATIVE ACTIVITY

Ceftolozane/Tazobactam

C/T is a combination of the renowned β-lactamase inhibitor tazobactam and an innovative anti-pseudomonal cephalosporin (5, 50). The FDA and the EMA have approved C/T for complicated urinary tract infection (cUTI) (51) and complicated intra-abdominal infection (cIAI) (52) at a dose of 1.5 g (ratio of 1.0 ceftolozane to 0.5 tazobactam) every 8 h, with double dosage (3 g; ratio of 2.0 ceftolozane to 1.0 tazobactam) in the phase III study ASPECT-NP for the treatment of NP (53). C/T is active in several multi-drug resistant (MDR) and extensively drug-resistant (XDR) *Enterobacteriales* strains, including ESBL-producting strains and *P. aeruginosa*. (54).

Regarding ESBL strains, C/T has differences depending on the pathogen concerned. C/T, in a study published by Tato et al. (55), seems to have higher activity against ESBL-producing *E. coli* than *Klebsiella* spp. Moreover, among ESBL pathogens, Castanheira et al. (56) have found that C/T retains a lower activity in bla_{SHV} isolates (61.1%) than $bla_{\mathrm{CTX-M}}$ strains (91.2%). All together, these data show that C/T is a major component of a carbapenem-sparing strategy, at least in the empiric setting, even if more evidence is needed to confirm the exact role as a targeted treatment of ESBL infections (57).

An increased risk of hospital mortality has been linked to inadequate initial antibiotic treatment for Pseudomonas aeruginosa BSI (58). From a microbiological perspective, 88 percent ($n = 615$) of isolates were susceptible to C/T in a major multi-center study carried out between 2012 and 2015 for meropenem-non-susceptible *P. aeruginosa* isolates in 32 US medically-based centers (59).

For contrast, additional single-center surveillance studies have shown a 60–94 percent C/T susceptibility of different *P. aeruginosa* isolate populations (60–62).

From a clinical point of view, Bassetti et al. (63) described one of the largest clinical trials using C/T in a multicenter cohort of 101 patients with documented *P. aeruginosa* infection. Sepsis and septic shock were present at diagnosis in 26.7 and 11.9% of patients, respectively, and concomitant *P. aeruginosa* bacteraemia was ruled out in only 15.8% of subjects (63).

The only independent predictor of clinical failure was sepsis (odds ratio [OR] = 3.02, 95% confidence interval [CI]: 1.01–9.2; $P = 0.05$) for patients with clinical success in comparisons to those suffering a clinical failure with multivariate analyses (odds ratio [OR] = 3.02, 95 per cent CI: [CI]: 1.01–9.2; $P = 0.05$) (63).

All together, these data show that C/T may be a valuable option to prevent nephrotoxicity with colistin- or aminoglycoside-sparing regimens including the risk of subtherapeutic dosages associated with reduced renal clearances (64, 65).

CEFTABUSE register (65) results showed a non-significant trend toward more favorable 14-day clinical cure rates in C/T patients than aminoglycoside or colistin (81.3 vs. 56.3%; $P = 0.11\%$). A similar pattern was found for crude deaths of 30 days (18.8 vs. 28.1%; $P = 0.73$) and acute kidney injury prevalence (0.0 vs. 25.0%; and $P = 0.04$), favors C/T vs. colistin or aminoglycoside.

Similarly, a retrospective multicenter observational cohort study [644] also found that C/T administration was independently associated with clinical cure (adjusted OR: 2.63; 95% CI: 1.31–5.30) and protected against AKI (adjusted OR: 0.08; 95% CI: 0.03–0.22) without any difference in in-hospital mortality. In addition, a systematic Maraolo et al. (62) study concluded that the therapy C/T could be useful even outside of an accepted setting of indication for difficult-to-treat *P. aeruginosa* infections: BSI is the third commonly indicated off-label (23/130; 17.7%).

Ceftazidime/Avibactam

C/A is an intravenous combination of a third-generation cephalosporin with the non-β-lactam/β-lactamase inhibitor avibactam (66), with activity against ESBL-producing bacteria, *P. aeruginosa* and KPC or OXA-48 carbapenemase producing bacteria. C/A has been approved in cIAI (67), cUTI (68), and NP (69) therapy as well as infection by microorganisms resistant to ceftazidime, according to a specific trial (RECLAIM, RECAPTURE, REPROVE, and REPRISE study, respectively) and, given the comparator mainly based on carbapenem drug administration, it is also a major component of a carbapenem-sparing strategy. Moreover, real-life data did show clinical efficacy in patients with KPC and OXA-48 carbapenemases. In a prospective study, Sousa et al. (70) described a cohort of 55 patients, 54% of whom presented with severe septic shock or sepsis; moreover, 54 of the 57 isolates were OXA-48-producing *K. pneumoniae*, 46% (26/55) of patients had confirmed bacteraemia, and C/A was mainly given as monotherapy (81%) with a mean duration of 13 days; The 14-day mortality rate was 14% (70). In systematic reviews and meta-analysis with infections by carbapenem-resistant bacteria, Fiore et al. (71) did not observe any difference in the mortality rates between C/A monotherapy or combination therapy ($N = 503$ patients; direct evidence OR: 0.96; 95% CI: 0.65–1.41), with similar findings in the unregistered systematic review by Onorato et al. (72).

A retrospective longitudinal study of 138 patients with KPC-Kp bacteraemia, in which a significantly lower mortality was observed in any patient with C/A than other drugs (36.5 vs. 55.8%, $P = 0.05$), and in which this was the only factor that was substantially correlated with survival, was addressed by Tumbarello et al. (73). In this complex analysis, the authors identified septic shock, neutropenia, Charlson Comorbidity Index ≥3, and recent mechanical ventilation as independent predictors of mortality, whereas C/A was the sole independent predictor of survival (73). In addition, Shields confirmed higher rates of clinical success ($P = 0.006$) and survival ($P = 0.01$) for C/A compared to other regimens and observed higher renal safety compared to aminoglycoside and colistin-containing regimens ($P = 0.002$) (74).

All together, data from official trials and real-life experiences showed that C/A is representing a major component in carbapenem-sparing strategies, including patients infected by KPC or OXA-48 carbapenemases producing bacteria in addition to *P. aeruginosa* and ESBL-producing bacteria (75).

Cefiderocol

Cefiderocol is an innovative siderophore cephalosporin that was produced to target carbapenem-resistant pathogens, including fermenting and non-fermenting (i.e., *P. aeruginosa* and *Acinetobacter baumannii*) GNB (76, 77). Among such pathogens is also included *Stenotrophomonas maltophilia*, which is intrinsically resistant to carbapenems (76, 78). In the CREDIBLE CR, cefiderocol was studied in a randomized, open-label, prospective, phase III clinical study in individuals with carbapenem-resistant gram-negative bacteria infections, regardless of species or infection site source and including sepsis and BSIs (ClinicalTrials.gov registration: NCT02714595) (79, 80). The clinical cure between cefiderocol and the comparator, defined as best available therapy (BAT), was similar for NP (50 vs. 53%) and BSI (43 vs. 43%). Besides, in complicated UTIs, cefiderocol was not inferior to the BAT group in microbiological eradication (53 vs. 20%) (80). In the cefiderocol group, more numerical deaths occurred, especially in the *Acinetobacter* spp subset, a finding which was not unequivocally explained. These results endorse cefiderocol as an alternative for treating patients with limited treatment options of carbapenem-resistant infections (80).

Wunderink et al. (81) recently published an APEKS-NP randomized double-blind, phase III, non-inferiority analysis in which 148 subjects and 152 subjects were allocated, respectively, for cefiderocol and meropenem. The authors suggested that cefiderocol was non-inferior in patients with Gram-negative NP equal to high dose extended-infusion meropenem and similar for all cases-mortality on Day 14 (12.4 vs. 11.6%) (81). The findings indicate that cefiderocol is a promising solution for treating NP, including those caused by MDR gram-negative organisms (81).

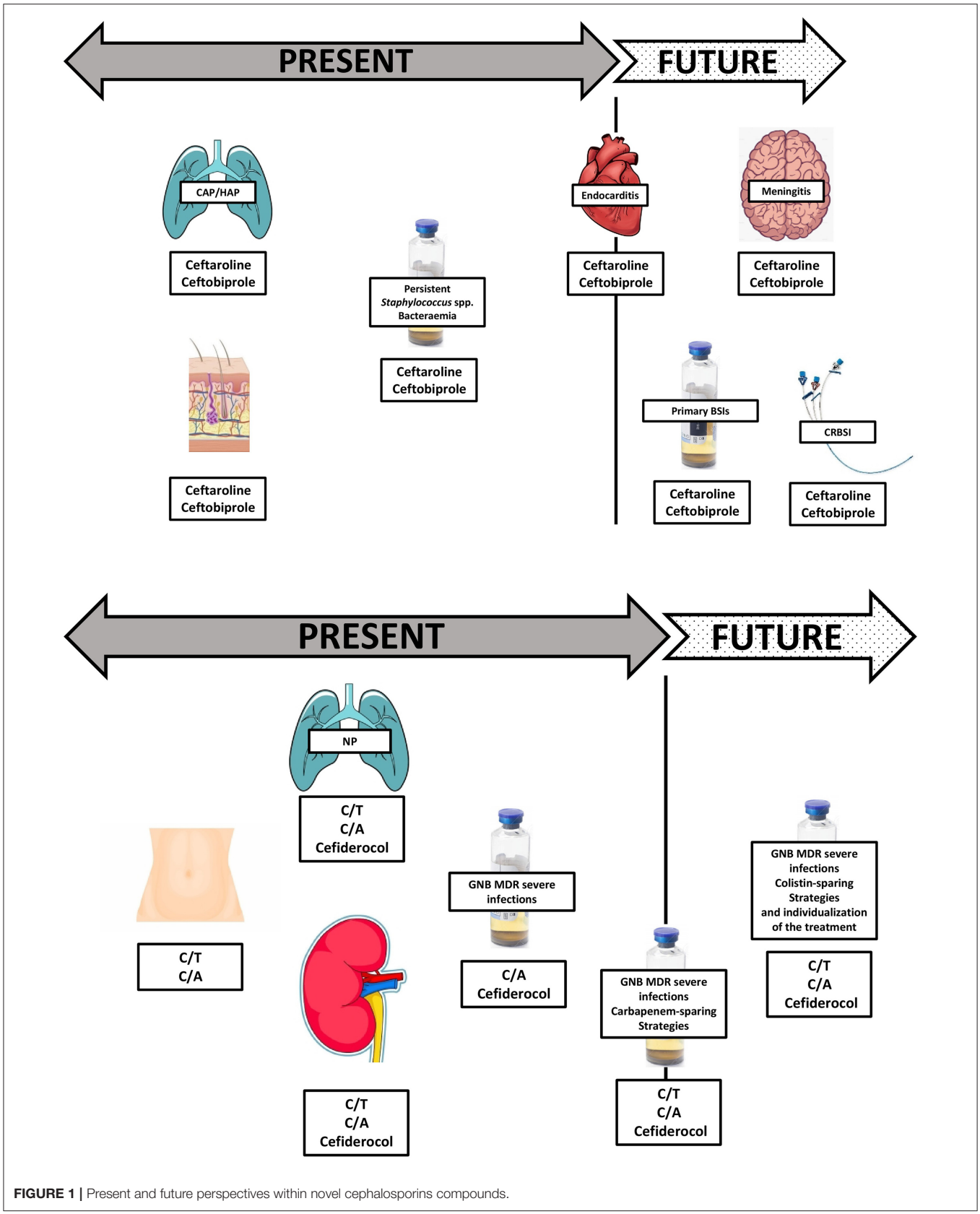


FIGURE 1 | Present and future perspectives within novel cephalosporins compounds.

TABLE 1 | Advantages of monotherapy and combination antimicrobial therapy.

PROs of Monotherapy	PROs of Combination Therapy
Low antibiotic pressure	Avoid resistance development in difficult-to-treat infections
Low risk of toxicities	Active on different mechanism
Improve de-escalation approach	In MDR infections to ensure sensitivity
Improve antibiotic stewardship bundle	Accelerate pathogen clearance high bacterial loads
Reduce the risk of antibiotic antagonism	Improve synergy between molecules
Improve diagnosis	Decrease the risk of inappropriate empiric antibiotic therapy

Hsueh et al. (82) measured *in vitro* cefiderocol, C/T, and C/A microbiological profile for *P. aeruginosa* and *S. maltophilia* and *A.baumannii* isolates in the bloodstream. In comparison with the C/T and C/A, Cefiderocol demonstrated much greater *in vitro* activity with MICs $\leq$ 4 mg/L for *P. aeruginosa* isolates resistant to colistin or imipenem (82).

Presently, in order to improve therapeutic effectiveness in serious CRE infections, high levels and combining methods which may have a new inhibitor β-lactam/β-lactamase are likely to be considered (77, 78, 83). Cefiderocol has been added to these therapeutic options, increasing the antimicrobial spectrum to *A. baumannii* and *S.maltophilia*, which in the last decade have been frequently omitted from the new molecules, theoretically allowing for enhanced individualization based on molecular resistance phenotypes, disease severity, susceptibility profiles, and patient characteristics in antimicrobial strategies (77, 78, 83, 84).

Besides, cefiderocol, from a pharmacokinetic point of view, is unique for its dosing regimen to include patients with an augmented renal clearance which is the primary cause for underdosing in very sick patients for beta-lactams.

DISCUSSION

The creation of three fifth-generation cephalosporins has made a major evolution in the last decade (i.e., ceftobiprole, ceftaroline, and ceftolozane), along with the development of cefiderocol, with its new "trojan horse" active transport mechanism for entering multi-drug resistant bacteria, as well as through novel therapeutic binomials (C/T and C/A) (5, 11, 44, 79). Data on novel cephalosporins may also be available, in addition to the official registration trials, in patients with bloodstream infections and severe infections as well as in infections by MDR bacteria including carbapenemases-producing bacteria from real-life settings. In addition, the future of antimicrobial stewardship in septic and bacteremic patients can be assessed through a multilevel evaluation from the microbiological, pharmacological, clinical, and financial perspectives (5, 12, 77, 78). Although the literature is clear on specific variability in gram-negative and gram-positive strains from various countries and cohorts, as well as different rates of carbapenem resistance, the *in vitro* susceptibilities of the novel cephalosporins are generally very promising. (5, 12, 77, 78). This variability makes it crucial to know their global and local epidemiologies, particularly in MDR gram-negatives with limited treatment options. Furthermore, there is a critical need for more pharmacological data to assess the best dosage and administration modalities in critically ill patients in order to avoid subtherapeutic levels of the drug (5, 12, 77, 78).

Theoretically, such new compounds allow a sparing approach in different antimicrobial classes, such as carbapenems, aminoglycosides, colistin, and also vancomycin for ceftaroline and ceftobiprole (12, 65, 77, 78). We are presented with molecules with an increasingly strong pathogen-specific identity, such as C/T for MDR/XDR *P. aeruginosa* and cefiderocol for difficult-to-treat CR strains, and we face a great change in the management of MRSA and endovascular bacteremia due to the evolving clinical data on ceftaroline and ceftobiprole (5, 11, 44, 79). Time seems to be on the side of these novel cephalosporins, making them useful in several severe infections (**Figure 1**). These molecules can also be used in therapeutic combinations, under particular circumstances. The primary rationale for using two distinct classes of antibiotics with activity against a single pathogen is, on the one hand, to potentiate pathogen clearance and, on the other, to assure the pathogen's susceptibility to the empiric therapy (85, 86). Conversely, monotherapy reduces the risk of antibiotic pressure, the rate of new infections, antibiotic antagonism, toxicity, and costs, though it may not cover MDR pathogens (**Table 1**) (85, 86). Combination antibiotic treatment was recommended in international guidelines for primary management of septic shock to provide appropriate empirical antibiotic coverage in a scenario with high risk of MDR pathogens (87). However, several other studies found no superiority of combination treatment, and some analyses showed an increased rate of side effects in patients receiving combination therapy (88–90). The desired approach to sepsis and serious infections would be presented to patients in the future based on their clinical condition, host characteristics, susceptibility profiles, and local epidemiology, which would fill the gaps in the use of new cephalosporins that currently exist. Finally, literature data emphasize the small spread of every study and also stress the importance of local monitoring. When determining early use of these agents in severely ill patients, careful consideration should be given to local susceptibility patterns.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

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

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Prognostic Significance of the Fission1/Parkin Ratio for Sepsis: A Prospective Cohort Study

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Introduction: Fission1 (Fis1) and parkin are key proteins related to mitochondrial fission and mitophagy, respectively. This study aimed to assess the prognostic value of the Fis1/parkin ratio as a biomarker in patients with sepsis.

Methods: Consecutive patients with sepsis ($n = 133$) or simple infection ($n = 24$) were enrolled within 24 h of arrival at the intensive care unit (ICU). Serum levels of Fis1, parkin, mitofusin2 (Mfn2), and peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) were measured by enzyme-linked immunosorbent assay (ELISA) upon ICU admission. Clinical parameters and standard laboratory test data were also collected. All patients received follow-up for at least 28 days.

Results: Patients with sepsis presented with significantly decreased serum levels of parkin, Mfn2, and PGC-1α, but an increased serum Fis1 level and Fis1/parkin, Fis1/Mfn2, and Fis1/PGC-1α ratios at ICU admission. Relative to patients with simple infections, the ratios were remarkably elevated in septic patients—particularly septic shock patients. The area under the receiver operating characteristic (ROC) curve of the Fis1/parkin ratio was greater than that of Fis1, parkin, Mfn2, and PGC-1α levels as well as that of the Fis1/Mfn2 and Fis1/PGC-1α ratios for prediction of 28-day mortality due to sepsis. All of the ratios were significantly higher in non-survivors than survivors at the 28-day follow-up examination. Fis1/parkin ratio was found to be an independent predictor of 28-day mortality in patients with sepsis.

Conclusions: The Fis1/parkin ratio is valuable for risk stratification in patients with sepsis and is associated with poor clinical outcomes for sepsis in the ICU.

Keywords: mitochondrial homeostasis, fission1/parkin ratio, sepsis, biomarker, prognosis

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INTRODUCTION

Sepsis is life-threatening organ dysfunction caused by a dysregulated host response to infection (1). Each year there are an estimated 48.9 million incident cases of sepsis worldwide and 11.0 million sepsis-related deaths, representing 19.7% of all global deaths (2). However, effective management of sepsis and resource allocation remain a challenge due to the inability to accurately diagnose the severity and risk of sepsis. Therefore, prognostic biomarkers are needed for early identification of patients at high risk of sepsis. Such patients could be transferred to the ICU and receive optimized hospital resources and therapies.

Among the complex mechanisms of sepsis and its heterogeneous nature, defective mitochondrial quality control (MQC) plays an important role in the severity of sepsis and sepsis-induced multiple organ dysfunction syndrome (MODS) (3–6). The MQC system aims to maintain mitochondrial homeostasis, allowing the mitochondrial network to segregate, recognize, and eliminate damaged mitochondria and to generate new mitochondria. MQC processes include mitochondrial biogenesis, mitochondrial dynamics (mitochondrial fission and fusion), and mitophagy (7, 8). Previous studies have demonstrated that sepsis is ameliorated by the recovery of mitochondria homeostasis (4, 9, 10). Therefore, indicators related to mitochondrial homeostasis may be useful for risk stratification and prognostic evaluation of patients with sepsis.

Fission 1 (Fis1), parkin, mitofusin2 (Mfn2), and peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) are four key proteins involved in mitochondrial fission, mitophagy, mitochondrial fusion, and mitochondrial biogenesis, respectively (10). It has been shown that recovery of mitophagy, mitochondrial fusion, and biogenesis can partly reverse organ failure under septic conditions, whereas worsening of sepsis is accompanied by activation of mitochondrial fission (3, 11–14). In other words, elevations of parkin, Mfn2, and PGC-1α appear to protect against organ dysfunction in animal models of sepsis, whereas Fis1 is associated with sepsis severity and multiple organ dysfunction.

The peripheral blood samples are an attractive tissue for biomarker discovery as they are easily obtained and analyzed. It has been proved that the expression of Fis1, Mfn2, parkin and PGC-1α in peripheral blood mononuclear cells (PBMCs) could give us some information about the mitochondrial quality control status (15–19), and considering the immune cell death during sepsis (20, 21), MQC-related proteins in the immune cells could continuously release into circulation, making the detection of these proteins in serum possible.

Simultaneous measurement of multiple biomarkers may be useful for overcoming the limitations of using a single biomarker. Assessment of multiple biomarkers associated with different sepsis-related pathways may be particularly useful. Thus, in the present study, we use the Fis1/parkin, Fis1/Mfn2, and Fis1/ PGC-1α ratios to reflect the severity of mitochondrial homeostasis disbalance. This study aimed to investigate the feasibility of using the Fis1/parkin, Fis1/Mfn2, and Fis1/ PGC-1α ratios to predict the prognosis of septic patients and to identify what ratio provided the best performance.

Abbreviations: MQC, mitochondrial quality control; PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1α; Fis1, fission protein 1; Mfn2, mitofusin2; ICU, intensive care unit (ICU); APACHE II score, Acute Physiology and Chronic Health Evaluation II score; CRRT, continuous renal replacement therapy; HR, heart rate; MAP, mean arterial blood pressure; MV, mechanical ventilation; NE, norepinephrine; SOFA, sequential organ failure assessment score; CVP, central venous pressure; Lac, lactic acid; PaO2, arterial partial pressure of oxygen; PI, perfusion index; Pv-aCO2, arteriovenous carbon dioxide partial pressure difference; ScvO2, central venous blood oxygen saturation; PCT, procalcitonin; cTnI, cardiac troponin I; NT-ProBNP, N-terminal pro-brain natriuretic peptide.

MATERIALS AND METHODS

Patients

This prospective study was carried out at Peking Union Medical College Hospital (PUMCH) between June 2019 and August 2020. The study was approved by the PUMCH institutional review board (approval number JS-2421) and informed consent was obtained from all enrolled patients or their relatives. Patient records were anonymized and deidentified before analysis.

The inclusion criteria were: (1) age ≥18 years and (2) diagnosis of sepsis [according to The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) (1)]. The exclusion criteria were: (1) age <18 years, (2) massive bleeding or pulmonary embolism, (3) heart attack or acute exacerbation of previous heart disease in the previous week, (4) heart surgery in the previous week, and (5) lack of informed consent by the patient or their relatives.

A total of 133 septic patients, who were followed for 28 days or until death, were enrolled in this study. Septic patients were divided into non-shock and shock subgroups. The criteria for inclusion in the shock subgroup were: a clinical construct of sepsis with persisting hypotension requiring vasopressors to maintain MAP ≥ 65 mmHg and a serum lactate level >2 mmol/L (18 mg/dL) despite adequate volume resuscitation on the day of ICU admission (1). Septic patients who did not meet these criteria were assigned to the septic non-shock group. Additionally, 24 patients with simple infection, but who did not meet the criteria for sepsis, admitted to the intensive care unit (ICU) served as a control group.

Baseline clinical and laboratory characteristics were obtained from medical records and routine ICU tests, including patient age, sex, hemodynamic parameters, blood chemistry, arterial blood gas analysis, Acute Physiology and Chronic Health Evaluation (APACHE) II score, and SOFA score.

Sample Collection and Measurement

Peripheral blood samples were collected within 24 h of ICU admission and centrifuged immediately. Serum was withdrawn and stored at −80°C until assessment by enzyme-linked immunosorbent assay (ELISA). The serum levels of Fis1, parkin, Mfn2, and PGC-1α were determined using commercially available ELISA kits following the instructions of the manufacturer (Fis1: Abbexa abx151559 Cambridge, UK; parkin: Abcam ab212159 Shanghai, China; Mfn2: Abebio AE33636HU Wuhan, China; PGC-1α: Cusabio CSB-E11761h Wuhan, China). According to the manufacturers' specifications, the ELISA assays were specific for native proteins, with no significant cross-reactivity with known analogs. Each well of the ELISA plate was loaded with 100 μL undiluted blood sample, and we did not measure the protein concentration before loading the plate.

Statistical Analysis

Results are presented as the mean and standard deviation or median and range (interquartile range). Group differences for continuous variables with a non-normal distribution were

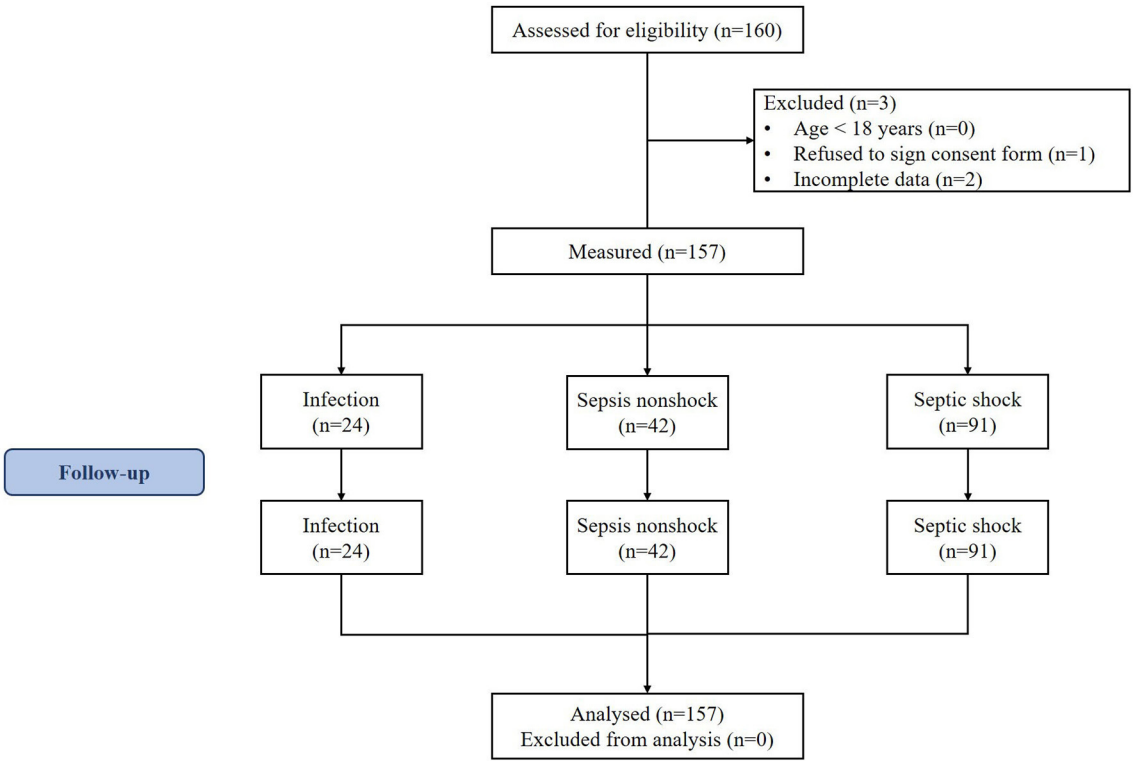


FIGURE 1 | CONSORT flow diagram of the study.

tested using the Kolmogorov-Smirnov test and Mann–Whitney *U* test. Categorical variables are reported as proportions and compared using the χ^2 or Fisher's exact tests. Logistic regression analysis was used to determine independent predictors of 28-day mortality. The receiver operating characteristic (ROC) curve was used to assess the accuracy of the variables for prediction of 28-day mortality. The Kaplan–Meier method was used to analyze the survival data. Comparisons between groups were performed using the log-rank test. $P < 0.05$ was considered to be statistically significant. All statistical analyses were performed with SPSS 22.0 software (IBM Inc.).

RESULTS

Patient Characteristics

Patient recruitment for this study is shown in **Figure 1**. A total of 133 septic patients and 24 infected patients (controls) were enrolled in the study.

There were no significant differences in age, central venous blood oxygen saturation (ScvO2), or arteriovenous carbon dioxide partial pressure difference (Pv-aCO2) between the two groups (infection and sepsis). APACHE II and SOFA scores differed between groups, with the sepsis group scoring significantly higher ($p < 0.001$). The basic characteristics all enrolled patients are listed in **Table 1**.

TABLE 1 | Characteristics of the study population.

Characteristics	Infection	Sepsis	*P*-value
Number of subjects	24	133	
Demographic data			
Age (yr)	62 (46–68)	65 (56–72)	0.092
Male, *n* (%)	21 (87.5%)	87 (65.4%)	0.032
Physiological data			
MAP (mmHg)	94.87 ± 14.18	85.73 ± 13.84	<0.001
HR (beats/min)	87 (77–100)	100 (86–114)	<0.001
PaO2/FiO2 ratio	356 (281–482)	278 (194–379)	<0.001
Lac (mmol/L)	1.0 (0.7–1.4)	1.6 (1.1–2.4)	0.002
Pv-aCO2 (mmHg)	4.6 (4.4–5.0)	3.7 (2.4–6.3)	0.368
ScvO2 (%)	69.2 (64.0–73.8)	74 (68.4–78.9)	0.252
SOFA score	0.5 (0–1)	12 (9–14)	<0.001
APACHE II score	13.5 (8–17)	20 (16–26)	<0.001
28-day mortality, *n*(%)	0 (0%)	18 (13.5%)	<0.001

Date was presented by mean ± standard deviation, n (%) or median (interquartile range). HR, heart rate; MAP, mean arterial blood pressure; Pv-aCO2, arteriovenous carbon dioxide partial pressure difference; ScvO2, central venous blood oxygen saturation; SOFA, sequential organ failure assessment; APACHE II, Acute Physiology and Chronic Health Evaluation II score.

Group Differences in Serum Levels and Ratios

Table 2 shows, for each study group, the serum Fis1, parkin, Mfn2 and PGC-1α levels; Fis1/parkin, Fis1/Mfn2, and Fis1/

PGC-1α ratios; and the PCT level. **Figure 2** presents the Fis1/parkin, Fis1/Mfn2, Fis1/ PGC-1α ratios and serum PGC-1α, Fis1, Mfn2, parkin levels for each subgroup (infection, sepsis nonshock and septic shock groups). Serum PCT, Fis1, parkin, Mfn2, and PGC-1α levels, as well as the Fis1/parkin, Fis1/Mfn2, and Fis1/ PGC-1α ratios, at ICU admission differed significantly among the groups. Relative to the control group, the sepsis group had significantly higher serum mediator levels and ratios ($p < 0.001$). Additionally, the Fis1/parkin, Fis1/Mfn2, Fis1/ PGC-1α ratios and serum PGC-1α, Fis1, Mfn2, parkin levels were markedly different among the sepsis non-shock, septic shock, and control groups ($p < 0.001$). Moreover, for patients with sepsis, we compared the ratios and serum levels between survivors and non-survivors. We found that all three ratios were significantly lower in survivors compared to non-survivors (**Figure 3**). Survivors had significantly higher serum parkin, PGC-1α levels and lower Fis1 level.

TABLE 2 | Mediators of the study population.

	Infection	Sepsis	*P*-value
Number of subjects	24	133	
Mediator levels			
PCT (ng/mL)	0.82 (0.56–2.20)	7.00 (2.10–25.00)	<0.001
Fis1 (pg/mL)	523.10 (427.57–609.59)	761.27 (642.26–837.11)	<0.001
Parkin (pg/mL)	249.60 (232.89–268.21)	181.44 (166.49–201.67)	<0.001
Mfn2 (pg/mL)	226.85 (205.96–240.59)	131.37 (113.54–161.88)	<0.001
PGC-1α (pg/mL)	386.66 (356.09–408.83)	283.20 (252.35–324.50)	<0.001
Fis1/Parkin ratio	1.98 (1.75–2.49)	4.28 (3.22–5.10)	<0.001
Fis1/Mfn2 ratio	2.37 (2.00–2.69)	5.90 (4.04–7.32)	<0.001
Fis1/PGC-1α ratio	1.36 (1.14–1.52)	2.69 (2.05–3.17)	<0.001

Date were presented by mean ± standard deviation, n (%) or median (interquartile range). PCT, procalcitonin; Fis1, fission 1; Mnf2, mitofusin2; PGC-1α: peroxisome proliferator-activated receptor γ coactivator 1α.

Value of Serum Levels and Ratios for Predicting 28-Day Mortality

Considering that the ratios differed significantly between survivors and non-survivors, we calculated the AUCs of the mediator levels and ratios as predictors of 28-day mortality using the ROC curve. The results are presented in **Table 3**. As shown, the Fis1/parkin ratio was a better predictor than serum PCT, Fis1, parkin, Mfn2, and PGC-1α levels or Fis1/Mfn2 and Fis1/ PGC-1α ratios (**Figure 4**). For serum Fis1/parkin ratio at ICU admission, the AUC to predict 28-day mortality was 0.792 (95% CI, 0.695–0.890, $P < 0.001$), and the optimal cut-off value was 4.0 (sensitivity 94.4%, specificity 49.6%).

Fis1/parkin Ratio as an Independent Predictor of 28-Day Mortality

Next, the SOFA score, APACHE II score, and Fis1/parkin, Fis1/Mfn2, and Fis1/PGC-1α ratios were included in a multivariate logistic regression model to determine the independent predictors of 28-day mortality (**Table 4**). A high Fis1/parkin ratio ($\beta = 1.347$, odds ratio [OR] $= 3.845$, $p = 0.021$) was a significant independent risk factor for 28-day mortality in septic patients.

Survival

We also performed a Kaplan–Meier survival analysis using the Fis1/parkin ratio cut-off (4.00) in septic patients (**Figure 5**). The results indicated that septic patients with a Fis1/parkin ratio above the cut-off value had a significantly lower survival rate ($p = 0.001$).

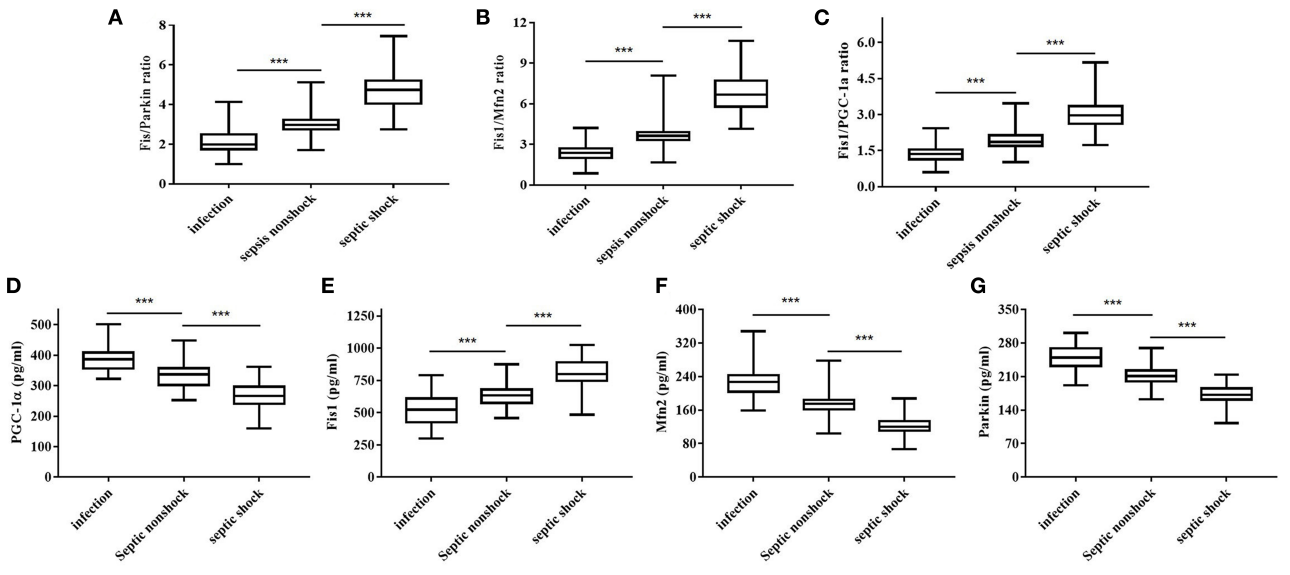


FIGURE 2 | Fis1/parkin ratio **(A)**, Fis1/Mfn2 ratio **(B)**, Fis1/PGC-1α ratio **(C)** and serum PGC-1α **(D)**, Fis1 **(E)**, Mfn2 **(F)**, parkin **(G)** levels at ICU admission in the patient subgroups.

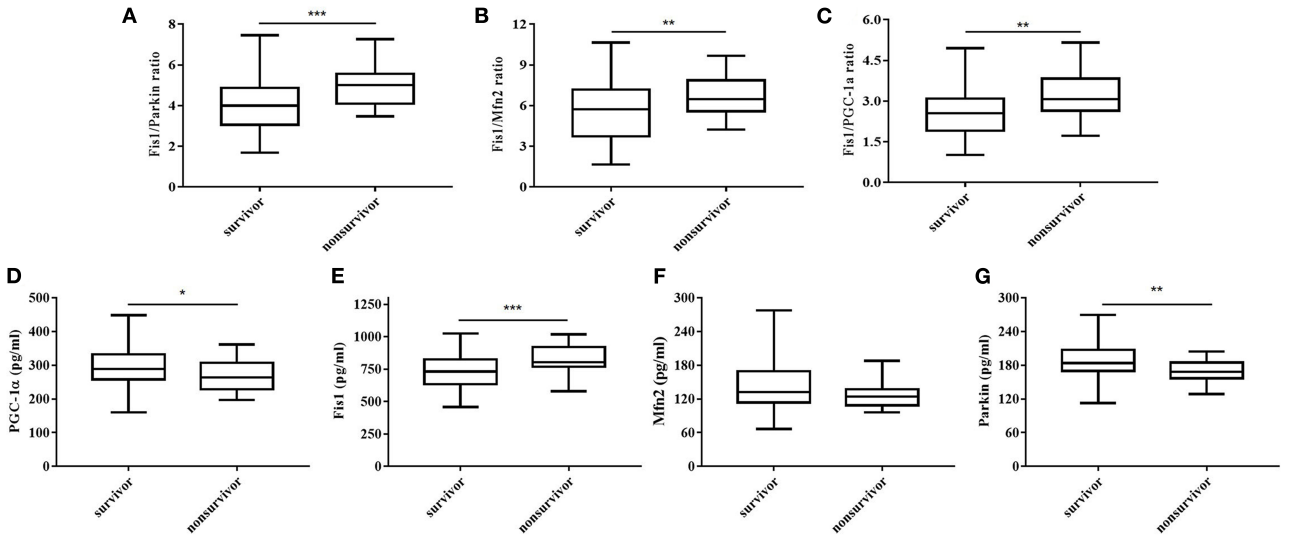


FIGURE 3 | Fis1/parkin ratio **(A)**, Fis1/Mfn2 ratio **(B)**, Fis1/PGC-1α ratio **(C)** and serum PGC-1α **(D)**, Fis1 **(E)**, Mfn2 **(F)**, parkin **(G)** levels at ICU admission in survivor and non-survivor groups of patients with sepsis at 28-day follow-up.

TABLE 3 | AUCs for predicting 28-day mortality in patients with sepsis.

	Variable	AUC	Standard error	*P* value	95% CI	
					Lower limit	Upper limit
28-day mortality	Fis1/Parkin ratio	0.792	0.050	0.000	0.695	0.890
	Fis1/Mfn2 ratio	0.708	0.056	0.005	0.598	0.817
	Fis1/PGC-1α ratio	0.725	0.063	0.002	0.601	0.848
	Fis1	0.740	0.056	0.001	0.630	0.849
	Parkin	0.231	0.055	0.000	0.124	0.338
	Mfn2	0.356	0.058	0.050	0.242	0.470
	PGC-1α	0.329	0.065	0.020	0.201	0.457
	PCT	0.692	0.066	0.009	0.563	0.821

DISCUSSION

There is an urgent need for biomarkers to improve early diagnosis and risk stratification for patients with sepsis. In our study, patients with sepsis presented with significantly decreased serum levels of parkin, Mfn2, and PGC-1α and significantly increased serum Fis1 level and Fis1/parkin, Fis1/Mfn2, Fis1/PGC-1α ratios. Notably, higher ratios were associated with increased severity of disease. Relative to ICU patients with other infections, septic patients showed remarkably elevated ratios, with septic shock patients having the highest ratios. It has been proved in the clinical and experimental studies, that the persistence of low mitochondrial mitophagy, biogenesis, fusion levels and a high level of fission during sepsis suggested a poor outcome (3, 6, 14, 22), which agrees with the changes observed in our study.

Our findings support that high ratios at ICU admission can identify the severity of sepsis. In addition, septic patients with poor outcomes (non-survivors) had higher Fis1/parkin, Fis1/Mfn2, and Fis1/PGC-1α ratios. The ratios were greater than the absolute Fis1, parkin, Mfn2, and PGC-1α levels for prognostic purposes and had significant prognostic value for 28-day mortality. The Fis1/parkin ratio performed best among all of the ratios, as reflected by the AUC. Additionally, Fis1/parkin ratio was proved to be an independent risk predictor for patients with sepsis. Septic patients with a higher Fis1/parkin ratio (>4.0) had a significantly lower survival rate. Therefore, we chosen Fis1/parkin ratio to represent the mitochondrial homeostasis, indicating the balance between risk factor and protective factor.

Mitochondria are highly dynamic organelles and frequently undergo fission and fusion to modulate mitochondrial morphology, number, and size. Fission (in which mitochondria divide) is essential for cell growth and division, as it promotes adequate numbers of mitochondria. Fission may also occur when there is significant mitochondrial damage, as fission will allow the cell to segregate the damaged portion (23). During sepsis, mitochondrial dysfunction results in activation of mitochondrial fission and inactivation of mitochondrial fusion, which each or both promote dysfunctional mitochondrial fragmentation (13). It remains uncertain whether the elevation of fission is a physiological adaptation or a manifestation of decompensation under septic conditions (4), although persistent elevation of fission during sepsis is associated with the deterioration of sepsis both *in vivo* and *in vitro* (13). The pretreatment with fission inhibitor (mdivi-1) significantly attenuated mitochondrial dysfunction and apoptosis in septic animal model (3). In this profile, the overactivation of fission during sepsis may be a risk

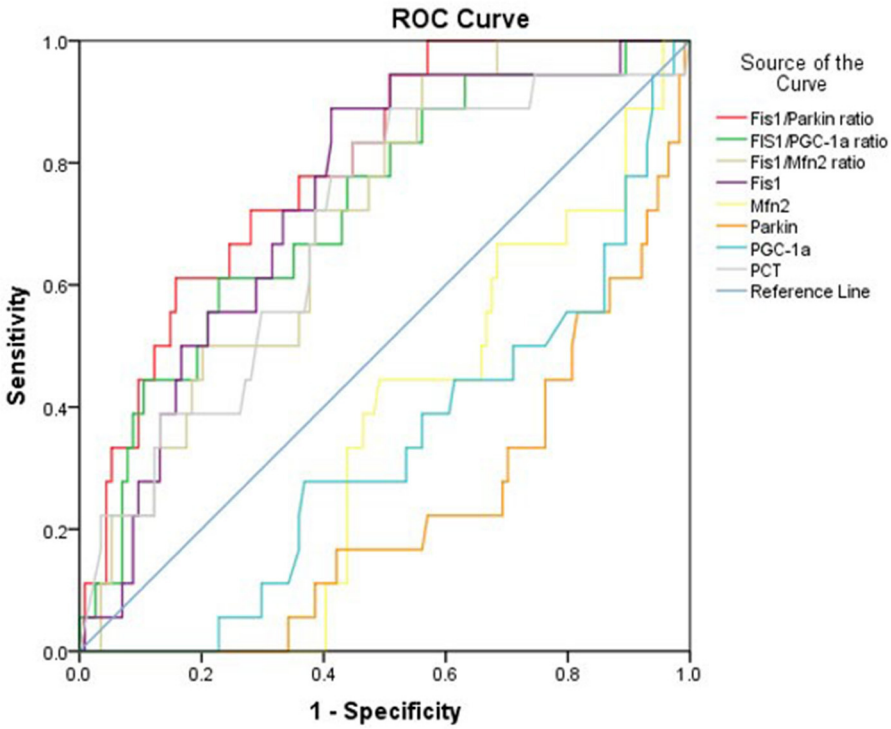


FIGURE 4 | ROC curves for predicting 28-day mortality in patients with sepsis.

TABLE 4 | Multivariate logistic regression analysis of factors predicting 28-day mortality in sepsis patients.

	β	SE	Wald	OR	*P*-value
Fis1/Parkin ratio	1.347	0.582	5.363	3.845	0.021
Fis1/Mfn2 ratio	−0.738	0.364	4.114	0.478	0.043
Fis1/PGC-1α ratio	0.407	0.564	0.522	1.503	0.470
SOFA score	0.164	0.085	3.708	1.178	0.054
APACHE II score	0.115	0.040	8.129	1.122	0.004

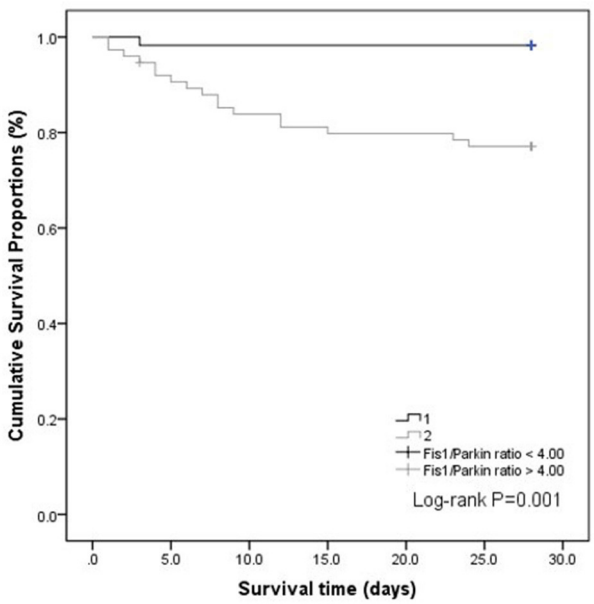


FIGURE 5 | Kaplan–Meier survival curves of the Fis1/parkin ratio for all patients with sepsis.

factor. Mitophagy refers to selective autophagy of mitochondria, and its main function is to recognize damaged mitochondria for degradation. In sepsis, mitochondrial dysfunction induces a loss of mitochondrial membrane potential that triggers mitophagy. Upregulation of mitophagy may improve organ function and reduce organ inflammation in response to lipopolysaccharide (LPS), whereas inhibition of mitophagy has been shown to increase mortality in septic mice (9, 22, 24). This phenomenon suggests a protective role of mitophagy during sepsis. Therefore, a higher Fis1/parkin ratio is expected to reflect a more severe imbalance of mitochondrial homeostasis. This would result in poorer outcomes for patients, which is consistent with our results. An elevation of Fis1/parkin ratio could be used to alert clinicians that mitochondrial dysfunction is existed, and clinical adjustments are needed to prevent the progression of sepsis. The reasons for the elevation of MQC-related biomarkers may be considered as: the persistent mitochondrial damage and the cell death (apoptosis, pyroptosis, or necroptosis) under septic exposure, which lead to continuous release of MQC-related

proteins into circulation. In a clinical study in multiple sclerosis (MS) patients, the serum levels of parkin are remarkably augmented and associated with disease activity, indicating the use of parkin as biomarker of mitophagy (15). The researchers had previously proved that parkin levels were increased within the CNS and at the systemic level in patients with MS compared to other neurological disorders and healthy individuals (16). Though these studies were not in septic setting, but suggesting the possibility of use serum parkin level as mitophagy related biomarker in septic patients. There are also some studies investigated the fission protein (Fis1, Drp1) in peripheral blood lymphocytes (PBL) and also the gene and protein expression of mitochondrial fusion (Mfn2)/fission (Fis1)/biogenesis (PGC-1α) in peripheral blood mononuclear cells (PBMCs) as biomarker to reflect the MQC status (17–19). Immune cells may be the main source of MQC-related proteins release. Of course, further reevaluation of the patient and repeated exploration for a possible source are still needed, and our research is ongoing.

Given the crucial role of mitochondria in sepsis (25, 26), numerous studies have evaluated other mitochondrial function biomarkers, such as electron transport chain (ETC) enzyme activity in platelets or circulating mitochondrial DNA (mtDNA) in plasma or serum, to monitor mitochondrial function. However, ETC has not been found to be useful for evaluating the prognosis of sepsis (27, 28). Quite a few studies have investigated the prognostic value of mtDNA in septic patients (29–33). Most studies that performed AUC analysis found a statistically significant association between mtDNA levels and mortality. However, steps need to be taken to standardize how mtDNA is measured to facilitate large, prospective, multicenter trials to better assess the ability of mtDNA to predict outcomes. In a previous study, we evaluated the potential utility of serum uncoupling protein-2 (UCP2) level as a mitochondrial function biomarker in septic patients (34). However, it did not outperform the Fis1/parkin ratio (the AUC of serum UCP2 level for predicting 28-day mortality was 0.704, compared with 0.792 for the Fis1/parkin ratio). There are also studies investigated the mitochondria in circulating cells, such as respiratory chain biochemistry in platelets (35) or the complex activity in platelets (28) or mitochondrial bioenergetic reserve in PBMCs (36, 37), aiming to provide insight into sepsis-associated temporal changes and how these changes relate to recovery. However, they were all required further validation. At present, monitoring of mitochondrial function is still limited to experimental work.

In a word, use of the serum Fis1/parkin ratio has several advantages: (1) Serum Fis1 and parkin levels can be accurately determined with high reproducibility using the ELISA technique, relative to biomarkers measured by western blot (WB) or polymerase chain reaction (PCR). (2) Serum samples are easier to obtain than tissue/organ samples or blood mitochondria containing cells (i.e., immune cells). (3) The Fis1/parkin ratio is specifically related to sepsis pathophysiology rather than more general inflammatory reactions. The use of this biomarker may help transform our understanding of sepsis from a "physiological syndrome" to a "group of distinct biochemical disorders" and lead to advances in the search for adjunctive sepsis therapies (38).

Limitations

Some limitations of this study merit consideration. First, an important limitation of the study is that the source of the proteins identified in the serum samples was not validated. Second, we did not compare the Fis1/parkin ratio with other classic biomarkers of mitochondrial function (i.e., mtDNA, complex of respiratory chain). Third, comparisons of Fis1 and parkin levels before and after treatment were not performed during patients' stays in the ICU. Fourth, complications of the patients' underlying diseases may have had an effect on the results. Lastly, this study was conducted at a single center with a relatively small sample size. Thus, larger studies at multiple centers are warranted to confirm our findings.

CONCLUSIONS

This study aimed to assess the use of the serum Fis1/parkin ratio as a biomarker of prognosis in septic patients. We found that the serum Fis1/parkin ratio is an independent risk factor and a predictor of 28-day mortality in patients with sepsis. The higher the Fis1/parkin ratio is, the severer the disease is. These results suggest that this ratio is valuable for rapid risk stratification when patients are admitted to the ICU. Further study is needed to verify the potential utility and beneficial effects of this biomarker to answer specific clinical questions.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by This study was approved by the Ethical Committee of PUMCH (approval number: JS-2421). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

WH conceived and designed the study, enrolled the patients, collected the blood samples, analyzed and interpreted data, performed the statistical analysis, and drafted the manuscript. XW conceived and designed the study, enrolled the patients, interpreted data and revised the manuscript. HZ enrolled the patients, analyzed data

and revised the manuscript. GW enrolled the patients, collected blood samples, and analyzed data. DL conceived and designed the study, interpreted data and revised the manuscript. All authors read and approved the final manuscript.

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REVIEW
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Sepsis—Pathophysiology and Therapeutic Concepts

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Sepsis is a life-threatening condition and a global disease burden. Today, the heterogeneous syndrome is defined as severe organ dysfunction caused by a dysregulated host response to infection, with renewed emphasis on immune pathophysiology. Despite all efforts of experimental and clinical research during the last three decades, the ability to positively influence course and outcome of the syndrome remains limited. Evidence-based therapy still consists of basic causal and supportive measures, while adjuvant interventions such as blood purification or targeted immunotherapy largely remain without proof of effectiveness so far. With this review, we aim to provide an overview of sepsis immune pathophysiology, to update the choice of therapeutic approaches targeting different immunological mechanisms in the course of sepsis and septic shock, and to call for a paradigm shift from the pathogen to the host response as a potentially more promising angle.

Keywords: sepsis, septic shock, immune pathology, inflammation, sepsis therapy

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INTRODUCTION

Sepsis is a life-threatening clinical condition with extensive physiological and biochemical abnormalities. The Third International Consensus (Sepsis-3) currently defines sepsis as "organ dysfunction caused by a dysregulated host response to infection", emphasizing for the first time the crucial role of the innate and adaptive immune response in the development of the clinical syndrome (1). Approximately 49 million people are affected by sepsis every year and it is estimated that 11 million deaths are caused by the syndrome, accounting for up to 19.7% of all deaths worldwide (2). Globally, mortality rates seem to be declining on average, however, up to 25% of patients still succumb to sepsis. In septic shock, a subgroup of sepsis characterized by profound circulatory, cellular and metabolic abnormalities, the hospital mortality rate approaches 60% (3).

Comprehensively defining "sepsis" has been subject of constant development and refinement over the last decades. Although our understanding of origin, pathophysiology, and immunological mechanisms of sepsis has made progress during the last three decades, our options of successful and specific therapeutic interventions remain restricted to non-existent. Only timely fluid resuscitation and early administration of broad-spectrum antibiotics have been shown to reduce mortality. A decisive factor is the time of correct diagnosis and the initiation of causal, supportive, and adjunctive measures. This implies that increasing awareness of sepsis and the promotion of quality improvement initiatives in the field of sepsis effectively improve patient survival, together with the development of novel diagnostics and interventions (4).

SEPSIS PATHOPHYSIOLOGY

In contrast to an uncomplicated and localized infection, sepsis is a multifaceted disruption of the finely tuned immunological balance of inflammation and anti-inflammation. The upregulation of pro- and anti-inflammatory pathways leads to a system-wide release of cytokines, mediators, and pathogen-related molecules, resulting in activation of coagulation, and complement cascades (5).

Recognition of pathogen-derived molecular patterns (PAMPs, e.g., endo- and exotoxins, lipids, or DNA sequences) or endogenous host-derived danger signals (damage-associated molecular patterns; DAMPs) is the starting signal. These molecules activate specific receptors (toll-like receptors, TLR) on the surface of antigen-presenting cells (APCs) and monocytes, thereby initiating the clinical syndrome of sepsis via transcription of genes involved in inflammation, cell metabolism, and adaptive immunity (6). While both pro-inflammatory and anti-inflammatory pathways are upregulated, the resulting inflammation leads to progressive tissue damage, finally causing multi-organ dysfunction. In many patients the concomitant immunosuppression, which is caused by downregulation of activating cell surface molecules, increased apoptosis of immune cells, and T cell exhaustion, leads to "immunoparalysis" in the later stages of the disease course and makes affected patients susceptible to nosocomial infections, opportunistic pathogens, and viral reactivation (**Figure 1**) (7, 8). Binding of PAMPs and DAMPs to TLRs on APCs and monocytes results in signal transduction, causing translocation of nuclear factor-kappa-light-chain-enhancer of activated B cells (NF-κB) into the cell nucleus. This leads to the expression of "early activation genes," including various pro-inflammatory interleukins (IL), e.g., IL-1, IL-12, IL-18, tumor necrosis factor alpha (TNF-α), and interferons (IFNs). These subsequently cause the activation of further cytokines (e.g., IFN-γ, IL-6, IL-8), complement and coagulation pathways, and, by negative feedback, downregulation of components of the adaptive immune system (9). These processes can be observed during the early stages of the septic disease by an increase in both pro-inflammatory and anti-inflammatory cytokines (8, 10, 11). The net effect on the immunological phenotype (hypo- vs. hyper-responsiveness) remains highly individualized and causes considerable diagnostic difficulties.

As part of the innate immune system, neutrophils form a significant part of the first line of defense against pathogens. Severe bacterial infections induce the release of both mature and immature forms of neutrophils from the bone marrow through emergency granulocyte maturation. When activated via PAMPs or DAMPs, immature neutrophils show reduced phagocytosis and oxidative burst capacity (13–15).

Clinical deterioration is often associated with the detection of elevated levels of these cells, which is in turn associated with increased spontaneous production and release of neutrophil extracellular traps (NETs) (16, 17). NETs are diffuse extracellular structures, consisting of decondensed chromatin with granular and nuclear proteins that have the potential to immobilize a wide range of pathogens. These include Gram-positive and Gram-negative bacteria, viruses, yeasts, but also protozoa and parasites that cannot be regularly phagocytized due to their size (18–20). The release of NETs is known to be triggered by cytokines and chemokines, but also by platelet agonists (i.e., thrombin, ADP, collagen, arachidonic acid) and antibodies (21). Increased occurrence of NETs either due to overproduction or to insufficient degradation has been shown to be associated with hypercoagulation and endothelial damage (17, 22, 23).

Sepsis-Induced Coagulopathy (and the Role of Endothelium in Sepsis)

Sepsis is frequently associated with coagulopathy, which is an important complication and contributes to the development of organ dysfunction. A recently published analysis of 1895 patients from Japan showed that 29% of critically ill patients with sepsis were diagnosed with sepsis-induced coagulopathy, which is synonymous with disseminated intravascular coagulation (DIC) (24). DIC was defined by the International Society on Thrombosis and Haemostasis (ISTH) as "an acquired syndrome characterized by the intravascular activation of coagulation with loss of localization arising from different causes. It can originate from and cause damage to the microvasculature, which, if sufficiently severe, can produce organ dysfunction" (25, 26). Sepsis-associated DIC is described as the systemic activation in coagulation with suppressed fibrinolysis that leads to organ dysfunction in combination with systemic inflammation. Thus, in the context of sepsis, the concept of DIC representing consumptive coagulopathy has been superseded by a more specific approach ("sepsis-induced coagulopathy"; SIC) focusing on the presence of organ dysfunction, decreased platelet count, and increased PT-INR (27).

Abbreviations: ADAMTS-13, "a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13"; AI, artificial intelligence; APC, antigen-presenting cell; CPFA, coupled plasma filtration adsorption; CRP, C-reactive protein; DAD, diffuse alveolar damage; DAMP, damage associated molecular pattern; DIC, disseminated intravascular coagulation; DNA, desoxyribonucleic acid; HLA-DR, human leukocyte antigen-D related; ICU, intensive care unit; IFN, interferon; IL, interleukin; Ig, immunoglobulin(s); ISTH, International Society on Thrombosis and Haemostasis; IVIG, intravenous immunoglobulin; IgGAM, immunoglobulin G/A/M; KIM-1, kidney injury molecule-1; MBC, minimum bactericidal concentration; MDSC, myeloid-derived suppressor cell; MHC, major histocompatibility complex; MIC, minimum inhibitory concentration; mPTP, mitochondrial permeability transition pores; MR-proADM, mid-regional proadrenomedullin; mtDNA, mitochondrial DNA; NET, neutrophil extracellular trap; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NGAL, neutrophil gelatinase-associated lipocalin; NK cell, natural killer cell; NO, nitric oxide; NOS, nitric oxide synthase; PAMP, pathogen-associated molecular pattern; PD-1, programmed death protein 1; PD1-R, programmed death protein 1-receptor; PICS, persistent inflammation, immunosuppression and catabolism syndrome; PMX, polymyxin B-immobilized fiber columns; PRR, pathogen recognition receptor; RCT, randomized controlled trial; RNS, reactive nitrogen species; ROS, reactive oxygen species; sa-AKI, sepsis associated acute kidney injury; SIC, sepsis-induced coagulopathy; TAMOF, thrombocythopenia-assiciated multiple-organ failure; TEC, tubular epithelial cell; T_{eff} cell, effector T cell; TF, tissue factor; TGF-ß, transforming growth factor-ß; T_H cell, helper T cell; TLR, toll-like receptor; TMA, thrombotic microangiopathy; TNF-α, tumor necrosis factor alpha; TPE, therapeutic plasma exchange; T_{reg} cell, regulatory T cell; TSS, toxic shock syndrome; vWF, von Willebrand factor; vWFCP, von Willebrand factor cleaving protease.

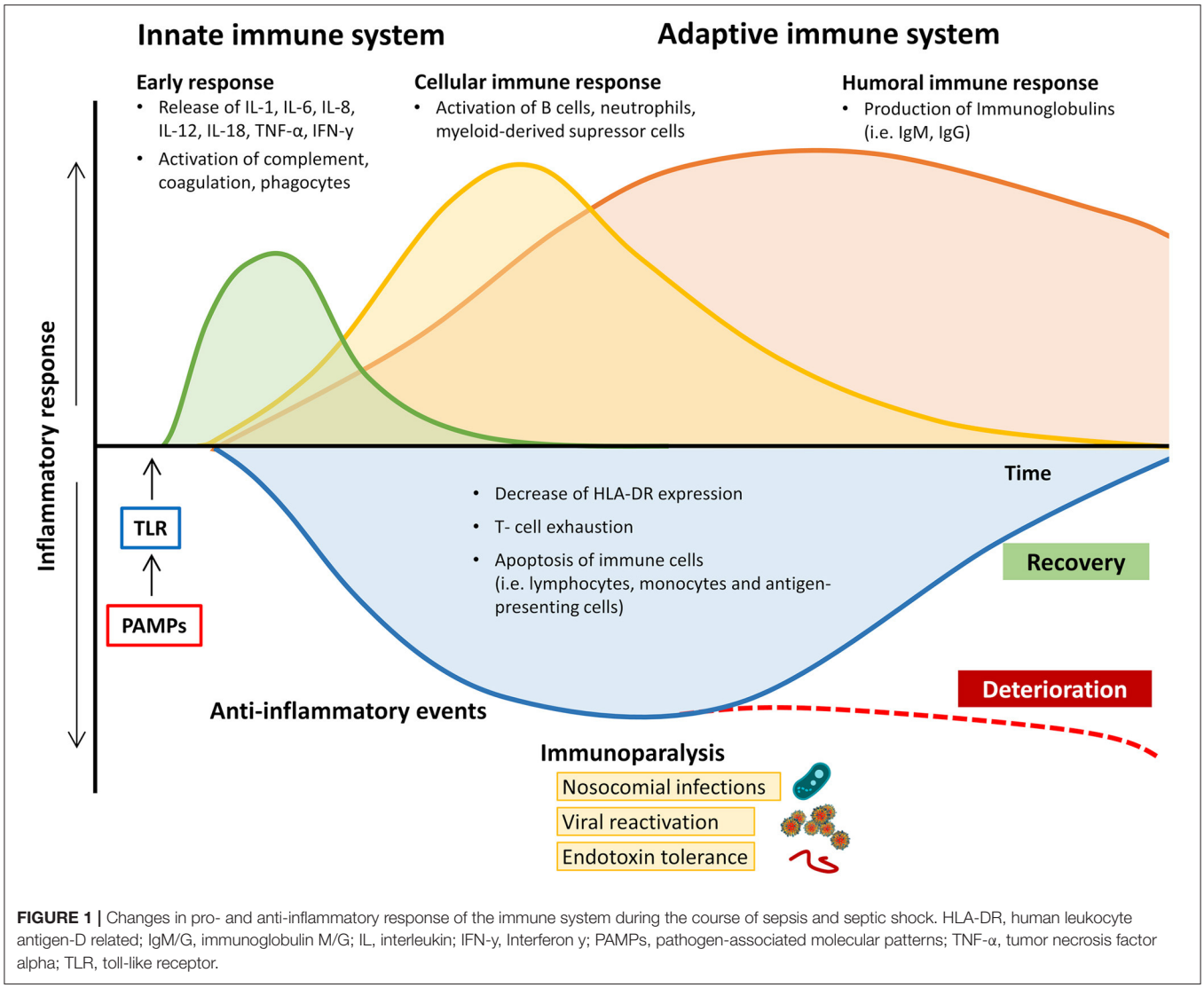


FIGURE 1 | Changes in pro- and anti-inflammatory response of the immune system during the course of sepsis and septic shock. HLA-DR, human leukocyte antigen-D related; IgM/G, immunoglobulin M/G; IL, interleukin; IFN-γ, Interferon γ; PAMPs, pathogen-associated molecular patterns; TNF-α, tumor necrosis factor alpha; TLR, toll-like receptor.

A large number of different pathogens and their products act on the endothelium via various pathomechanisms. Several predominantly proinflammatory responses of the cell to pathogen-induced stimulation have been identified. In addition to direct pathogen-associated activation, non-specific stimulation of endothelial cells by products of the host response (DAMPs) plays an essential role in the inflammatory process. In the context of some hemorrhagic fevers or acute phases of systemic, exuberant, proinflammatory host response (e.g., sepsis), it is postulated that this damage to the endothelium may be crucial to the course of the disease. In addition, the endothelium contributes significantly to the aggravation of inflammation through the release of proinflammatory substances, recruitment of inflammatory cells, procoagulant activity, and hyperpermeability (28).

Endothelial cells lose their anticoagulant function after proinflammatory stimulation and promote coagulation by decreased expression of thrombomodulin and heparan sulfate on the cell surface and increased expression of tissue factor (TF). Together, increased TF expression by pathogen-activated endothelium, adherent tissue factor-loaded monocytes, and leukocytic microparticles may activate the coagulation cascade. Finally, the pro-inflammatory serine protease thrombin activates the G-protein coupled protease-activated receptor-1 of endothelial cells, enhancing endothelial responses such as hyperpermeability, adhesion molecule expression, and cytokine production (29).

The Complement System in Sepsis

Complement activation products (such as the anaphylatoxins C3a, C4a, and C5a) are elevated in the early stages of sepsis (30). Physiologically, C5a is associated with the chemotaxis of neutrophils to the site of infection. By binding C5a to the C5a receptor (C5aR), neutrophils develop into migratory cells with the ability to enter inflamed tissue and remove pathogens and debris (31). Here, PAMPs and DAMPs induce the release of NETs, granular enzymes, and reactive oxygen species (ROS) during the oxidative burst, which, in turn, shifts the coagulation balance

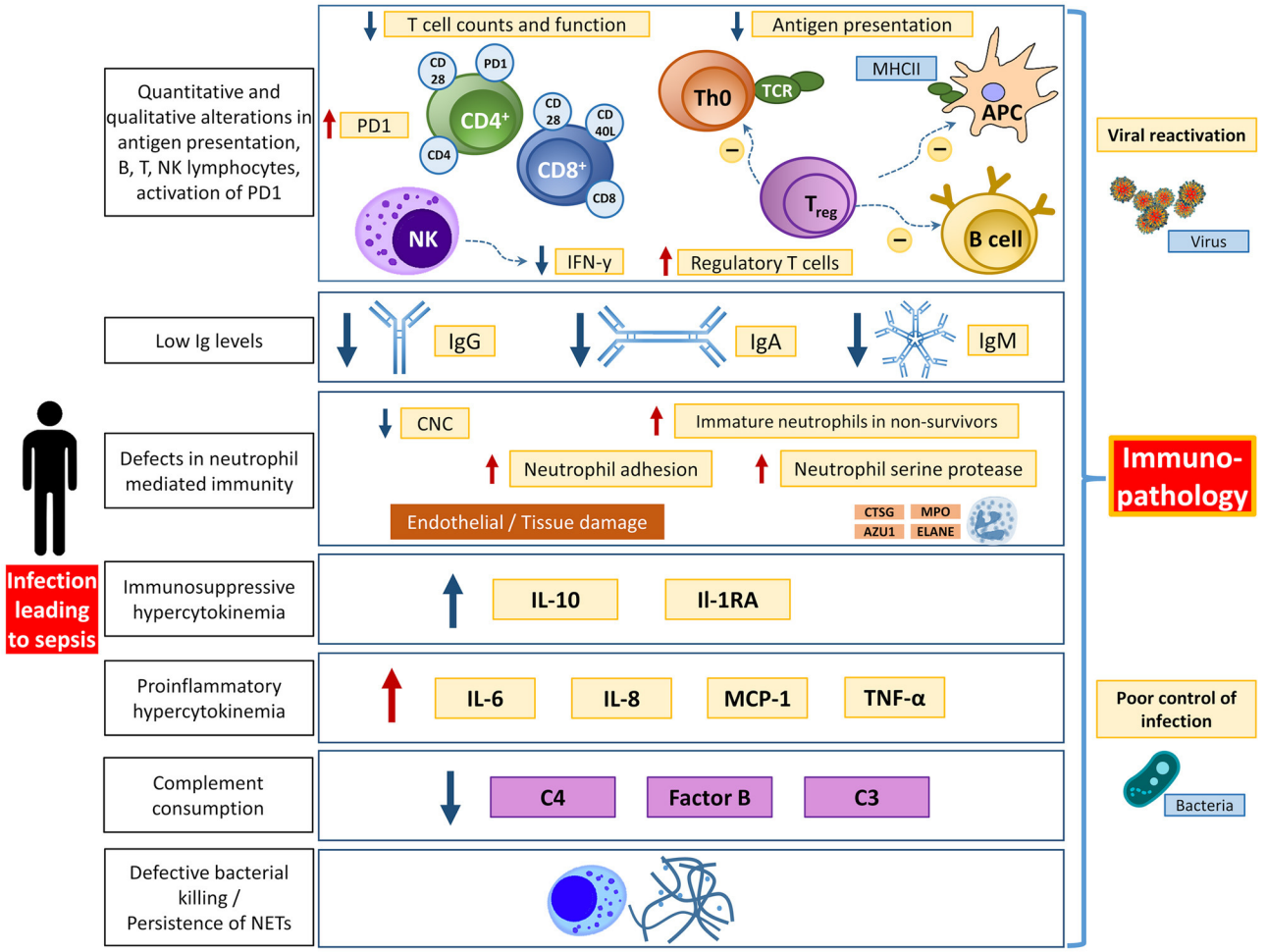


FIGURE 2 | Overview of different aspects of immunological dysfunction with details of the affected entities. APC, antigen presenting cell; AZU1, azurocidine 1; CNC, circulating neutrophils count; CTSG, cathepsin G; ELANE, elastase; IFN-γ, interferon γ; Ig, immunoglobulin; MHCII, major histocompatibility complex II; MPO, myeloperoxidase; PD1, programmed death protein 1; TCR, T cell receptor. Adapted from Bermejo-Martin JF (12) with permission.

toward prothrombotic activity, whilst fibrinolysis is inhibited. As a result, disseminated microvascular thrombosis is initiated, and consumption of clotting factors occurs, which is the hallmark of overt DIC (27). An excessive activation of C5a in sepsis causes aggravation of systemic inflammation, progressive apoptosis of lymphocytes, and even dysfunction of neutrophils (32). Overwhelming levels of C5a during sepsis lead to downregulation of C5aR with adverse effects on the further course of the disease. Homing of neutrophils into the microvasculature, further tissue damage, thrombosis, and ultimately multi-organ failure take place. In a mouse model, the blockade of C5a or C5aR inhibits the development of sepsis. Significantly increased survival has been shown in models of mild to moderate sepsis of C5aR-deficient mice, accompanied by improved pathogen clearance and largely preserved liver function (33). In patients with sepsis, however, downregulated levels of C5aR correlate with a poor prognosis when C5a levels are simultaneously elevated (34).

In summary, C5a as well as C5aR are key players in many acute and chronic inflammatory conditions, making C5a a highly attractive pharmacological target. The important involvement in sepsis-related inflammation makes both C5a and C5aR promising starting points for the development of novel therapeutic approaches. With Vilobelimab (anti-complement C5a) and Avdoralimab (anti-receptor C5aR monoclonal antibody; NCT04371367) the respective first-in-class monoclonal antibodies are currently tested both in clinical sepsis trials and in COVID 19 (35).

Sepsis-Induced Immunosuppression and Persistent Inflammation, Immunosuppression and Catabolism Syndrome

Although the hallmark of sepsis is generally considered to be the early systemic inflammatory response, there is a significant component of immunosuppression that also occurs in both early and late stages of the disease (9, 36–38) (**Figure 2**). In the early stages of sepsis, the depletion of B and T

lymphocytes can be observed in addition to an increased apoptosis rate of stromal cells and APCs (36, 39–44). The mechanisms underlying sepsis-induced lymphopenia are not yet fully understood but may be caused by increased migration into the tissue, increased apoptosis, and reduced production since in emergency hematopoiesis the release of neutrophils and monocytes is given priority (45, 46). The persistence of lymphopenia, and the decrease of immunoglobulin levels during sepsis, are associated with increased mortality (47, 48).

Even though many details about the function of B lymphocytes in sepsis have been revealed, their role goes beyond the production and secretion of immunoglobulins. B lymphocytes also produce cytokines, act as APCs, and modulate the innate immune response (49, 50). Through interaction with dendritic cells, macrophages, T and other B lymphocytes, clonal expansion is induced, which ultimately leads to the synthesis of highly specific antibodies. After differentiation into high-affinity, antibody-secreting plasma cells, B lymphocytes contribute significantly to effective host protection by producing antibodies (51). At the onset of sepsis, B cells can be activated by pathogens directly via interaction with pathogen recognition receptors (PRRs), which leads to an initial immune response by innate-like B cells (49, 52, 53). In septic shock, non-survivors have recently been shown to have pronounced functional impairment of B lymphocytes, resulting in decreased IgM production following stimulation and an overall decreased level of IgM (54). The ratios of different peripheral B cell subgroups (immature/transitional B cells, naive B cells, tissue-like memory B cells, resting memory B cells, and activated memory B cells) in septic shock differ significantly from those of healthy control patients (55). Sepsis survivors also have a significantly higher number of circulating B lymphocytes than non-survivors, especially in the first 24 h after the onset of sepsis (54). This effect can be attributed to the release of IgM, a natural antibody that is particularly important in the fight against Gram-negative bacteria (49). The hypothesis of B lymphocyte protection by secreted IgM is supported by the observation that in survivors of sepsis or septic shock elevated levels of circulating IgM antibodies have been detected in comparison to non-survivors just in the first 24 hours of the disease (54). Interestingly, critically ill patients who did not suffer from septic infection showed a similar picture (56). However, up to now, there is insufficient data justifying the routine use of IgM levels or B lymphocyte counts in the early stages of sepsis for prognostic purposes.

Apart from sepsis-induced lymphopenia, an increased rate of apoptosis of APCs and monocytes is a common observation during sepsis, which is also associated with a significant reduction of pro-inflammatory cytokines (36, 37, 39–44, 57–59). At the same time, there is decreased expression of human leukocyte antigen DR (HLA-DR) on the surface of the remaining monocytes and dendritic cells, resulting in pathogen recognition impairment and a reduction of opsonization with T cell receptor proteins. This leads to disruption of the Th1- and Th2-response as an essential component of the adaptive immune response (60). The inability of monocytes to restore normal levels of HLA-DR expression during the course of the disease has been shown to be a negative predictor for the outcome of sepsis, as well as endotoxin tolerance in the early stages of sepsis (61–63).

In addition to the loss of pro-inflammatory cytokine production due to the reduction of APCs and monocytes, acute infection leads to significantly increased granulopoiesis, whereby immature myeloid cells migrate into the peripheral blood and become functionally active. These myeloid-derived suppressor cells (MDSCs) release anti-inflammatory cytokines (e.g., IL-10 and transforming growth factor ß, TGF-ß), which significantly aggravates immunosuppression (9, 64). In the context of malignant diseases, the immunosuppressive properties of MDSCs are the focus of extensive research. Recently, Darden et al. published the results of a pilot study in which they used single-cell RNAseq to demonstrate different subsets of MDSCs, which are associated with the various courses of sepsis and may thereby be used as prognostic factors (65). Since knowledge of the mechanisms involved in sepsis remains limited, MDSCs seem to be another promising target for future research (66, 67).

In sepsis, the expression of inhibitory immune checkpoint molecules such as programmed death protein 1 (PD-1) is increased on the surface of T cells, APCs and peripheral tissue epithelial cells, which binds to the inhibitory programmed death protein 1-receptor (PD1-R) expressed on B and T lymphocytes (68). Binding to PD1-R suppresses leukocyte function and leads to apoptosis of immune cells, which contributes to the further depletion of T and B cells, APC dysfunction, and expansion of regulatory T cells (T_{reg}) (9, 41, 69–72). Although controlled apoptosis of cells of innate and adaptive immunity is initially advantageous for the host, the simultaneous downregulation of the inflammatory response in sepsis leads to the extensive loss of immune cells and the inability of the host to continue to defend itself against invading pathogens. Inhibiting apoptosis of immune cells has been shown to be beneficial in sepsis (73).

In general, acquired immunosuppression in sepsis is caused by epigenetic and metabolic mechanisms resulting in reprogramming of immune cells. After activation of pro-inflammatory genes in early sepsis, histone-mediated alterations lead to conversion of euchromatin to silent heterochromatin (74, 75). These epigenetic processes are linked to metabolic pathways, such as glycolysis or oxidative phosphorylation, which can lead to the accumulation of metabolic products such as acetyl-coenzyme A (Acetyl-CoA) and nicotinamide adenine dinucleotide (NAD) during the course of sepsis. Acetyl-CoA as well as NAD act as cofactors for the epigenetic enzymes histone acetyltransferase and histone deacetylases sirtuin-1which negatively influence gene transcription (73, 76).

In addition, post-translational gene control is provided by non-coding RNA such as microRNA (miRNA). This evolutionarily conserved, non-coding single-stranded RNA plays an important role in gene silencing and in fine tuning of protein expression (77).

miRNAs are usually transcribed and processed within the nucleus through the complex interaction of multiple factors, including RNA polymerase 2, RNase III and the DiGeorge syndrome critical region 8 (DGCR8) complex (78). After shifting into the cytoplasma and final processing steps, miRNA can be guided to messenger RNA (mRNA) for post-transcriptional

regulation by the RNA-induced silencing complex (RISC). RISC is a functional conglomerate of RNAse III, transactivation-responsive RNA-binding protein and Argonaute-2 proteins.

Furthermore, also direct interaction to target mRNA via complementary base sequences can lead to functional modification and even degradation of mRNA, thereby adjusting protein expression levels or inhibiting translation. Each mRNA is under the control of numerous miRNAs, and, conversely, each miRNA controls hundreds of mRNAs (79).

miRNA have been detected in several body fluids, such as saliva and urine, but also in plasma. Due to their resistance to temperature, pH and RNAses, miRNA have a system-wide influence on cellular functions (80, 81). The signaling cascade downstream of the activated TLR, e.g., is modified by the action of miRNA so that excessive inflammation in response to an infection is attenuated (82).

Back in 2012, Gentile et al. described the clinical phenotype of persistent inflammation, immunosuppression, and catabolism syndrome (PICS) in surgical patients with a prolonged (>10 days) ICU stay (83). This condition, which had previously been described as "late MOF," "CARS," or "complicated clinical course," typically evolves after an initial and pronounced septic or inflammatory insult. It is characterized by persistent inflammation and acquired immunosuppression, prolonged ICU stay, and is usually associated with poor outcome. PICS may also occur after major trauma, and in elderly patients with sarcopenia and immunosenescence experiencing trauma, major surgery, or sepsis (84). Early diagnosis and advanced organ support in sepsis have substantially decreased mortality for many patients admitted to the ICU. However, a significant proportion of sepsis survivors develops chronic critical illness (CCI) with ongoing organ dysfunction. A subset of CCI patients will develop PICS, predisposed to a poor quality of life and indolent death (85). With an aging population in many developed countries, many sepsis survivors will eventually develop CCI after successful initial resuscitation, which is a debilitating condition with profound personal and social costs.

Acute Respiratory Distress Syndrome in Sepsis

When critically ill patients develop pulmonary dysfunction, there is often an associated primary pulmonary affection, such as pneumonia, exacerbation of chronic obstructive pulmonary disease (COPD), aspiration, pulmonary embolism, or pulmonary contusion. A progression of lung injury often leads to acute respiratory distress syndrome (ARDS), as defined by the Berlin criteria (86). In septic shock, the incidence of severe ARDS is reported to be up to 40%, and is more frequent in the presence of a pulmonary focus (87, 88). The occurrence of ARDS may also lead to the development of dysfunction in other organs, such as kidneys, liver, cardiovascular and central nervous system, which often persists until late in the course of the disease.

In case of sepsis induced multiple organ dysfunction syndrome (MODS), the lungs are the predominant organ system affected, and a primary pulmonary pathology is absent in many cases. Sepsis-induced ARDS is caused by an uncontrolled and complex interaction between inflammatory cytokines and cellular mediators that damage the alveocapillary unit and can be classified in three overlapping phases (89):

- exudative phase, characterized by edema and alveolar hemorrhage within the first days.
- proliferative phase, marked by organization and repair.
- fibrotic phase, usually after 3–4 weeks after the onset of ARDS and characterized by collagenous fibrosis.

Direct or indirect damage to the pulmonary epi- and endothelium leads to increased alveolar capillary permeability, resulting in progressive exudate of protein-rich fluid. Plasma proteins in this fluid inactivate surfactant factor, and *de-novo* production of surfactant is reduced by ongoing damage to type 2 pneumocytes. The resulting surfactant deficiency leads to an increase in intraalveolar surface tension, thus causing diffuse microatelectasis (90). Additional injury to the alveolar capillary membranes is exacerbated by neutrophil entrapment in the pulmonary microcirculation. The local release of inflammatory mediators by neutrophils and macrophages migrating into the alveoli and interstitial space contributes to diffuse endothelial cell injury and destruction. In parallel, there is deposition of leukocytes and platelets and progressive destruction of type I alveolar pneumocytes, whereas type II alveolar pneumocytes show hyperplasia. In the advanced stage of these changes, a morphological condition called diffuse alveolar damage (DAD) develops (91).

If successfully addressed at an early stage, lung injury is almost fully reversible. However, if there is a persistent exudate of protein-rich fluid and further infiltration by neutrophils, mononuclear cells, fibroblasts and lymphocytes, respiratory failure progresses, and pulmonary fibrosis completely transforms the lungs. Collagen is accumulated and microcystic honeycomb, traction bronchiectasis and fibrosis of the alveolar ducts occur as well as abnormally enlarged air spaces with an abnormal increase in dead space (92).

Sepsis-Induced Acute Kidney Injury

The pathophysiology of the development of sepsis-associated acute kidney injury (sa-AKI) is still poorly understood. Progress in research is slow and often based on extrapolations from postmortem observations, cell cultures, and animal models. The prevailing pathophysiological concept primarily identifies decreased renal blood flow resulting in tubular epithelial cell necrosis as the probable cause for AKI as a consequence of hypoperfusion and shock (93, 94). However, recent findings suggest that not only hypoperfusion but also other factors must play a role: sa-AKI may both occur in stable (macro)circulatory conditions and during increased renal blood flow (95). Histopathological findings of postmortem human and animal tissue samples do not allow to draw a direct line between severity of renal parenchymal damage and functional changes. These observations lead to the conclusion that different mechanisms are involved in the development of sa-AKI.

Sepsis is usually accompanied by the co-occurrence of inflammation, microcirculatory dysfunction, and metabolic reprogramming. Inflammatory mediators, DAMPs and PAMPs are released into the intravascular space and, upon binding to membrane-bound receptors such as TLR, cause the synthesis and release of additional proinflammatory molecules. Specific subtypes of these receptors, such as TLR-2 and TLR-4, are also expressed by renal tubular epithelial cells (TECs). Here, binding of damage- or pathogen-associated molecular patterns cause an increase in oxidative stress and mitochondrial damage. Paracrine signaling also occurs in an attempt to protect neighboring cells from damage by inactivation, but this also leads to a decline in organ function. In addition to endotheliopathy and glycocalyx damage, activation of the coagulation cascade and autonomic nervous system signaling can also impair the microcirculation (96, 97). This results in functional occlusion of capillaries by leukocytes and platelets, and damage to the endothelium is accompanied by vasodilation and endothelial leakage. The resulting edematous peritubular distension reduces the oxidative supply to TEC due to the prolonged diffusion distance. The mechanisms described above in the course of sepsis lead to an intrarenal redistribution of blood flow, with hypoperfusion of the renal medulla.

Early diagnosis and initiation of appropriate therapeutic measures in sa-AKI is time critical. Advanced and more sensitive markers for kidney damage or AKI risc prediction are needed, such as neutrophil gelatinase-associated lipocalin (NGAL), urinary kidney injury molecule-1 (KIM-1) and the combination of the regulatory proteins IGFBP7 and TIMP-2 (98, 99). NGAL is released by renal TECs and was shown in early studies to be a good tool for predicting RRT need and in-hospital mortality. However, because NGAL is also released by activated neutrophils, it may also be elevated in non-renal causes (100, 101). KIM-1 is released from proximal TECs after nephrotoxic and ischemic damage and was also shown to be a good predictor of AKI. In sepsis, survivors versus non-survivors had significantly lower KIM-1 levels at 24 and 48 h. In a study of 86 children with circulatory collapse, KIM-1 was able to predict impending AKI before changes in serum creatinine as well as GFR (102, 103). So far, these promising approaches with NGAL and KIM-1 have not been proven in sufficiently powered clinical trials. The combination of urinary IGFBP7 and TIMP-2 showed good prediction of AKI in several studies, with non-renal organ failures not leading to a change in their levels (104–106). As regulatory proteins of G1 cell cycle arrest, both have a protective effect during cellular stress and quantify an individual patient's risk for impending AKI (105). Because of its involvement in endothelial dysfunction and capillary leakage, mid-regional proadrenomedullin (MR-proADM) is also of great interest. In a secondary analysis of SISPCT trial, which enrolled more than thousand severe sepsis and septic shock patients, it was shown that MR-proADM within the first seven days of sepsis provided a more accurate prediction for requirement for RRT than urine output and creatinine (107, 108). These results could recently be confirmed in patients with COVID-19 by our group, suggesting that MR-proADM may be a useful predictor for requirement of RRT during ICU stay (109).

Cardiac Dysfunction

The term sepsis-induced cardiac dysfunction is used to describe a variety of acute cardiac disorders caused by sepsis. Septic cardiomyopathy has become the focus of much research in recent decades and is associated with significantly increased mortality of up to 50% (110). Septic cardiomyopathy represents a complex cardiac dysfunction affecting both ventricles. Apart from non-specific conditions (age, obesity), no specific risk factors are known to date. Clinically, it presents with all the signs of circulatory failure associated with systemic infection. The differences to the clinical manifestation of cardiac dysfunction in patients with decompensated heart failure of non-septic etiology lie in the features of altered global hemodynamic parameters (preload, afterload, microcirculation). Unlike other myocardial pathologies, septic cardiomyopathy therefore requires a multimodal approach to diagnosis and therapy.

The underlying pathophysiological mechanisms can be broadly classified into three groups: impaired myocardial circulation, direct cardiac depression, and impaired cardiac mitochondrial function (111).

A balanced intravascular fluid status is a major prerequisite for cardiac function, and loss of vascular tone due to arterial vasodilation is one of the main causes of hemodynamic instability secondary to sepsis. The development of sepsis-induced endothelial dysfunction also plays a major role. A mere maintenance of coronary blood flow does not protect against the development of myocardial dysfunction, since endothelial damage may cause profound microcirculatory maldistribution of blood flow (112).

Direct myocardial depression is based partly on sepsis-related decrease in myocardial adrenergic response due to downregulation of ß-adrenergic receptors and their components, caused by pro-inflammatory mediators. In particular, IL-1ß and TNF-α appear to have a pronounced direct effect on myocardial contractility *in vitro* (113). Il-1 stimulates increased synthesis of nitric oxide (NO) via NO synthase (NOS) and thereby enhances its effects in the cardiovascular system (114, 115).

Due to the effect of NO on both cardiac ß-adrenergic receptors (where it leads to suppression of adrenergic response) and on mitochondria (inducing functional impairment), NO was shown to be associated with the severity of cardiac dysfunction and with increased mortality (116). Further, increased levels of prostanoids (e.g., prostacyclin and thromboxane) appear to influence coronary endothelial function. Therapeutic approaches to reduce the effect of prostanoids by using anti-inflammatory agents such as cyclooxygenase inhibitors (e.g., ibuprofen, indomethacin) have not shown efficacy in clinical studies.

Another mechanism of sepsis-induced cardiac dysfunction is the influence of the complement system. Activation of complement factor C5 (C5a) as a strong proinflammatory mediator promotes the release of granular enzymes, the release of further cytokines and ROS and increases chemotaxis of neutrophils. In addition, cardiomyocyte-expressed C5a-receptors mediate further C5a-induced cardiodepression, thereby making it a potential target for anti-C5a antibodies (117).

Since the beating heart is highly dependent on the continuous supply of ATP, metabolic dysfunction of myocardial

mitochondria has also been identified as a key mechanism in the development of septic cardiomyopathy (118, 119). Cardiomyocytes contain a large number of mitochondria, which explains the close relationship between sepsis-induced cardiac dysfunction and outcome in case of mitochondrial dysfunction (120). Increasing levels of ROS and reactive nitrogen species (RNS) have a negative impact on oxidative phosphorylation and directly inhibit mitochondrial respiration, which may lead to apoptosis in addition to other direct damage to cellular components (121, 122). Consumption and deficiency of antioxidants (ascorbic acid, a-tocopherol, uric acid, and others) during sepsis may amplify oxidative stress and are associated with the development of organ failure in sepsis. However, none of these agents so far have been convincingly demonstrated to bring about clinically meaningful benefits (123–125).

A balanced distribution of Ca^{2+} is fundamentally linked to the regeneration of ATP. However, cytokines and other mediators lead to Ca^{2+} overload of mitochondria in sepsis by disrupting the Ca^{2+} storage function of the sarcoplasmic reticulum. Ca^{2+} overload leads to the opening of mitochondrial permeability transition pores (mPTPs) and subsequently to caspase protein-induced mitochondrial damage (126, 127). With the assistance of mPTPs, circular mitochondrial DNA (mtDNA) can be released from mitochondria (128). Acting as a DAMP, mtDNA can activate the immune response via TLR-9, and plasma levels have been shown to be significantly lower in sepsis survivors than in non-survivors (129–131).

Although sepsis-induced cardiac dysfunction is closely related to prognosis and has been the focus of many research projects, effective treatment options are lacking.

The Role of Immunoglobulins

Immunoglobulins are produced and released by differentiated B cells (plasma cells) (132). The variable regions of these glycoproteins allow non-covalent cross-linking with bacterial and other antigens, whereby the constant region signals antigen binding. Within the human humoral immune system, IgA, IgG, and IgM are the most important classes. The main function of IgA is mucosal immunity, while IgG brings about opsonisation and complement activation in addition to secondary antibody reactions. In addition to the primary antibody response, the main function of IgM is complement activation. The antigen binding affinity of natural IgM antibodies is typically lower when compared to IgG, but their polyvalence allows for high avidity binding and efficient engagement of complement to induce complement dependent cell lysis (133). IgA, IgG, and IgM are known to behave synergistically in sepsis and septic shock, and the simultaneous occurrence of low plasma levels of these antibodies is associated with reduced survival in patients at the onset or during sepsis or septic shock (54, 134–141). The etiology of low plasma levels of immunoglobulins in sepsis is not fully understood but is most likely the result of multifactorial events such as endothelial dysfunction with subsequent vascular leakage, redistribution to inflamed tissue, complement consumption, excessive catabolism, and downregulated production and secretion due to secondary immunosuppression (12, 132, 135, 136, 142).

THERAPEUTIC CONCEPTS IN SEPSIS AND SEPTIC SHOCK

In 2004, the Surviving Sepsis Campaign (SSC) was initiated as a campaign to advance the worldwide treatment of sepsis comprehensively to improve survival through joint efforts. From the beginning, "sepsis bundles," i.e., a set of procedural measures to be taken within a prescribed time window, have been the cornerstones for the successful treatment of sepsis and septic shock. Strict adherence to and consistent application of these bundles have reduced the relative risk of mortality by up to 25%, although the evidence for the efficacy of individual measures remains controversial (4, 143, 144).

Today, standard therapy for sepsis consists mainly of attempting to eliminate the focus like interventional radiology or surgical measures for source control and timely administration of empirically targeted antibiotics (causal therapy). Further, additional intensive care measures are used for individual organ support like vasopressor administration, mechanical ventilation, and renal replacement therapy (supportive therapy). Parallel to these standards, adjunctive treatments can be used.

Causal Therapy

It is widely accepted that an early start of interventions is crucial for success. The "Hour-1-Bundle" was introduced in response to new evidence based on the 2016 guidelines and replaces the previous recommendations of the 3- and 6- h bundles with the explicit intention of starting fluid resuscitation and sepsis management measures immediately. The "Hour-1-Bundle" consists of 5 clinical interventions: blood cultures prior to antibiotics, administration of broad-spectrum antibiotics, administration of IV fluid, application of vasopressors, and measurement of lactate levels.

Aiming to eliminate the source of infection as the underlying cause of the continuing immune imbalance is of fundamental importance for successful sepsis therapy. If this fails or is incomplete, the probability of survival will be reduced (145). In addition to surgical interventions, the removal of existing intravascular catheters and devices should also be considered. Blood cultures and other biologic samples should be obtained, but this should not delay antimicrobial therapy (146). The causal treatment of a (suspected) underlying infection and hemodynamic management must be performed simultaneously. In 2014, the MEDUSA trial showed that delay in antimicrobial therapy and source control was associated with increased mortality in sepsis and septic shock patients (147).

The second mainstay in the therapy of sepsis and septic shock is antibiotic therapy. For this, the best possible knowledge of the pathogen epidemiology and the presumed anatomical focus are prognostically important. Due to the substantial variability of pathogens between different countries, regions and hospitals, the local pathogen and resistance situation should be known, and regular updates should be scheduled.

Broad-spectrum antibiotics, which empirically cover the expected pathogen spectrum, are the first choice and should be administered at the earliest possible point in time. However,

to what extent does the classification of the pharmaceutical agents matter, does it make a difference whether bactericidal or bacteriostatic drugs are chosen? And what are the differences between these two categories? Contrary to what is implied by the literal designation, it is rather a matter of definition. To classify an antibiotic agent, knowledge of two parameters is essential. First, the minimum inhibitory concentration (MIC), which defines the concentration of agent that prevents the visible growth of bacteria under defined conditions (e.g., growth media, temperature, CO_2 concentration). Second, the minimum bactericidal concentration (MBC) of the agent, which that results in a 1,000-fold reduction in bacterial density within 24 h under the same specific conditions. Based on the ratio of MIC and MBC, the classification of the respective antibiotic agent is formally defined: a ratio of MBC-to-MIC >4 is determined bacteriostatic, whilst a ratio MBC-to-MIC ≤4 is determined bactericidal (148). In a systematic review of a total of 56 randomized controlled trials (RCTs) published between 1985 and 2017 comparing bactericidal and bacteriostatic antibiotics, Wald-Dickler et al. found no evidence for the superiority of bactericidal antibiotics. Of the evaluated RCTs, which investigated a broad spectrum of underlying infections, six showed a statistically significant difference in favor of bacteriostatic agents, and only one publication showed an advantage for a bactericidal antibiotic (however, the bacteriostatic antibiotic was dosed suboptimally) (149). No compelling link to the clinical course of infection and no prediction with regard to outcome could be identified, as long as the choice of the agents used and their respective dosage was based on evidence-based data.

Following an initial loading dose, further dosage of antimicrobial agents should be adapted according to drug properties and pharmacokinetics as well as pharmacodynamic principles, since the function of almost every organ system is significantly disturbed during sepsis or septic shock (increased distribution volume due to aggressive fluid resuscitation and capillary leakage, hypotension, restricted renal and hepatic function). As soon as the causative pathogen is identified, narrowing of the initial therapy is recommended (150).

Use of Lactate as Marker for Severity and Disease Progression

Lactate production and clearance are influenced by numerous factors. In the context of DO_2/VO_2 mismatch during critical illness, elevated lactate levels can have various causes, such as an anaerobic metabolic response due to tissue hypoxia, decreased hepatic clearance, or pronounced ß-adrenergic stimulation of Na/K-ATPase leading to a consecutive increase in aerobic glycolysis (151). A persistent elevation of serum-lactate above 2 mmol/l in critically ill patients is an independent predictor of mortality across different groups of ICU patients with sepsis, trauma, organ failure, and shock due to septic, cardiogenic, and hemorrhagic etiology, among others (152). A retrospective analysis of 400 patients with severe hyperlactatemia (>10 mmol/l) showed markedly increased ICU mortality compared with the overall cohort of ICU patients, with wide variation in mortality among the different groups (153). If severe hyperlactatemia persisted for more than 24 h, it was associated with extremely high mortality (>95%); if it lasted more than 48 h, none of the patients survived. In a recent evaluation of 781 ICU patients, Hayashi et al. showed that maximum arterial lactate concentration within 24 h provided robust prediction of both in-hospital mortality and 90-day survival, comparable to the predictive power of APACHE III in unselected ICU patients (154).

Due to its availability and strong association with disease severity and patient outcome, lactate has an outstanding role as a diagnostic marker and as a marker of disease progression. This holds true for both the absolute values and for the kinetics over time (lactate clearance) (155). It is also part of the current "sepsis-3" definition, where a lactate value >2 mmol/l despite adequate volume substitution in conjunction with the need for vasopressor therapy to maintain a mean arterial pressure ≥65 mmHg defines septic shock. Because of its easy availability, close monitoring of the lactate value whilst >2 mmol/l (e.g., every 1–2 h) is a recommended parameter to guide volume therapy and hemodynamic management (146, 156).

Supportive Therapy

Fluid Resuscitation

An essential component of the "Hour-1-bundle" is adequate volume therapy to treat sepsis-induced tissue hypoperfusion and to counteract absolute and/or relative hypovolemia caused by vasodilatation, external fluid loss and capillary leakage. Immediately after identification of a septic patient with hypotension and/or elevated lactate levels, the treatment should be initiated. This has been repeatedly shown to reduce mortality (157, 158). Following the current paradigm, 20–40 ml/kg of crystalloid fluid should be administered within the first 3 h in accordance with SSC guidelines. The use of fluids other than crystalloids for initial resuscitation and intravascular volume replacement is currently not recommended in patients with sepsis and septic shock. If hypotension persists despite adequate fluid resuscitation, the use of catecholamines is indicated to ensure adequate perfusion of vital organs and to maintain mean arterial pressure above 65 mmHg. The guidelines recommend norepinephrine as the vasopressor of choice, according to current data, with moderate evidence.

Although there is general consensus that high-dose fluid replacement is indicated in the first hours of septic shock for rapid normalization of oxygen delivery and circulatory function, there is general uncertainty about the further continuation of fluid administration and appropriate target parameters. There is increasing concern that a continued positive fluid balance will have a negative impact on prognosis (159–162). At the same time, there is evidence that significantly earlier use of vasopressors may be beneficial, contrary to what is recommended by current guidelines (163, 164). At what point vasopressors should be started and fluid therapy stopped is still unclear. Thus, prospective studies evaluating the impact of early vasopressor therapy on the development of multiorgan dysfunction and the total volume of resuscitation fluids required during early septic shock are clearly needed.

Steroids

The use of corticosteroids as a supportive measure in the treatment of sepsis and septic shock has been a matter of debate for decades. Currently, SSC guidelines recommend the use of hydrocortisone only in patients with vasopressor-dependent refractory septic shock, who do not respond to fluid resuscitation. It is recognized that there are no data demonstrating a survival benefit from continued use of hydrocortisone in sepsis therapy. In 2008, a European multi-center study in a cohort of almost 500 patients showed no improvement in 28-days mortality when using hydrocortisone in septic shock. In addition to an increase in secondary infections, an increased incidence of hypernatremia and hyperglycemia was observed, and as a result, hydrocortisone was no longer recommended as standard therapy in septic shock (165). In 2018, the influence of adjuvant glucocorticoid therapy on 90-days mortality was investigated in 3,800 patients with septic shock (ADRENAL). Although there was a more rapid hemodynamic stabilization and also a shortening of the duration of mechanical ventilation, no significant difference in 90-days mortality was found (166). However, the results of yet another study (APROCCHSS) from 2018 partially contradict these findings. Annane et al. were able to demonstrate a significant reduction in 90-days mortality (43.0 vs. 49.1%). $p = 0.03$) in favor of intervention when hydrocortisone plus fludrocortisone was used in adult septic shock patients, but the study population differed from the first cohort with a lower proportion of surgical patients, abdominal infections and a higher proportion of renal replacement procedures (167). In 2017, Marik et al. demonstrated within a retrospective before-after study that moderate doses of hydrocortisone in combination with early administration of IV vitamin C and thiamine can effectively prevent progressive organ dysfunction, including acute kidney injury. Compared to a control cohort, they demonstrated a dramatic reduction in mortality in patients with sepsis and septic shock (8.5 vs. 40.4%, $p < 0.001$). Unfortunately, these promising results could neither be confirmed in a further retrospective evaluation nor in a multicenter randomized open-label study after enrollment of 216 patients with septic shock. No significant change in survival or vasopressor-free time over 7 days could be demonstrated after a triple therapy of vitamin C, thiamine and hydrocortisone vs. hydrocortisone alone (168, 169). In toxic shock syndrome (TSS) caused by staphylococcal or streptococcal exotoxins acting as superantigens, the use of steroids is not recommended; there is only anecdotal and outdated evidence for beneficial effects (170, 171). However, the administration of IVIG in TSS has repeatedly been suggested (cf. 3.3.4 Immunoglobulins in sepsis).

Ventilation

In the field of mechanical ventilation as a supportive measure for patients with sepsis and respiratory insufficiency, only moderate progress has been achieved so far. The goals of mechanical ventilation include improving gas exchange and reducing work of breathing, as well as preventing high airway pressures and further iatrogenic damage to the lung tissue. In sepsis-induced acute respiratory distress syndrome (ARDS), the recommendation for lung-protective ventilation with a tidal volume of 6 ml/kg standard body weight with an upper limit for the plateau pressure of 30 cm H_2O remains valid. Prone position in ARDS is also highly recommended and recognized to lower mortality. Despite low complication rates, a significant reduction in driving pressure and an improvement in oxygenation, an international observational prevalence study showed in 2018 that prone positioning was only used in 32.9% of patients with severe ARDS (172, 173).

Meduri et al. demonstrated in an RCT in patients with refractory ARDS that prolonged administration of methylprednisolone was associated with clinical improvement and reduced mortality (174). The rate of infections was comparable in the study groups. Overall, the evidence for the regular use of steroids in ARDS is still insufficient.

Adjunctive Therapies

Over the last decades, the knowledge about the manifold and complex immunological interactions, the pro- and anti-inflammatory pathways as well as the disorders of the complement and coagulation system has improved. Unfortunately, however, it has not been possible to translate this knowledge into therapeutic approaches for the treatment of sepsis. Many of the common procedures used in daily intensive care medicine cannot be considered to be fully based on criteria of evidence-based medicine so that up to date, no adjuvant therapy for sepsis and septic shock has proven to be effective for sepsis and septic shock (9, 175).

Extracorporeal Blood Purification

Besides preventing a continued activation of the pro- and anti-inflammatory pathways by an early reduction of PAMPs and DAMPs with source control measures, controlling excessive levels of cytokines and mediators by blood purification methods may be a reasonable approach. Extracorporeal blood purification techniques (BPTs) consist of different approaches and methods, most of which have their origin in renal replacement therapy (RRT). Examples are high volume hemofiltration (HVHF) and plasmapheresis, but also the use of special filters such as high cut-off (HCO) membranes and methods for the adsorption of endotoxin and cytokines or combinations of these methods such as coupled plasma filtration adsorption (CPFA). An overview of different techniques of extracorporeal blood purification is illustrated in **Figure 3**.

Although extracorporeal blood purification therapies have been shown to remove both inflammatory mediators and bacterial toxins, there is still a lack of evidence for their efficacy in sepsis therapy (176). Technically, HVHF does not differ from conventional RRT, as no additional components have to be added to the circuit. However, an increased convective target dose of well above 35 ml/kg/h is used. The procedure is easy to use if experience in the implementation of continuous renal replacement therapies is available. With HVHF, inflammatory mediators are removed from the bloodstream by convection. The effect on the outcome of sepsis and septic shock has been investigated in numerous studies, in which different convective target doses and continuous vs. ntermittent application were examined (177–180). A recent meta-analysis could demonstrate both hemodynamic improvement (lower HR and higher MAP),

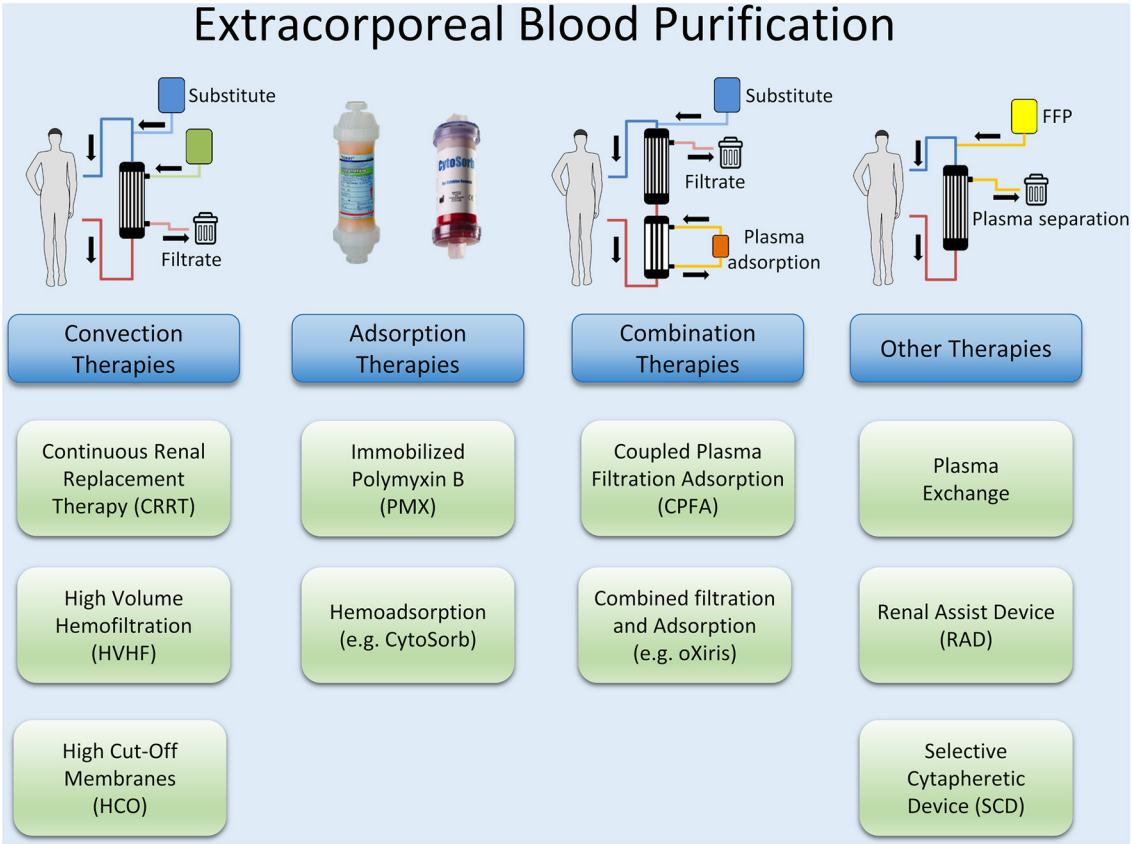


FIGURE 3 | Currently available blood purification methods.

and lower mortality of critically ill patients, but no substantial influence on oxygenation Index or disease severity. Also, most RCTs included in the meta-analysis were not of high quality and there was no uniform observation period concerning mortality (181).

The use of high-cut-off (HCO) membranes with an increased pore size (20 nm vs. 10 nm for the standard high-flux membrane) should offer a more effective elimination of inflammatory mediators. In one clinical trial, improved elimination of the inflammatory mediators IL-1, IL-6, and TNF-α was demonstrated in patients with sepsis-induced renal failure, but at the same time there was also significant albumin loss (182). Other studies were terminated prematurely due to the lack of difference in 28-days mortality, vasopressor requirements, ventilation days and ICU length of stay compared to conventional membranes (176).

Recently, there has been renewed interest in plasmapheresis for patients with severe refractory septic shock, the suggested rationale being (apart from blood purification) a rapid substitution of consumed protective plasmatic factors to support microvascular barrier function and microcirculation (177). To date, however, only sparse data are available on the use of therapeutic plasma exchange (TPE) in sepsis. In a recent meta-analysis, Putzu et al. showed that the use of plasmapheresis was associated with decreased mortality compared with standard therapy (183). In addition to a recent pilot RCT with 40 patients, which demonstrated a reduction in catecholamine requirements in patients with septic shock, the EXCHANGE trial is another prospective multicenter study with 352 participants, which investigates the efficacy of therapeutic plasma exchange in septic shock (NCT03065751) (184).

Various proteins and receptors balance the interaction between the endothelium of the vessels and circulating cells. Von Willebrand factor (VWF), with its multimeric structure, is a key protein in platelet-vessel wall interaction. The sensitive balance is controlled by a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS-13, also known as von Willebrand factor-cleaving protease; VWFCP). Reduced ADAMTS-13 activity can lead to markedly elevated levels of large VWF multimers, resulting in thrombocytic microangiopathy (TMA). Extreme but also typical forms of this are thrombocytopenic thrombotic purpura (TTP) and thrombocytopenia-associated multiple-organ failure (TAMOF). Sepsis is often associated with ADAMTS-13 deficiency due to immune-mediated antibodies, and the severity of this deficiency appears to be associated with outcome (185, 186). In addition to replacing ADAMTS-13 with recombinant proteins, the therapeutic armamentarium also consists of TPE, potentially

eliminating circulating pathogens or (auto-) antibodies in addition to replacing missing or depleted proteins (187, 188).

Coupled plasma filtration adsorption (CPFA), which was developed as a treatment for sepsis in the 1990s, is a combination of blood purification methods (189). After separation of plasma from cellular blood components with a highly permeable filter, adsorption within the plasma component by styrene polymer resin is performed before the purified plasma is returned to the cellular components and subjected to conventional hemofiltration. By avoiding direct contact between blood cells and the adsorption material, improved biocompatibility is described (190). The largest RCT to date, with 192 patients, was terminated prematurely in 2014 due to futility, without demonstrating any difference in terms of hospital mortality or ICU-free days (191). The follow-up studies COMPACT 2 (NCT01639664) and ROMPA (NCT02357433) were terminated prematurely in 2017, since the COMPACT 2 study detected a significantly increased mortality for the therapy group within the first 72 h after enrolment (192). This finding ultimately led to the discontinuation of ROMPA. At this time, no further studies are known to investigate the effect of plasmapheresis in sepsis therapy.

Adsorption Techniques

In Gram-negative sepsis, endotoxin (lipopolysaccharide (LPS) and its fragments trigger the activation of different cell types (monocytes, endothelial cells, polymorphonuclear neutrophils, and tissue-resident cells) and plasma systems (complement and coagulation pathways). It seemed logical to devise extracorporeal systems that could remove the triggering stimulus. Polymyxin B, a cyclic lipophilic peptide antibiotic, is the ligand most studied for neutralizing LPS because of its high affinity for the lipid A moiety in endotoxin. Two randomized controlled trials have evaluated a device using hemoperfusion through polymyxin B-immobilized fiber columns (PMX) in sepsis or septic shock with abdominal focus, but have shown contradictory results in terms of mortality reduction: Cruz et al. (2009) showed a trend toward mortality reduction, however, this result could not be confirmed by Payen et al. (193, 194). In another clinical trial the impact on mortality in patients in septic shock and high endotoxemia should also be investigated (195). However, after enrolment of 450 patients and completion of the study, it was shown that the primary endpoint of 28-days mortality was not reached by "per-protocol analysis" (196). A subsequent *post-hoc* analysis of the data revealed that patients with high endotoxin levels had a significant reduction in mortality, significant improvements in mean arterial pressure and an increase in ventilator-free days (197). Further evaluation of the data suggests that there may be an upper limit of endotoxin load for successful treatment with PMX.

Besides specific adsorption of endotoxin in Gram-negative sepsis, a broader approach of cytokine adsorption might be more promising. Hemadsorption using the CytoSorb® adsorber column is a non-selective and concentration-dependent method by which a spectrum of cytokines and inflammatory mediators like IL-1β, IL-6, IL-8, IL-10, and TNF-α are adsorbed from the bloodstream. In addition, free hemoglobin, myoglobin, bilirubin, bile acids and bacterial toxins (except endotoxin), activated complement and some drugs are eliminated. This technique could therefore be a suitable approach in the context of an excessive pro-inflammatory and anti-inflammatory response, especially in the early phase of sepsis (198–200). Despite widespread clinical use, the available evidence for this technique showing a positive impact on outcome in septic patients is still limited.

In 2017, a prospective single-center study with 20 consecutive patients with refractory septic shock was published in which cytokine adsorption was used as a rescue therapy (201). The study showed a significant reduction of vasopressor requirement and an increase of lactate clearance resulting in the resolution of septic shock in 13 patients (65%). Another case series of 26 patients with septic shock and renal replacement therapy also demonstrated that cytokine adsorption was associated with rapid stabilization of hemodynamic parameters, a reduced need for vasopressors, and a reduction of serum lactate (202). Compared to mortality prediction by the APACHE II score (Acute Physiology And Chronic Health Evaluation II), this study showed reduced observed mortality for patients in whom cytokine adsorption was initiated within 24 h after onset of sepsis, however, no control group was included.

In 2015 an international registry was established to evaluate the use of cytokine adsorption under real-life conditions. According to the last published interim evaluation after enrolment of more than 600 critically ill patients, in 60% of these patients the indication to use CytoSorb® was sepsis and septic shock (203). Analysis of the completed patient data sets ($n = 495$) showed a significant reduction in IL-6 levels and an observed 28-days mortality of 62.5% compared to an expected mortality rate of 71.3% as predicted by APACHE-II. There was a trend indicating that patients with the highest disease severity benefitted most from the intervention. Further, no adverse events were recorded in septic patients. The inherent absence of a control cohort and patient heterogeneity are reasons why these results cannot uncritically be adopted into clinical practice. Further randomized controlled trials are currently underway in patients with sepsis and septic shock, but also in patients with severe COVID-19.

Immunotherapy

One of the main causes of the high mortality in intensive care units continues to be sepsis-induced immunosuppression. As a hallmark, there is often a remarkable reduction in the number of circulating lymphocytes, including $CD4^+$- and $CD8^+$- T cells and B cells at the onset of sepsis, which lasts up to 28 days and is significantly correlated with mortality (40, 47). Major advances in proteomics, metabolomics and genomics as well as in point-of-care diagnostics enable a novel approach in the therapy of sepsis linked to the term "-OMICS" (204). Successful interventions to influence and reposition the immune system of the host with the help of immunomodulating substances might be transferred from cancer therapy, where the application of immunomodulating therapies is already part of the therapeutic armamentarium (205).

Antibodies against programmed cell death 1 (PD-1) receptor and the corresponding ligand (PD-L1) as well as Interleukin-7

are considered promising candidates for the treatment of sepsis and its immunological consequences. PD-1 and PD-L1 modulate as key components ("checkpoints") in a negative costimulatory pathway the duration and amplitude of the normal T cell immune response toward infectious stimuli. The activation of PD-1 enhances immunosuppressive signals and reduces effector function in both the innate and adaptive immune system (206). While PD-1 is only expressed on activated immune cells such as T cells, PD-L1 is expressed by a variety of other cells like antigen-presenting cells and tumor cells. The binding of PD-1 to PD-L1 results in a reduced release of cytokines, a growth arrest of T cells, and even to apoptosis. Exhausted T cells regularly show surface expression of PD-1 and PD-L1. Increased expression of PD-1 and PD-L1 is also found on circulating monocytes and $CD4^+$ lymphocytes in patients in septic shock, which is associated with the occurrence of secondary (nosocomial) infections and increased mortality (69, 207).

In patients with sepsis-related immunosuppression, addressing PD-1 and/or PD-L1 appears to be an option with potential clinical benefit, especially since checkpoint inhibitors have already been successfully used in cancer immunotherapy (208). In *ex-vivo* studies in human cells, the use of monoclonal antibodies blocking either PD-1 or PD-L1 led to an increase of cytokine production and secretion by T cells and monocytes (209). Nivolumab is a human IgG4 monoclonal antibody that binds to the programmed cell death 1 (PD-1) receptor and prevents the receptor from interacting with its PD-L1 and PD-L2 ligands. Nivolumab has been shown to improve viral clearance in the treatment of chronic hepatitis C (210). In the treatment of sepsis and septic shock, a phase I clinical trial was completed in 2018. After enrolment of 38 patients, there were no unexpected safety findings, and no increase in pro-inflammatory cytokines (211).

As an anti-apoptotic cytokine, Interleukin 7 (IL-7) is necessary for clonal expansion and lymphocyte survival and induces the proliferation of $CD4^+$ and $CD8^+$ T cells. *Ex-vivo* experiments had shown that IL-7 increases T cell cytokine production and normalizes peripheral blood lymphocyte metabolism in patients with septic shock (212). Due to its key role in the development, maturation, expansion and homeostasis of B and T lymphocytes and its manifold effects on innate and adaptive immunity, IL-7 has been called the "maestro of the immune system" (213). Its efficacy in the treatment of viral infections has already been demonstrated in clinical studies with HIV patients (214). In combination with further *ex-vivo* results, the potential to restore important immunological defects in patients with sepsis could be demonstrated (215). In 2018, "IRIS-7" was published as a prospective, randomized, double-blind, placebo-controlled study in 27 patients with septic shock and severe lymphopenia (216). This study aimed for the first time at defects in adaptive immunity in the context of immunoadjuvant therapy. The use of recombinant human IL-7 increased the absolute lymphocyte count and the numbers of circulating $CD4^+$ and $CD8^+$ T cells, and there was no evidence for an increased pro-inflammatory response or a worsening of organ dysfunction.

In a recent *ex-vivo* study comparing patients with sepsis due to multi-resistant bacteria and critically ill but non-septic patients, septic patients showed increased expression of PD-1 and PD-L1 (217). Administration of both anti-PD-L1 and IL-7 resulted in increased IFN-γ production by T cells. Patients whose T cells could not be stimulated to increase production of IFN-γ showed a trend toward increased mortality.

The results obtained so far show that immunomodulation to restore and reorganize adaptive immunity may become a powerful tool for the future treatment of sepsis. However, further immunological phenotyping of critically ill patients with sepsis is needed to identify target groups. For this, robust diagnostic tools must be developed to identify this group of patients quickly and reliably (218). Also, meaningful prospective studies to validate biologically plausible hypotheses are still pending, as well as randomized controlled studies that show clinical benefit of these interventions (219).

Immunoglobulins

On the assumption that immune dysregulation and acquired immunosuppression are significant factors during sepsis and septic shock, stimulation of the immune response and/or substitution of individual immune system components might be a promising therapeutic approach. Within the usually well-balanced network of interacting and regulating factors of the immune system and the inflammatory response, polyvalent intravenous immunoglobulins (IVIG) might be an effective intervention to positively affect both pro- and anti-inflammatory processes (**Figure 4**) (220, 221). Experimental data show that polyvalent immunoglobulins can neutralize exo- and endotoxin, interact with complement factors, and improve pathogen phagocytosis by opsonization (222–225). For fulminant TSS, which is caused by staphylococcal or streptococcal superantigens, and for Kawasaki disease the use of adjunctive polyclonal IVIG preparations is recommended with moderate evidence (226–229).

In a *post-hoc* analysis of the CIGMA trial, which evaluated the efficacy of an IgM- and IgA-enriched polyclonal antibody preparation in patients with severe community-acquired pneumonia, a significant relative reduction in all-cause mortality of 54–68% was shown in a subgroup with high CRP, low IgM and a high CRP/low IgM ratio at baseline compared to placebo (230). Currently, the only available IgGAM preparation is Pentaglobin®, in which the content of IgM and IgA is enriched to 12% each. The formulation also contains neutralizing and toxin-binding antibodies against numerous Gram-positive and Gram-negative bacteria and modulates the effect of other pro-inflammatory (IFN-y, IL-6) as well as anti-inflammatory cytokines (IL-10) during lymphocyte response (225, 231, 232).

Although IVIG is widely used in the treatment of neurological, immunological and hematological diseases, the Surviving Sepsis Campaign (SSC) guidelines do not recommend the use of classical IVIG preparations containing almost exclusively IgG for sepsis and septic shock (145). In 2007, a randomized controlled multi-center study with 653 patients showed no survival benefit for the use of iv-immunoglobulin G (233). Even though generally well-tolerated, the administration of IVIG is not completely free of risks. In addition to side effects like hyperviscosity syndrome with thromboembolic events in some

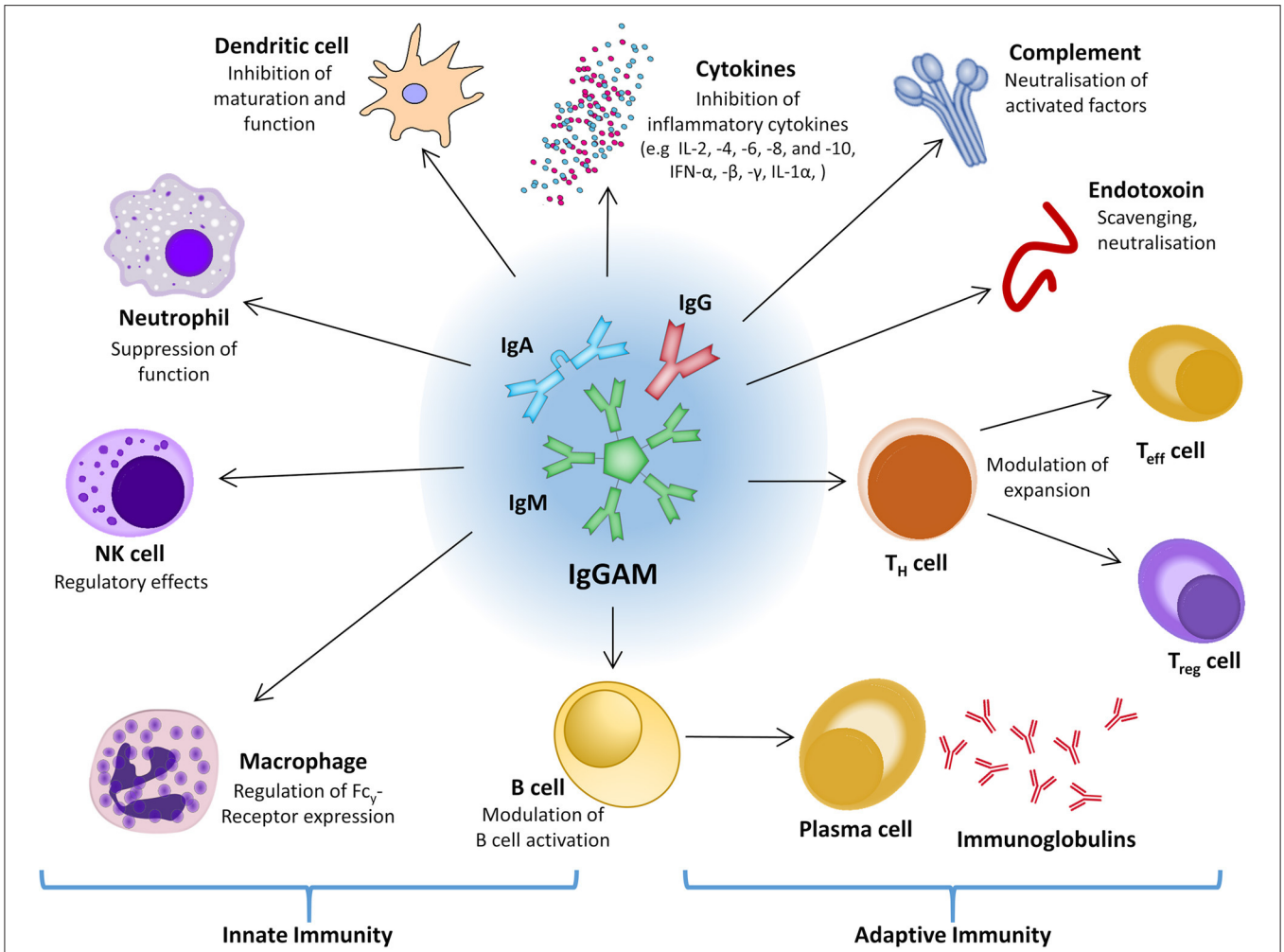


FIGURE 4 | The central role of IgGAM in the innate and adaptive immune response. IFN, interferon; Ig, immunoglobulin; IgGAM, immunoglobulin G/A/M; IL, interleukin; NK cell, natural killer cell; T_{eff} cell, effector T cell; T_H cell, helper T cell; T_{reg} cell, regulatory T cell.

patients, cases of acute renal failure have been described, which are, however, presumably due to added stabilizers in the IVIG preparations (234).

In summary, the use of IVIG in the treatment of sepsis and septic shock remains controversial. Up to now, there is little reliable data due to highly variable study protocols, patient heterogeneity and inconsistency in the spectrum of analyzed laboratory parameters (235). It is expected, however, that ongoing RCTs (e.g., PEPPER-Trial; Personalized medicine with IgGAM compared with standard of care for treatment of peritonitis after source control) will provide more conclusive information (236).

Use of Artificial Intelligence in Sepsis, Gene Expression

Early identification of prognostic indicators from the vast amount of clinical and biochemical data is difficult and inconclusive. Survivors of sepsis often suffer from multiple long-term sequelae that can affect their quality of life and significantly shorten their life expectancy (237).

Current research is aimed at identifying biomarkers to help identify a possible severe clinical course at an early stage and to improve outcome through individualized therapy management. A landmark paper by Davenport et al. demonstrated in 2016 substantial heterogeneity in the individual host response to sepsis when investigating the transcriptome. At least two distinct sepsis response patterns (SRS1 and SRS2) could be identified with SRS1 being characterized by relative immunosuppression, endotoxin tolerance and metabolic derangement. These features were significantly associated with higher short-term mortality (238).

Using existing datasets of genetic expressions of septic patients, artificial intelligence (AI) systems are trained to recognize disease progression and clinical outcomes. In a recent publication, Banerjee et al. describe the use of a dataset of 228 pediatric patients with gene expression profiles collected within 24 h of ICU admission, through which an AI system was trained by the use of machine learning in multiple phases (239). In several steps, 20 differentiated expressed genes already

associated with prediction of complicated course outcomes were identified. Based on further processing and training steps of this system, it was finally possible to identify 8 biomarkers that are known to be associated with an overshooting innate immune system. These biomarkers have previously been associated with sepsis mortality, now, however, show a predictive association with the severity of the disease course, even in surviving patients.

For example, matrix metalloproteinase 8 (MMP8) and resistin (RETN) have been identified to be associated with the release of TNF-α (240). Primarily expressed by macrophages and mononuclear cells, MMP8 itself shows beneficial anti-inflammatory activity in animal studies. MMP8 knockout mice show decreased NET activity, whereas in septic patients NET activation leads to NETosis, which in turn is positively correlated with severity (241, 242). This machine learning approach further identified lipocalin-2 (LCN2), which is known to be involved in microbiome homeostasis, in particular in protection of intestinal epithelia against oxidative stress. This immunosuppressive protein is considered a "hot candidate" for therapeutic use in abdominal sepsis (243, 244). Kangelaris et al. investigated genetic expression changes in septic patients with ARDS, and identified membrane metalloendopeptidase (MME) and hydroxycarboxylic acid receptor 3 (HCAR3) as candidates (245). Genes overexpressed in ARDS are frequently associated with poor outcome in sepsis, including MMP8 and RETN. Also, overexpression of MMP8, olfactomedin 4 (OLFM4), and interleukin 1 receptor type 2 (IL1R2) is associated with disease severity and the occurrence of organ failure in patients with AKI (246, 247).

In 2019, Seymour et al. published a paper describing the application of machine learning to readily available clinical data (rather than the genome or transcriptome analysis) (248). Data sets containing data from a total of 20,189 patients fulfilling sepsis-3 definition within 6 h of hospital admission were investigated. Based on the analysis of 29 sepsis-related variables (including demographics, vital signs, inflammation markers and markers of organ dysfunction), four distinct sepsis phenotypes (α, β, γ, and δ) could be differentiated. These phenotypes differed multidimensionally in terms of demographics, organ dysfunction, and laboratory values, but showed similarities of immune response, clinical outcome and response to therapy within the respective subclass. Using conventional analysis of sepsis subcategories such as site of infection, severity of illness, or organ dysfunction, these phenotypes cannot be captured. The early availability of the clinical parameters upon which this analysis was based may enable early identification of the respective phenotype and thus a potentially individualized therapy.

In summary, the approach using genome-wide association studies together with the application of AI using machine learning methods to available clinical data most probably may help to identify further markers and patient subclasses which are associated with severity and outcome. It is expected that suitable panels derived from clinical signs and peripheral blood samples will enable prognosis at an early stage and with little effort.

CONCLUSION

Today, the cornerstones in therapy of sepsis and septic shock still consist of early focus control, timely administration of anti-infective drugs and hemodynamic stabilization through fluids and vasopressors. Over the last decades, a paradigm change is taking place, shifting the focus from the pathogen to the host when examining sepsis pathophysiology. Clinical understanding is continuously developing toward an immunological perspective. Complex pro- and anti-inflammatory pathways and disorders of the complement and coagulation system have been elucidated, thus revealing the heterogeneity and complexity of the syndrome. Alas, it has not yet been possible to transform knowledge into evidence-based practice for the effective treatment of sepsis.

Despite progress in the (further) development of innovative therapeutic approaches, such as targeted immune modulation, the use of novel anti-infective substances or methods for extracorporeal blood purification, there are still no effective adjunctive measures for which sufficient evidence has been provided. Since the search for drug-based sepsis therapies has proven unsuccessful in recent years, more focus should be placed on methodologically innovative approaches of research. The previous approach of using exclusively anti-inflammatory therapies has been disappointing, and the investigation of strategies aiming at re-balancing the profound immune dysregulation during sepsis and septic shock seems to be a promising goal.

AUTHOR CONTRIBUTIONS

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

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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.

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Clinical Profile, Prognostic Factors, and Outcome Prediction in Hospitalized Patients With Bloodstream Infection: Results From a 10-Year Prospective Multicenter Study

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Background: Bloodstream infection (BSI) is one of the most common serious bacterial infections worldwide and also a major contributor to in-hospital mortality. Determining the predictors of mortality is crucial for prevention and improving clinical prognosis in patients with nosocomial BSI.

Methods: A nationwide prospective cohort study was conducted from 2007 until 2016 in 16 teaching hospitals across China. Microbiological results, clinical information, and patient outcomes were collected to investigate the pathogenic spectrum and mortality rate in patients with BSI and identify outcome predictors using multivariate regression, prediction model, and Kaplan–Meier analysis.

Results: No significant change was observed in the causative pathogen distribution during the 10-year period and the overall in-hospital mortality was 12.83% (480/3,741). An increased trend was found in the mortality of patients infected with *Pseudomonas aeruginosa* or *Acinetobacter baumannii*, while a decreased mortality rate was noted in *Staphylococcus aureus*-related BSI. In multivariable-adjusted models, higher mortality rate was significantly associated with older age, cancer, sepsis diagnosis, ICU admission, and prolonged hospital stay prior to BSI onset, which were also determined using machine learning-based predictive model achieved by random forest algorithm with a satisfactory performance in outcome prediction.

Conclusions: Our study described the clinical and microbiological characteristics and mortality predictive factors in patients with BSI. These informative predictors would inform clinical practice to adopt effective therapeutic strategies to improve patient outcomes.

Keywords: bacterial bloodstream infection, mortality, pathogenic spectrum, prediction model, prognostic factors

INTRODUCTION

Despite the great advances in medical diagnosis and therapy over the past decades, bloodstream infection (BSI) remains a major cause of infectious disease morbidity and mortality in both low- and middle- or high-income countries (1, 2). Several studies have reported that BSI was the seventh most common cause of death and the leading cause of death caused by infections (1, 2). It is estimated that at least 23 per 100,000 people die each year shortly following an episode of BSI (2). Immunocompromised, chemotherapies, intravascular catheters, and high consumption of antibiotics rendered hospitalized patients highly vulnerable to bacterial colonization, local infection, and even systemic infection (3). A previous study showed that different bacterial species had a significant impact on the prognosis of bacteremia, but the pathogenic spectrum responsible for BSI varied substantially over time and by region (4). Moreover, bloodstream infection can lead to sepsis, an extreme systemic response to infection, which is associated with increased mortality and length of hospital stay and additional medical costs (5). Previous efforts have demonstrated that rapid assessment and intervention is crucial for the prognosis of BSI patients, especially in the emergency department and ICU, because implementing timely and effective infection treatment can significantly reduce the incidence of BSI-associated deaths (6, 7).

Accurate identification of predictors associated with mortality in patients with BSI is critical to informing clinical interventions and improving clinical outcomes. Although some prognostic factors have been identified as potential predictors for BSI mortality, most previous reports particularly focused on a single group of people, such as children or the aged, or with specific clinical conditions including cancer and trauma as well as causative organisms equipped with multidrug resistance (8–11). In addition, multiple machine learning approaches have been developed and increasingly used in predicting unfavorable outcomes and in identifying predictors of mortality for different types of disease, with better performance than the classical multivariate regression analysis method (12). Previous studies have explored the use of the random forest model, one of the machine learning approaches, in the prediction of multidrug-resistant bacterial infection and mortality due to sepsis in the emergency department and in providing significant clinical outcome predictors based on permutation importance of different variables (13, 14). Until now, however, very few studies have characterized the feasibility of machine learning technology for the purpose of predicting all-cause mortality of hospitalized patients with BSI.

In the present study, we sought to describe the trends of the incidence of key bloodstream pathogens and BSI-associated mortality over time for the period 2007–2016, which were collected by a national prospective surveillance program. Independent factors for all-cause mortality in hospitalized patients with BSI were also assessed. These results might facilitate a physician's decision-making process concerning rational treatment for high-risk individuals with bacteremia and optimize clinical resources.

MATERIALS AND METHODS

Study Design

This study was an investigative and predictive analysis based on BSI patients' clinical data from the CARES study (Chinese Antimicrobial Resistance Surveillance of Nosocomial Infections), which is a nationwide, longitudinal, prospective study encompassing 16 tertiary-care teaching hospitals in 10 provinces in China between 2007 and 2016 (15–17). Each hospital has at least 1,200 beds and one infectious disease department and infection control committee with specialist doctors, nurses, and microbiological laboratory personnel. Clinicians can be informed immediately by telephone with the positive blood culture as a critical value. Our aim in this study was to analyze the pathogenic spectrum of bacteremia and further elucidate the independent predictors for all-cause

TABLE 1 | Distribution and incidence of bloodstream bacterial isolates during the study period.

Rank	Isolates (%) during					
	2007–2008 (1,080*)	**2009–2010 (1,061*)**	**2011–2012 (808*)**	**2013–2014 (866*)**	**2015–2016 (893*)**	**Ten years (4,708*)**
1	*E. coli* (29.07)	*E. coli* (26.58)	*E. coli* (30.07)	*E. coli* (30.72)	*E. coli* (30.24)	*E. coli* (29.21)
2	*K. pneumoniae* (11.57)	*K. pneumoniae* (10.65)	*K. pneumoniae* (13.99)	*K. pneumoniae* (15.13)	*K. pneumoniae* (12.99)	*K. pneumoniae* (12.70)
3	*S. aureus* (10.83)	*S. aureus* (10.08)	*S. aureus* (10.02)	*S. aureus* (8.55)	*S. aureus* (9.18)	*S. aureus* (9.79)
4	*P. aeruginosa* (6.02)	*P. aeruginosa* (7.45)	*A. baumannii* (8.91)	*A. baumannii* (7.27)	*A. baumannii* (8.06)	*A. baumannii* (7.03)
5	*E. faecium* (5.46)	*A. baumannii* (6.41)	*P. aeruginosa* (6.31)	*S. epidermidis* (5.54)	*P. aeruginosa* (6.16)	*P. aeruginosa* (6.33)
6	*S. epidermidis* (5.37)	*S. hominis* (5.37)	*S. epidermidis* (6.06)	*P. aeruginosa* (5.54)	*E. faecium* (4.82)	*S. epidermidis* (4.91)
7	*A. baumannii* (5.19)	*E. faecium* (4.81)	*E. faecium* (4.58)	*E. faecium* (4.27)	*S. hominis* (4.37)	*E. faecium* (4.82)
8	*S. hominis* (3.98)	*S. epidermidis* (4.52)	*E. cloacae* (4.08)	*E. cloacae* (3.35)	*E. cloacae* (3.92)	*S. hominis* (3.99)
9	*E. cloacae* (2.59)	*E. cloacae* (4.15)	*S. hominis* (3.34)	*E. faecalis* (2.54)	*S. epidermidis* (3.14)	*E. cloacae* (3.59)
10	*E. faecalis* (2.59)	*E. faecalis* (2.83)	*E. faecalis* (2.85)	*S. hominis* (2.54)	*E. faecalis* (2.69)	*E. faecalis* (2.70)

**Number of isolates.*

mortality at 28 days among hospitalized patients with BSI. Cases were eligible for this study if they had a positive blood culture for gram-negative or gram-positive bacteria and sufficient documentation in the electronic health records to assess therapy and outcomes within 28 days of the positive blood culture. This study was approved by the Research Ethics Board at Peking University People's Hospital, which waived the need for informed consent, because of the observational nature of the study.

Clinical Data Collection

All cases considered in this study were hospitalized patients aged ≥18 years and had at least one documented isolation from positive blood culture during their hospitalization. In order to identify the clinical predictors of BSI mortality, each patient was included only once at the time of the first bacterial isolation from blood culture during the study period. All elements in demographic data, antibiotic administration records, laboratory and microbiological results, and clinical information, including potential predictors for mortality, were extracted from electronic health records (EHRs) by trained reviewers. The primary outcome variable was in-hospital mortality within the first 28 days after drawing the positive blood cultures. Additionally, patients with missing observations or treated on an outpatient basis were excluded retrospectively. All bloodstream isolates were transferred to a reference laboratory (Peking University People's Hospital) and identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Cases with coagulase-negative *Staphylococcus* isolated from a single blood culture without any clinical evidence of infection were also excluded.

Prognostic Factor Analysis

Cox multivariable regression analysis was performed to identify independent predictors for BSI 28-day mortality. We conducted univariate logistic regression analysis for each candidate variable using Pearson's chi-square test or Fisher's exact test, with a $P < 0.10$ being the criterion for further analysis in the backward, conditional stepwise multivariable regression model. The Hosmer–Lemeshow goodness-of-fit test was used to assess model fit. Hazard ratios, 95% confidence intervals, and associated *P*-values were also reported.

Statistical Analysis

For univariate analysis, normally distributed continuous variables were expressed as means ± standard deviations (SD) and compared using *t*-test or Mann–Whitney *U* test. Categorical variables and their relative frequencies were expressed as absolute numbers and compared using Pearson's chi-square test or Fisher's exact test. The multivariate regression analyses were performed to identify independent predictors using IBM SPSS software (version 24.0) for Windows. The chi-square test for trend in proportions was performed to determine significant variations in etiology and mortality during the study period. All reported *P*-values are two-sided and statistical significance was set as $P < 0.05$. In addition, all cases and potential factors were used to develop the random forest model and export strong predictors for BSI-related mortality using the R package randomForest. We also quantified the discriminative performance using the area under the ROC curves (AUC), sensitivity, specificity, positive predictive value, and negative predictive value. For variables significantly associated with mortality in both multivariate analysis and random forest model, a Kaplan–Meier curve was plotted to show the survival probabilities at 28 days.

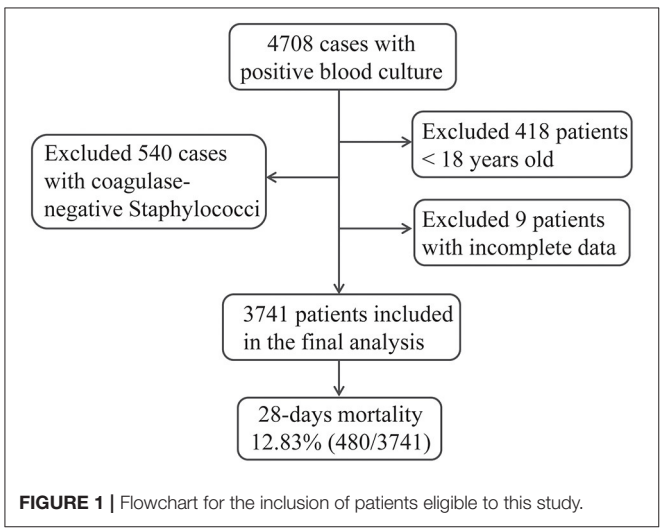


FIGURE 1 | Flowchart for the inclusion of patients eligible to this study.

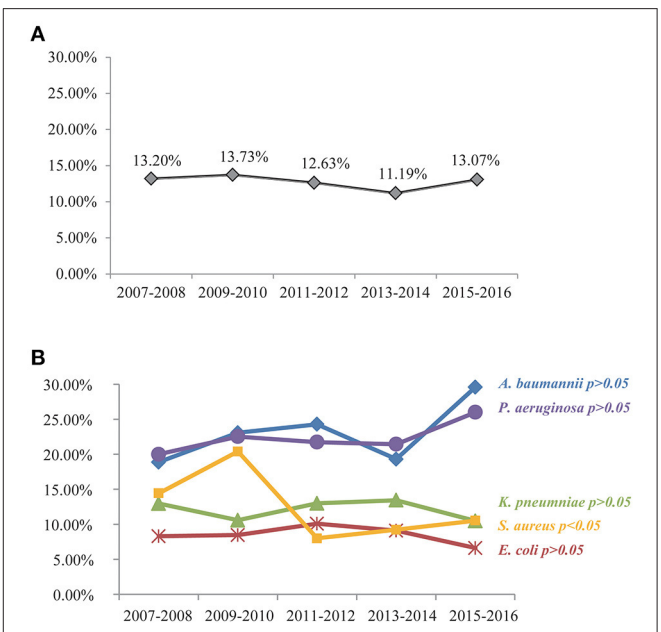


FIGURE 2 | All-cause mortality among patients with nosocomial BSI by a 2-year period **(A)** and 10-year trend in mortality related to different causative bacteria **(B)**. *S. aureus*-related mortality rate has dropped significantly during the 10-year period ($P < 0.05$). Upward trends in *A. baumannii*- and *P. aeruginosa*-related mortalities were noted but with no statistical significance as assessed by the chi-square test ($P > 0.05$). *E. coli*- and *K. pneumoniae*-associated mortality remained stable and relatively lower ($P > 0.05$).

RESULTS

Distribution and Incidence of Bacteremia Isolates

From 2007 to 2016, 4,708 patients with positive blood culture were documented in 16 tertiary-care teaching hospitals, and each first bacterial isolate was enrolled in microbiology analysis. Among these isolates, the proportion of gram-negative isolates was higher than that of gram-positive ones (70.33 vs. 29.67%). Overall, *Escherichia coli* (29.21%, 1,375/4,708) and *Klebsiella pneumoniae* (12.70%, 598/4,708) were the most common BSI-causing pathogens followed by *Staphylococcus aureus* (9.79%, 461/4,708), *Acinetobacter baumannii* (7.03%, 331/4,708), and *Pseudomonas aeruginosa* (6.33%, 298/4,708). Notably, *E. coli*, *K. pneumoniae*, *S. aureus*, and *A. baumannii* remained predominant as the top 4 pathogens responsible for BSI during the study period except *P. aeruginosa* that substituted *A. baumannii* in 2009–2010. The 10 most common pathogens are listed in **Table 1** by a 2-year period.

Demographic and Clinical Characteristics

For the purpose of identifying predictors of BSI-associated mortality in the present study, a total of 3,741 hospitalized patients fulfilled our inclusion criteria and were included in the final analysis (**Figure 1**). The overall 28-day mortality rate was 12.83% (480/3,741) during the 10-year study period and did not vary significantly among different years (**Figure 2A**). However, the BSI-associated mortality varied somewhat over time by

TABLE 2 | Demographics, comorbidities, and clinical treatments of patients with BSI.

Variables	Total, *N* = 3,741 (100%)	Survived, *N* = 3,261 (87.17%)	Died, *N* = 480 (12.83%)	*P*-value
Demographics				
Age >65 years	1,188 (31.76%)	948 (79.80%)	240 (20.20%)	<0.001
Male	2,248 (60.09%)	1,943 (86.43%)	305 (13.57%)	0.098
Comorbidity				
Malignancy	1,078 (28.82%)	922 (85.53%)	156 (14.47%)	0.056
Diabetes mellitus	601 (16.06%)	513 (85.36%)	88 (14.64%)	0.147
Hypertension	522 (13.95%)	461 (88.31%)	61 (11.69%)	0.399
Cardiovascular disease	607 (16.23%)	515 (84.84%)	92 (15.16%)	0.061
Cerebrovascular disease	359 (9.60%)	304 (84.68%)	55 (15.32%)	0.138
Liver disease	521 (13.93%)	445 (85.41%)	76 (14.59%)	0.196
Renal disease	422 (11.28%)	365 (86.49%)	57 (13.51%)	0.659
Respiratory disease	452 (12.08%)	383 (84.73%)	69 (15.27%)	0.099
Immunocompromised	34 (0.91%)	27 (79.41%)	7 (20.59%)	0.174
Invasive procedures				
Central venous catheter	591 (15.08%)	503 (85.11%)	88 (14.89%)	0.103
Peripheral intravenous catheter	2,978 (79.60%)	2,608 (87.58%)	370 (12.42%)	0.142
Urinary catheter	1,114 (29.78%)	955 (85.73%)	159 (14.27%)	0.086
Invasive mechanical ventilation	204 (5.45%)	179 (87.75%)	25 (12.25%)	0.800
Surgery within the past 14 days	975 (26.06%)	833 (85.44%)	142 (14.56%)	0.060
Condition prior to infection onset				
Preinfection length of stay >14 days	1,139 (30.45%)	933 (81.91%)	206 (18.09%)	<0.001
Prior hospitalization within 90 days	1,245 (33.28%)	1,069 (85.86%)	176 (14.14%)	0.092
Antibiotic exposure within 2 months	1,685 (45.04%)	1,485 (88.13%)	200 (11.87%)	0.111
Primary site of infection				
Urinary tract	305 (8.15%)	276 (90.49%)	29 (9.51%)	0.070
Respiratory system	351 (9.38%)	297 (84.62%)	54 (15.38%)	0.133
Central line-associated	224 (5.99%)	187 (83.48%)	37 (16.52%)	0.089
Intra-abdominal	587 (15.69%)	510 (86.88%)	77 (13.12%)	0.821
Skin and soft tissue	140 (3.74%)	125 (89.29%)	15 (10.71%)	0.445
Unknown	2,134 (57.04%)	1,866 (87.44%)	268 (12.56%)	0.566
Clinical symptoms				
Presentation with sepsis	773 (20.66%)	574 (74.26%)	199 (25.74%)	<0.001
Temperature <35 or >40°C	407 (10.88%)	336 (82.56%)	71 (17.44%)	0.003
Treatments				
ICU admission	862 (23.04%)	653 (75.75%)	209 (24.25%)	<0.001
Immunosuppressive therapy	394 (10.53%)	337 (85.53%)	57 (14.47%)	0.305
Inappropriate empirical treatment	760 (20.32%)	640 (84.21%)	120 (15.79%)	0.006

different causative organisms. Mortality due to *S. aureus*-related BSI declined from 20.39% in 2009–2010 to < 10% in 2015–2016, while that associated with *A. baumannii* and *P. aeruginosa* increased between the years 2007–2008 and the years 2015–2016. *E. coli*- and *K. pneumoniae*-associated mortality remained stable and relatively lower than that of other bloodstream isolates (**Figure 2B**).

Demographic and clinical characteristics of bacteremia patients as well as the results of univariate analysis of the comparison between survived and died groups are shown in **Table 2**. The mean age of all BSI cases was 56 years (SD = 17.33, range = 18–99) and patients were predominantly male (60.09%). The most common underlying condition was malignancy (28.82%) and the source of the BSI was primary (unknown origin) in 57.04% of the cases. Mortality varied according to comorbidities, type of catheter, and clinical therapy. The highest mortality was accompanied with sepsis symptoms (25.74%) and ICU admission (24.25%). Multiple statistically significant predictors ($P < 0.05$) were identified in the univariate analysis. Compared with survived patients, dead patients with BSI were more likely to be >65 years of age, their length of hospital stay prior to BSI was >14 days, sepsis was present, intermittent temperature was <35 or >40°C, they were admitted to the ICU, and inappropriate empirical treatment was provided.

Independent Mortality Predictors

All variables that were statistically significant ($P < 0.10$) in the univariate analysis were included in further Cox regression analysis. In the multivariate analysis, factors independently associated with higher mortality in patients with BSI included age >65 years (HR, 2.219; 95% CI, 1.745–2.822; $P < 0.001$), malignancy (HR, 1.301; 95% CI, 1.005–1.683; $P = 0.046$), pre-infection length of stay >14 days (HR, 1.615; 95% CI, 1.271–2.052; $P < 0.001$), ICU admission (HR, 6.261; 95% CI, 1.859–21.079; $P < 0.001$), and presentation with sepsis (HR, 3.973; 95% CI, 3.202–4.929; $P < 0.001$).

Besides, we also identified potential predictors with the highest coefficients based on permutation importance using the random forest algorithm. It is found that ICU admission [variable importance (VI), 53.89], presentation with sepsis (VI, 21.66), age >65 years (VI, 11.58), inappropriate empirical treatment (VI, 9.29), temperature <35 or >40°C (VI, 8.92), pre-infection length of stay >14 days (VI, 8.32), malignancy (VI, 5.62), cardiovascular disease (VI, 4.42), surgery within the past 14 days (VI, 4.28), and central line-associated (VI, 3.31) were the top 10 important predictors in the random forest model. These results were consistent with what is found in the multivariate analysis.

In addition, we also evaluated the model's ability to discriminate outcome based on all clinical data collected. The sensitivity (0.81), specificity (0.74), negative predictive value (0.95), and AUC (0.856) showed high to moderate predictive performance, while the positive predictive value is only 0.32 (**Figure 3**).

Survival Curve Analysis

To evaluate the trends of in-hospital mortality, five predictors identified both in multivariate regression analysis and random forest predictive model were selected to construct survival curve analysis (**Figure 4**). Kaplan–Meier curves demonstrated that 28-day survival distributions were significantly different in patients with age >65 years ($P < 0.001$), pre-infection length of stay >14 days ($P < 0.001$), ICU admission ($P < 0.001$), and presentation with sepsis ($P < 0.001$). Although BSI patients with malignancy tended to have a worse outcome, the log-rank test was not significant ($P = 0.061$) and the two survival curves crossed early at around 4 days. All survival curves run parallel until the first week and start to diverge, with a continuously higher death rate among patients with corresponding prognostic factor.

DISCUSSION

Globally, the incidence of bacteremia remains high and continues to contribute to increased patient morbidity and mortality, as well as medical costs (1). In this study, a total of 4,708 BSI cases were obtained from the well-studied nationwide dataset over a 10-year period, and we reported that the 28-day all-cause mortality rate among hospitalized patients with BSI in China was 12.83%, which was slightly greater than that reported in a recent large multicenter study (12%) in the USA (18). Demographics, comorbidity, and clinical treatment information were investigated in our study to evaluate the predictors of mortality. The multivariate analysis showed that a total of five independent predictors for BSI mortality were identified in the dataset, which is associated with older age, malignancy, hospital length of stay, clinical symptom, and ICU admission. These predictive factors were also identified by a machine learning model and survival curve analysis.

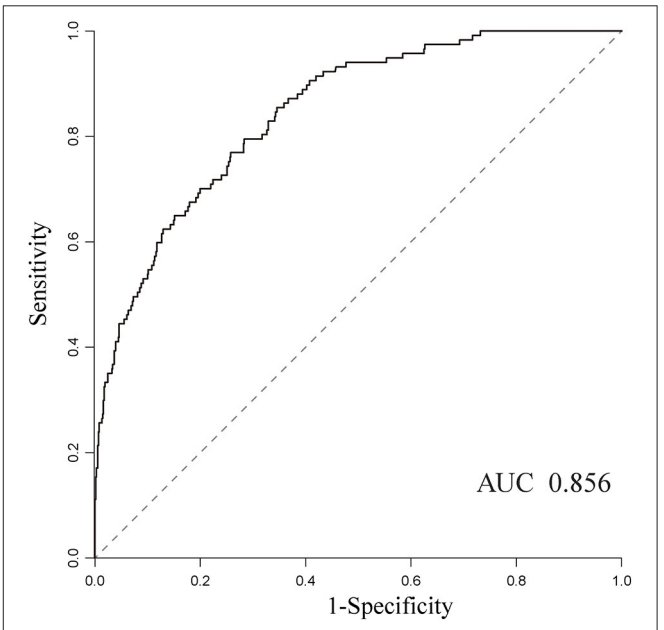


FIGURE 3 | The receiver operating characteristic curves for predicting mortality using the random forest algorithm and the AUC was 0.856.

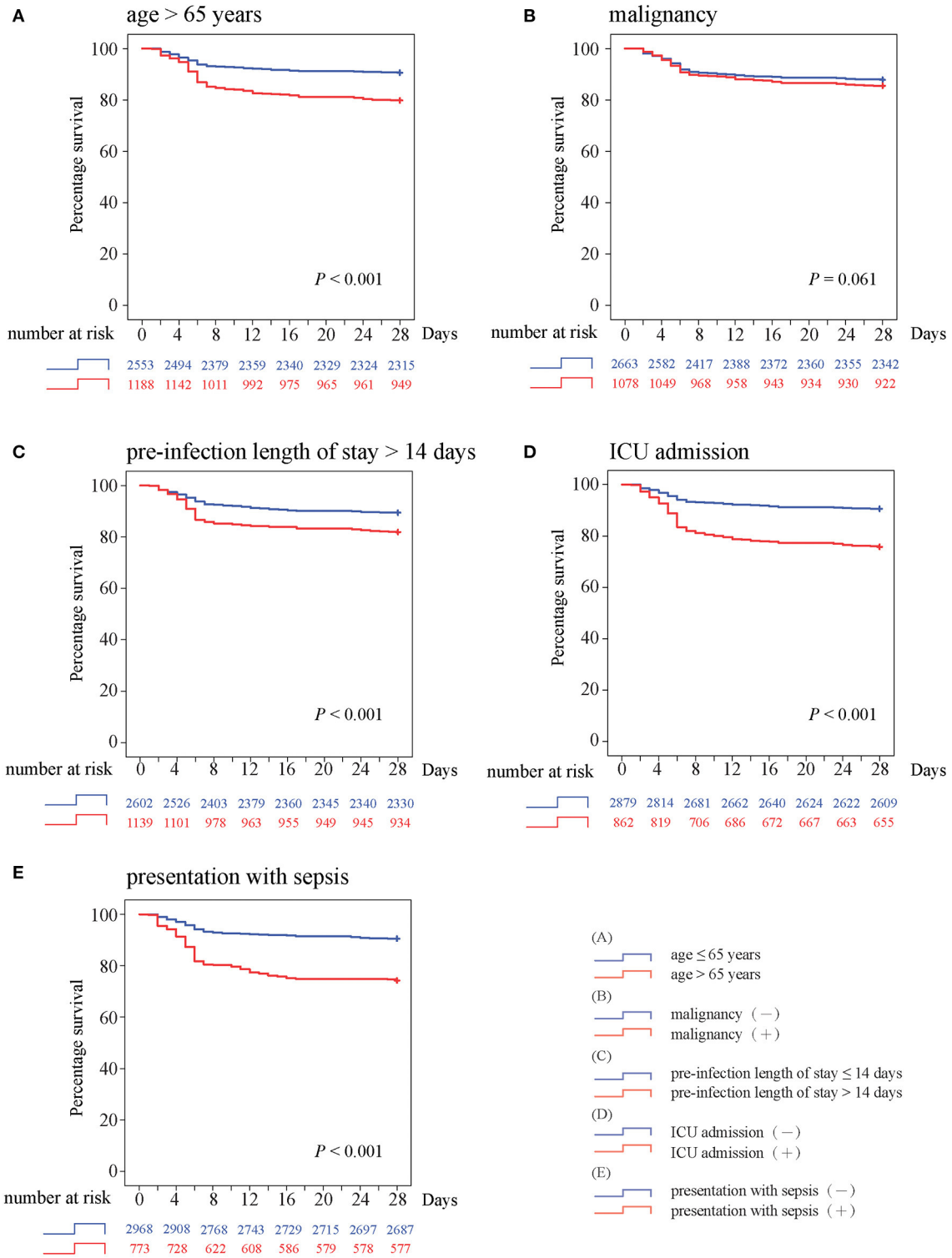


FIGURE 4 | Kaplan–Meier survival curves with log-rank test for **(A)** age >65 years, **(B)** malignancy, **(C)** preinfection length of stay >14 days, **(D)** ICU admission, and **(E)** presentation with sepsis.

Patients at increased risk of death after bacteremia could be identified in real time according to prognostic factors. Previous studies have reported that multiple clinical factors, including underlying medical conditions, previous antibiotics exposure, and severity of bacteremia, were independently associated with poor outcome in patients with BSI (19, 20).

Patient-related factors, including older age, female sex, and recent hospitalization, were additional significant predictors of mortality (21). However, most of these studies focused on a specific subpopulation group suffering from BSI or those individuals infected with multidrug-resistant pathogen (21), while the current study extracted the predictive factors from a general patient population. The diverse population could increase the generalizability of the identified predictors. More complicated clinical manifestation could be available in the analysis, and these predictors might be more broadly applicable in clinical practice.

Previous studies proved that machine learning techniques are capable of harnessing a mass of clinical variables and the interaction between these factors and, ultimately, predicting clinical outcomes of interest with a satisfactory accuracy in real time (12). In the field of infectious disease prediction, the machine learning model has mostly been limited to the use of predicting infection with multidrug-resistant organism and sepsis in the ICU and emergency department (22–25). In this study, we made an attempt to utilize the machine learning prediction model to predict mortality among hospitalized patients with BSI, and it performed satisfactorily with an AUC value of 0.856. The prediction model also exported important predictors for BSI mortality, including ICU admission, presentation with sepsis, inappropriate empirical treatment, etc. These variables could aid in the physician's judgment and provide clinicians with real-time prognostic information to assist in decision-making and reduce preventable BSI-related adverse events. Moreover, no parameter optimization was performed for this model in order to simplify the application of machine learning approaches in the healthcare settings in our study. It suggests that parameter optimization could further improve predictive performance. In addition, other machine-learning-based models, such as the support vector machine, artificial neural networks, or deep learning, may also be constructed with this dataset and compared with the random forest model in this study.

Based on the nationwide culture-confirmed BSI cohort, we found that the overall mortality of BSI patients during the 10-year period was relatively stable, but the mortality of patients with BSI due to different causative pathogen presented different changing trends. *P. aeruginosa* and *A. baumannii*, two clinically important non-fermenters, were linked to increased mortality during the study period. Conversely, *S. aureus*-related mortality rate showed a gradually decreasing trend. A possible explanation for the observed phenomena was the extremely limited therapeutic options for bloodstream infections due to carbapenem-resistant *P. aeruginosa* and *A. baumannii* that have spread increasingly in recent years, while vancomycin- or daptomycin-resistant *S. aureus* were relatively rare in China.

In conclusion, our study determined the overall mortality rate of patients with bloodstream infection during a 10-year period and identified multiple predictors associated with poorer outcomes using multivariable-adjusted analysis and random forest predictive model. These clinically important predictive factors, including abnormal body temperature, longer hospital stay, and presentation with sepsis, could aid clinicians in identifying patients at high risk of death and lead to timely medical interventions to improve patient outcomes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Research Ethics Board at Peking University People's Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

LJ and HW conceived and designed the study. CZ, HL, RW, and QW led the data collection and analysis. LJ wrote the manuscript. HW critically reviewed and edited the manuscript. All authors have read, commented, and approved the final version of the article.

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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.

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REVIEW
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Neonatal Sepsis: The Impact of Carbapenem-Resistant and Hypervirulent *Klebsiella pneumoniae*

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The convergence of a vulnerable population and a notorious pathogen is devastating, as seen in the case of sepsis occurring during the first 28 days of life (neonatal period). Sepsis leads to mortality, particularly in low-income countries (LICs) and lower-middle-income countries (LMICs). *Klebsiella pneumoniae*, an opportunistic pathogen is a leading cause of neonatal sepsis. The success of *K. pneumoniae* as a pathogen can be attributed to its multidrug-resistance and hypervirulent-pathotype. Though the WHO still recommends ampicillin and gentamicin for the treatment of neonatal sepsis, *K. pneumoniae* is rapidly becoming untreatable in this susceptible population. With escalating rates of cephalosporin use in health-care settings, the increasing dependency on carbapenems, a "last resort antibiotic," has led to the emergence of carbapenem-resistant *K. pneumoniae* (CRKP). CRKP is reported from around the world causing outbreaks of neonatal infections. Carbapenem resistance in CRKP is largely mediated by highly transmissible plasmid-encoded carbapenemase enzymes, including KPC, NDM, and OXA-48-like enzymes. Further, the emergence of a more invasive and highly pathogenic hypervirulent *K. pneumoniae* (hvKP) pathotype in the clinical context poses an additional challenge to the clinicians. The deadly package of resistance and virulence has already limited therapeutic options in neonates with a compromised defense system. Although there are reports of CRKP infections, a review on neonatal sepsis due to CRKP/ hvKP is scarce. Here, we discuss the current understanding of neonatal sepsis with a focus on the global impact of the CRKP, provide a perspective regarding the possible acquisition and transmission of the CRKP and/or hvKP in neonates, and present strategies to effectively identify and combat these organisms.

Keywords: neonatal sepsis, *Klebsiella pneumoniae*, carbapenem resistance, hypervirulence, CR-hvKP, antibiotics, treatment, lower-middle-income countries

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INTRODUCTION

The infiltration of sterile regions of the body with microorganisms and the manifestation of a reaction to it thereof, is known as sepsis. When this occurs within the first 28 days of life consequences can be dire, as the newborns first encounter a world of pathogens. Their immune system has not yet developed to equip them in this battle. Thus, the total number of neonatal deaths due to sepsis is a staggering half-million per year (1, 2).

The pathogens that a neonate encounters are essentially present everywhere, starting with the birth canal, the crib, the hands of the nurse, or even the nasogastric tube. For the ones who are premature or low-birth-weight and require prolonged hospitalization or life support systems, the chances of infection are very high (3). In low-income countries (LICs) and lower-middle-income countries (LMICs), where nearly all resources are inadequate, breaches in care or infection control can lead to sepsis.

If one had to name an organism that readily becomes resistant to antibiotics, can harbor numerous plasmids, can survive in the environment & within the human gut, and is a dread in the neonatal intensive care units (NICU) in LMICs, it would be none other than *Klebsiella pneumoniae* (4, 5). This organism has increasingly shown various facets of a successful pathogen. Resistance to several antibiotics at a low fitness cost makes it capable of causing outbreaks in neonatal units. *K. pneumoniae* is resistant to a repertoire of antibiotics. Resistance to carbapenems, considered as the "last resort" against serious infections caused by Gram-negative bacilli, has limited therapeutic options immensely. As options of treatment were slowly failing, resistance to carbapenems was a cul-de-sac, because carbapenem-resistant *K. pneumoniae* (CRKP) are also resistant to several other antibiotics. Carbapenem-resistant genes are frequently harbored on plasmids that can spread from one species to the other. These resistance genes code for enzymes that efficiently hydrolyze carbapenems and all other β-lactam antibiotics. Further, most are also resilient against inhibition by the commercially viable β-lactamase inhibitors. In addition to carbapenem resistance, *K. pneumoniae* also had another ace up its sleeve, hypervirulence. Hypervirulent *K. pneumoniae* (hvKP) possess features that arm them to evade the host immune system to cause infections in immunocompetent hosts. hvKP are invasive and can disseminate to multiple sites.

With the World Health Organization (WHO) guidelines of treatment inadequately poised to tackle CRKP and hvKP in the neonatal population, this review presents different aspects of CRKP and hvKP and their impact on the newborn.

NEONATAL SEPSIS: A DISEASE THAT CAN'T BE IGNORED

According to the "Levels and Trends in Child Mortality report, 2019" the estimated global rate of neonatal deaths was 17 per 1,000 live births, and worldwide ∼6,700 neonates died each day in 2019 (6). A notable disparity exists between high-income countries (HICs) and LMICs in terms of the rate of neonatal death (1, 6). Eighty percent of the entire global burden was from two regions: Sub-Saharan Africa (42%) and Central and Southern Asia (37%). In both these regions, the neonatal mortality rate is around 24 deaths per 1,000 live births whereas in North America and Europe the rate is three deaths per 1,000 live births (6). Conditions related to infections such as sepsis and pneumonia as well as conditions not related to infections such as preterm birth complications, intrapartum-related events (e.g., birth asphyxia), and congenital anomalies are the predominant causes of neonatal death (1).

Sepsis is a dysregulated host response to systemic infections leading to shock and multi-organ dysfunction (7, 8) and may or may not be associated with a positive blood culture (9). When a pathogen can be isolated from the blood or cerebrospinal fluid of a neonate (a child within 28 days from birth) with noticeable hemodynamic changes, it is defined as neonatal septicemia (3). Overall, the rate of neonatal sepsis varies between 6.5 and 38 per 1,000 live births (hospital born only) in LMICs with bloodstream infections (BSIs) ranging between 1.7 and 33 per 1,000 live births (10). These rates are 3–20 times higher than the rates of the industrialized countries which ranges between 1 and 5 per 1,000 live births (4). The neonatal sepsis rates from underdeveloped countries are not exactly represented in the above figures because in these countries many children are born at home. In the present COVID-19 situation, hospitalization for delivery has decreased further (11). Conversion of health management facilities to COVID-19 hospitals or shutting down of medical facilities due to COVID-19 spread has caused non-availability of proper treatment to all other critical life-threatening conditions (12, 13), including neonatal care (11, 14, 15).

In the most widely accepted notion, if sepsis is manifested within 72 h of life, it is defined as Early-onset sepsis (EOS), in which case, infections are conventionally thought to be transmitted from the mother. Any sepsis presenting after 72 h of life is defined as Late-onset sepsis (LOS) and the infection in such cases is thought to be hospital or community-acquired (3, 8). Premature and low-birth-weight neonates are more susceptible to infections caused by microorganisms and thus, for low-birth-weight neonates, every infection should be considered as hospital-acquired (3). In a recent study, a significant difference observed between EOS and LOS was associated with gestational age, as premature neonates showed higher rates of LOS and it is well-known that they are at higher risk of exposure to nosocomial infections as these neonates require longer hospital stays, central venous access, and often mechanical ventilation (16). In the same study, it was also noted that birth by cesarean section was more associated with LOS than birth by normal vaginal delivery (16). In LICs and LMICs, EOS and LOS cannot be properly distinguished. Due to poor hygienic practices in labor rooms and nurseries, every infection regardless of the time of onset can be hospital-acquired (10).

The disparity between LMICs and HICs is also reflected in the etiological agents causing the infections. In LMICs like India or Jordan, the causative agents of EOS are similar to causative agents of LOS (17–19). Studies show that Gram-negative bacteria, mainly *K. pneumoniae* and *Acinetobacter baumannii* play a pivotal role in causing neonatal septicemia along with *Escherichia coli* and Gram-positive *Staphylococcus aureus* in resource-poor settings (17–21). Whereas, in developed countries, group B *Streptococcus*, *E. coli,* and *S. aureus* are the major pathogens causing neonatal septicemia (3, 8).

THE BUG

Klebsiella pneumoniae is a Gram-negative, non-motile, and usually encapsulated bacillus of the Enterobacteriaceae family. This organism is omnipresent in the environment;

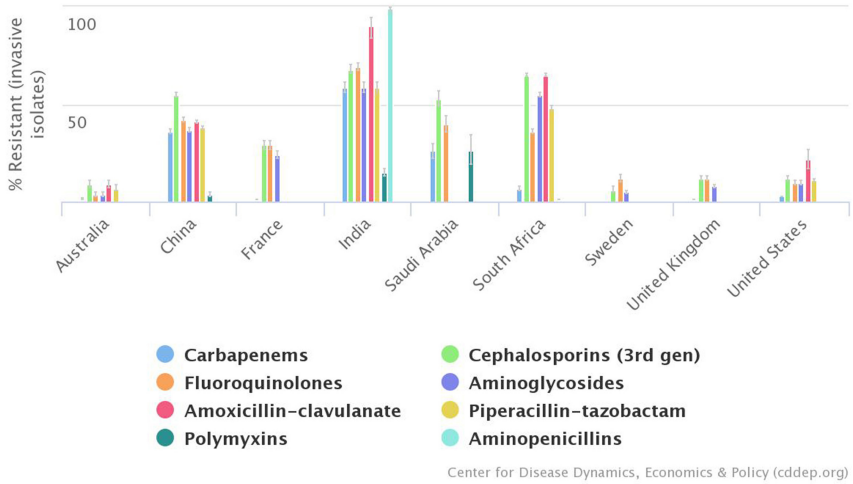


FIGURE 1 | Graphical representation of existing disparity between high-income countries (HICs) and lower-middle-income countries (LMICs) regarding antimicrobial resistance profile of *K. pneumoniae*; according to Center for Disease Dynamics, Economics, & Policy (CDDEP) data (37).

K. pneumoniae is found in the soil, water, plants, and sewage. *K. pneumoniae* is also a part of the microbiome of the nasopharynx and gastrointestinal (GI) tract of healthy human beings (22, 23). It is an opportunistic pathogen and causes both hospital-acquired and community-acquired infections (24). In hospitals, *K. pneumoniae* causes both endemic infections and outbreaks of epidemic strains; chances of acquisition of *K. pneumoniae* in nasopharynx, GI tract, and skin increases with longer hospital stays and use of invasive devices (22). *K. pneumoniae* is one of the most predominant pathogens isolated from the intensive care units (ICUs) (23) and causes infections such as bacteremia, respiratory tract infections, urinary tract infection (UTI), invasive liver abscesses, endophthalmitis, and endocarditis (22, 25). Its capability of biofilm formation in the catheter enables it to cause catheter-associated UTI (26).

K. pneumoniae is also the predominant causative pathogen of neonatal sepsis (19, 27–30). Often a localized infection or colonization of the urinary tract, GI tract, or respiratory tract disseminates into the blood and leads to sepsis (26). The role of *K. pneumoniae* in causing neonatal sepsis is discussed later in detail.

Typing of strains is an integral part of epidemiological studies and presently multi-locus sequence typing (MLST), a method of distinguishing strains based on DNA sequences of internal fragments of multiple house-keeping genes, is prevalent. In *K. pneumoniae*, MLST is based on seven conserved housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*) (31). The extensive drug-resistant (XDR) epidemic clones of *K. pneumoniae* are ST11, ST14, ST15, ST17, ST37, ST101, ST147, ST258, ST512, and these are reviewed elsewhere (32). *K. pneumoniae* can also be serotyped based on its capsular antigens (78 K antigens) (33).

Virulence of *K. pneumoniae* is essentially linked with its capsule which serves a dual purpose in the cell: it protects the cell from phagocytosis mediated by polymorpho-nuclear granulocytes and serum resistance by inactivation of one of the complement components (C3B). The pili or fimbriae is another component of the bacterial cell that helps in pathogenicity by mediating the adhesion of the pathogen to the mucosal layer and/or epithelial cells of the lower urinary tract, respiratory tract, and GI tract. Type 1 pili mediate adherence and then colonization of urinary and respiratory tract. Mannose-resistant Klebsiella-like hemagglutinin (MR/K-HA), a Type 3 pili, helps in the adhesion to Bowman's capsule, renal vessels, and tubular basal membranes of the human kidney (22). *K. pneumoniae* often possess large virulence plasmids (pLVPK) which harbor *rmpA*, *rmpA2*, and aerobactin biosysnthesis genes (34). With the possession of such virulence traits such as hypercapsule production, aerobactin and yersiniabactin synthesis, the bacterial cell becomes hypervirulent (34, 35).

For more than two decades, the increase in septicemia and meningitis in newborns caused by *Klebsiella* has been a matter of concern (22). Acquired resistance to critical antibiotics and hypervirulence in this fast-evolving pathogen is bound to make the existing scenario even more unmanageable.

THE RESISTANCE OF THE BUG TO THE DRUGS

The indiscriminate use of antimicrobials has inadvertently lead to the emergence of resistance to different drugs (32, 36). The stark difference between antibiotic resistance rates of *K. pneumoniae* between HICs and LMICs is evident in **Figure 1**.

Klebsiella pneumoniae is notorious for its ability to acquire antibiotic resistance determinants and it belongs to the 'critical' category in the WHO global priority pathogen list (38). It is one of the ESKAPE pathogens [*Enterococcus faecium, Staphylococcus aureus, K. pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp.*] which are mostly responsible for the spread of antibiotic resistance in hospital-acquired infections (36). The presence of *Klebsiella* in both the environment and the human body allows them to acquire a large variety of antibiotic resistance determinants. *K. pneumoniae* has a repertoire of around 400 antibiotic resistance genes which is almost double that of other pathogens (24). As the soil and the gut are both hot-spots for the inter-genus transfer of antibiotic resistance, *K. pneumoniae* has a selective advantage as it dwells in both these niches (24). Most of the antibiotic resistance determinants either appear first in *K. pneumoniae* or they are quickly acquired by this organism. *K. pneumoniae* also show higher variability in the G+C contents of its genomes than its other counterparts, indicating that it acquires external DNA from varied sources (24).

As with other Enterobacteriaceae, the majority of antimicrobial resistance (AMR) determinants are plasmid-mediated in *K. pneumoniae* (24, 36). Most pathogenic *K. pneumoniae* carry three or more AMR plasmids and the stability of the plasmids are relatively more in this organism compared to *E. coli* (24). The segment of the plasmid responsible for replication control to maintain a specific plasmid copy number is called a replicon (32). PCR-based replicon typing method (39) and advances in genomics have helped in recognizing plasmid types of *K. pneumoniae*. Various replicons have been found in *K. pneumoniae,* either alone or in combinations, which are IncFIIK, IncN1, IncX3, IncA/C, IncR, IncHI1-FIA, IncHIB-FIB, IncHI2, IncL (32). One of the replicons found frequently in *K. pneumoniae,* IncFIIK replicon, is present in multi-replicon plasmids which also possess IncFIB replicons (32). IncFIIK plasmids have a narrow host range and are rarely found outside this genus. Whereas, other replicons, IncR, IncA/C, IncX3, IncHI1 are of broad host range and thus act as a shuttle for inter-genus horizontal gene transfer (32). Apart from the transfer of genes via plasmids, mobile genetic elements such as insertion elements (e.g., IS*26*), transposons (e.g., Tn*4401a)*, and integrons (e.g., Integron1), present in the plasmids, mediate mobilization of the resistance genes (often gene cassettes) between different plasmids or between chromosome and plasmids (32).

THE CRKP MENACE

In the 1980s, extended-spectrum β-lactamases (ESBLs) producing *K. pneumoniae* emerged and spread throughout the world (22, 40). This led to the use of the carbapenems (meropenem, imipenem, ertapenem), a β-lactam antibiotic, which became the antibiotic of choice to treat infections caused by ESBL-positive *K. pneumoniae* (40, 41). Eventually, with use of carbapenems, a new group of enzymes emerged- the carbapenemases, which could hydrolyze most of the β-lactam antibiotics including the carbapenems. The first plasmid-mediated carbapenemase IMP-1 was identified in *K. pneumoniae* in 1991 (42). KPC-1-producing *K. pneumoniae* was reported from the USA in 1996 (43). This carbapenemase was named KPC-1, for *K. pneumoniae* carbapenemase. Since then other carbapenemases have also emerged and CRKP has rapidly spread worldwide (23, 44).

Carbapenem resistance in *K. pneumoniae* or other bacteria occurs by two main mechanisms: (i) production of carbapenemases (45), and (ii) porin loss (OmpK35 and OmpK36) combined with the presence of AmpC cephalosporinases or ESBLs such as CTX-M-15 and/or overexpression of efflux pumps (21, 46, 47).

As carbapenemases are primarily mediated via mobile genetic elements contributing to the spread of carbapenem resistance, a more detailed discussion of these enzymes are done here. Carbapenemases belong to the molecular class A (e.g., KPC, GES, IMI), class B (e.g., IMP, VIM, NDM), and class D (e.g., OXA-48-like) of β-lactamases according to the Ambler classification. Molecular class A and D enzymes are called serine carbapenemases as they contain serine molecule in their active site and molecular class B enzymes are called metallo-β-lactamase (MBLs) as they contain two Zn^{2+} ions in their active site. MBLs cannot hydrolyze the monobactam aztreonam. Class A serine carbapenemases are predominantly inhibited by tazobactam. Class B MBLs are inhibited by EDTA, dipicholinic acid, or 1,10-o-phenanthroline *in vitro*. Class D carbapenemases (e.g., OXA-48-like) which hydrolyze oxacillin, cloxacillin, and carbenicillin, are inhibited *in vitro* by NaCl (46). As different carbapenemases have emerged over time, many have several enzymatic variants (such as KPC-2, KPC-3, NDM-5, NDM-7, OXA-48, OXA-232, IMP-4, IMP-8, etc.) with higher catalytic efficiency, stability, or better metal ion binding capacity (47). All such carbapenemases are harbored on plasmids which have shown both intra- and inter-species transmission.

KPC is a plasmid-mediated molecular class A serine carbapenemase. bla_{KPC} gene is found within a unique transposon Tn*4401* variant which has led to the mobilization of the gene to different types of plasmids and through the plasmids to other organisms (32). This carbapenemase has spread vastly in Italy, Greece (48), and the USA (49). Currently, there are 75 alleles reported in the NCBI pathogens database (https://www.ncbi.nlm.nih.gov/pathogens/refgene/#blaKPC), of which KPC-2 and KPC-3 are prevalent. Association of the KPC-2 and KPC-3 with epidemic clone ST258 is the pivotal factor for the spread of these genes.

New Delhi metallo-β-lactamase (NDM-1) is the most widely disseminated class B metallo-β-lactamase. It was first reported in a *K. pneumoniae* and *E. coli* in 2009 and was recovered from a Swedish patient returning from India (50). Although NDM-1 was the most prevalent variant to date, variants such as NDM-4, NDM-5, NDM-7 which are more stable in the zinc-deprived condition due to an M154L mutation have rapidly emerged (51, 52). Currently, there are 31 alleles of bla_{NDM} reported in the NCBI pathogens database (https://www.ncbi.nlm.nih.gov/pathogens/refgene/#blaNDM). bla_{NDM} is found across various sequence types of *K. pneumoniae*, no association with any particular ST was reported (53). Co-existence of many other antibiotic resistance determinants like *armA*, *rmtB*

(aminoglycoside resistance), *qnrB*, *qnrS*, *aac(6′)-Ib-cr* (plasmid-mediated fluoroquinolone resistance), and $bla_{CTX-M-15}$ (ESBL) (21, 28, 54) is often noticed in these strains. bla_{NDM-1} is present in varied broad host plasmids (e.g., IncX3, IncA/C, IncHIB-M/FIB-M) (32). The bla_{NDM} gene is almost always found bracketed by a truncated or entire IS*Aba125* element (upstream) and a ble_{MBL} gene (downstream) (55).

Molecular class D carbapenemases such as OXA-48 is also a very potent transferable carbapenemase emerging in Enterobacteriaceae (45). It was first reported in 2001 from Turkey in a multidrug-resistant (MDR) *K. pneumoniae* isolate which possessed MBLs and lacked outer membrane proteins (53). OXA-48 has now spread to all continents except Antarctica (56). OXA-48 and OXA-48-like carbapenemases (e.g., OXA-181 and OXA-232) cannot hydrolyze extended-spectrum cephalosporins and can selectively hydrolyze carbapenems (imipenem and ertapenem). Although, bla_{OXA-48} is associated with diverse STs, epidemic STs such as ST101, ST147, ST15, and ST395 are more common than others. bla_{OXA-48} is generally bracketed by IS*1999* in transposon Tn*1999* and $bla_{OXA-181}$ is associated with IS*Ecp1* (57). OXA-181 differs from OXA-232 by a single amino acid substitution and both have a similar genetic environment, suggesting that $bla_{OXA-181}$ is the probable progenitor of $bla_{OXA-232}$. OXA-181 was first reported from Indian hospitals and is endemic to Indian subcontinent. It is now reported from Asia, Africa, Middle East, Europe, North America, and Oceania (56). OXA-232 was first isolated in France from three patients who just returned from India. The $bla_{OXA-232}$ gene was carried in a ColE2 plasmid, situated within a Tn*2013* transposon, downstream a IS*Ecp1* element (58). OXA-232 has been majorly associated with ST14, ST15, and ST16. OXA-232 is endemic in India and has now been reported from other parts of Asia, USA, Africa, and Europe (56).

IMP and VIM are two other plasmid-mediated MBLs. IMP (imipenemase) carbapenemase was first reported in the year 1991 from Japan from an *Serratia marcescens* strain (59). Followed by its identification in *K. pneumoniae* strains from Japan and Singapore, IMP-4-positive *K. pneumoniae* was reported from Australia in 2002 and IMP-8-positive *K. pneumoniae* was reported from Taiwan 2001–2002 and later from various other countries. VIM (Verona integron-encoded metallo-β-lactamases) are spread in Southern Europe and also in other countries (23). VIM-1 and VIM-2 were discovered in *P. aeruginosa* and later found in Enterobacteriaceae. IncN plasmid carrying bla_{VIM-1} was later reported from *K. pneumoniae* in Greece (60). Both these enzymes are associated with class 1 integrons and various insertion sequences such as IS*26*, IS*6100* which are associated with specific plasmid types (61).

DRUG-RESISTANT BUG AND THE NEWBORN

Various studies have been published regarding the spread of carbapenem-resistant Enterobacteriaceae but the data on neonatal sepsis is infrequent. Here we present the studies related to neonatal sepsis caused by CRKP (**Table 1**). We have focused on the major carbapenemases such as KPC, NDM, and to some extent OXA-48, IMP, and VIM. The genetic aspects of these carbapenemases are already discussed in the above section.

Over the last few years, KPC-producing *K. pneumoniae* have been reported in neonates from various countries such as Egypt (75), Jordan (17), China (71, 74, 77), and India (76). bla_{KPC-2}-positive *K. pneumoniae* caused infections in China and India. The sequence types of the corresponding strains from China and India were however different: strains from China belonged to ST11 whereas, strains from India belonged to ST147 (76). bla_{KPC} was harbored in large plasmids along with other resistance determinants such as $bla_{CTX-M-15,TEM,SHV,OXA-1}$, *rmtB*, *aac(6′)-Ib-cr*, *qnrB,* and *qnrS*. The genetic environment of bla_{KPC-2} in ST147 strains from India corroborated with the genetic environment of the other bla_{KPC-2}-possessing strains recovered from adults, where bla_{KPC-2} was associated with IS elements IS*Kpn6* and IS*Kpn7*, plasmid type IncFII, and transposon Tn *4401*. All neonates in this study had an overlapping stay in the hospital so a chance of transmission from one neonate to another was predicted (76). In one of the above studies from China, three of the four infants died due to the infection caused by KPC-producing *K. pneumoniae*. Only one neonate responded to the therapy of amikacin in combination with imipenem (74). The other study from China mentioned the isolation of KPC-producing CRKP not only from blood but also from sputum, urine, aspiration catheter, and hospital environment, indicating that the hospital environment can harbor CRKP strains which may cause disease later (71). The study from Jordan interestingly showed that infection due to KPC-producing *K. pneumoniae* and *Acinetobacter spp.* led to higher mortality and previous exposure to carbapenems and vancomycin significantly increase this risk (17). Further, the study from Egypt showed that neonatal mortality was inversely related to gestational age and birth-weight. The same study also showed that neonates who eventually succumbed to the infection had a significant reduction in platelet count and hemoglobin levels (75).

Shortly after the report of bla_{NDM-1} in an adult patient in 2009, bla_{NDM-1}–possessing *K. pneumoniae* causing neonatal septicemia was identified in 2011 from India (62). Three retrospective studies from the same unit showed that carbapenem-resistant *K. pneumoniae* not only persisted in the unit but gradually became the most predominant carbapenem-resistant organism causing septicemia (21, 28, 55). One of these studies also reported the *in vivo* interspecies plasmid transfer event of bla_{NDM-1} in a neonate from whom *Enterobacter cloacae* was isolated initially and *E. coli* later. The study showed that the bla_{NDM-1} plasmid in both the species was identical indicating its possible transmission between *Enterobacter cloacae* and *E. coli* (55). The study of Datta et al. reported that male sex, low birth weight, birth at extramural centers were significantly associated with sepsis caused by NDM-1-positive isolates. However, sepsis caused due to these isolates did not result in a higher mortality rate (21). NDM-positive *K. pneumoniae*-mediated neonatal infections are now reported from India (51, 85), Nepal (65), China (68–70, 72, 77), Nigeria (66), Colombia (63), and Turkey (67). Although bla_{NDM-1} was the most prevalent allele to date, alleles such as bla_{NDM-4} and bla_{NDM-5} are slowly emerging

TABLE 1 | Carbapenem-resistant K. pneumoniae causing neonatal septicemia or intestinal colonization.

Country	Study timeline	Year of publication	Clinical presentation	Source(s)	Sequence Types (STs)	Carbapenemases identified	Other resistance genes identified	Plasmid type(s) and integrons	References
India	NA	2011	Sepsis	Endotracheal aspirate and Blood	ND[a]	NDM-1	$bla_{CTX-M-15,TEM-1,OXA-1,SHV-1}$	NA[b]	(62)
Colombia	Aug 2011–Jan 2012	2013	Hypoxic-ischemic encephalopathy, respiratory distress syndrome, necrotizing enterocolitis, and sepsis	Blood	ST1043	NDM-1	*qnrA* and $bla_{SHV.}$	IncA/C, *Intl*1	(63)
India	2007–2011	2014	Septicemia	Blood	ND	NDM-1	$bla_{CTX-M-15,TEM-1,OXA-1,CMY,SHV-1,}$ *armA, rmtB, rmtC, aac(6')-Ib,* and *aac(6')-Ib-cr*	IncN, IncHIB-M/IncFIB-M, IncFIIK, IncR	(21, 55)
India	2012	2014	Sepsis	Blood	ND	NDM-1	$bla_{CTX-M-15}$	ND	(64)
Nepal	Aug 2011–June 2012	2014	Suspected sepsis	Blood or Cerebro-spinal fluid.	ST15	NDM-1	$bla_{CTX-M-15,SHV-28,TEM-1,OXA-1}$, *qnrB1, aac(6')-Ib,* and *aac(6')-Ib-cr,*	Multireplicon plasmid IncHI1B/IncFIB	(65)
Nigeria	Sept 2012–Sept 2016	2014	Sepsis	Blood.	ST476	NDM-5	$bla_{CTX-M-15}$, bla_{OXA-1}, $bla_{OKP-B-6}$ bla_{TEM-1}, *aac(6')-Ib-cr*, ble_{MBL}, *qnrB1*, and *sul2.*	IncX3	(66)
Turkey	2013	2014	Colonizer	Rectal swab	ND	NDM-1	$bla_{CTX-M-15,CTX-M-3,SHV-1,SHV-27,OXA-1,}$ and *rmtC*	NA	(67)
China	2012–2013	2015	Neonatal sepsis, neonatal pneumonia, necrotizing enterocolitis, and respiratory distress syndrome	Blood	ND	NDM-1	*qnrS* and $bla_{CTX-M-15,CMY-4,TEM-1,SHV-1.}$	ND	(68)
China	Apr 2011–Oct 2013	2017	Neonatal pneumonia	Sputum, blood, Umbilical secretion	ST 20, ST54, ST705, and ST290	NDM-1, IMP-4, IMP-8	$bla_{CTX-M-14,CTX-M-15,TEM-1,DHA-1}$	ND	(69)
China	June 2016–Aug 2016	2018	Sepsis, respiratory distress syndrome	Blood Sputum	ST234 and ST1412	NDM-1	*qnrB4* and $bla_{CTX-M-14,SHV-148,}$	ND	(70)

(Continued)

TABLE 1 | Continued

Country	Study timeline	Year of publication	Clinical presentation	Source(s)	Sequence Types (STs)	Carbapenemases identified	Other resistance genes identified	Plasmid type(s) and integrons	References
China	2015	2018	NA	Blood, urine, sputum, aspiration catheter, and radiant warmer	ST11, ST20, and ST888	NDM-1 and KPC-2	$bla_{CTX-M-14,CTX-M-15,TEM-1}$	ND	(71)
China	May 2014–Aug 2014	2018	Septicemia, pneumonia	Blood, Sputum, and Urine	ST1419 and ST101	NDM-1	$bla_{SHV-12,CTX-M-15,TEM-1}$	ND	(72)
India	Dec 2015–Jan 2017	2018			ND	NDM-1, NDM-4, and NDM-5	bla_{OXA}, bla_{CMY}, and bla_{SHV}.	IncFIA, IncFIC, IncF, IncK, IncFIB, IncY, IncFIIA,	(51)
India	Jan 2012–June 2014	2019	Septicemia	Blood	ND	NDM-1	*qnrB, qnrS, aac(6')-Ib, aac(6')-Ib-cr,* $bla_{CTX-M-15,TEM-1,OXA-1,SHV-1,}$ *armA, rmtB,* and *rmtC.*	IncFIIK and IncHIB-M	(28)
India	July 2016–Dec 2017	2019	Septicemia	Blood	ST29, ST347, ST1224, and ST2558	NDM-1	$bla_{CTX-M-15}$, *qnrS1, qnrB1, aac(6')-Ib,* and *aac(6')-Ib-cr.*	IncFIIK	(73)
China	June 2010–Sept 2010	2013	Sepsis	NA	ST11	KPC-2	$bla_{CTX-M-14,CTX-M-15,TEM-1,SHV-11,SHV-12,}$ *rmtB, aac(6')-Ib-cr,* and *qnrS*	ND	(74)
Jordan	Jan 2012–Dec 2015	2018	Sepsis	Blood	ND	KPC	ESBL genes	ND	(17)
Egypt	Feb 2019–Sept 2019	2020	Sepsis	Blood	ND	KPC, VIM, and NDM	$bla_{CTX-M,}$ bla_{OXA-1}, qnrS, and *qnrB.*	ND	(75)
India	2013–2016	2020	Sepsis	Blood	ST147	KPC-2	bla_{CTX-M}, $bla_{TEM,SHV,OXA}$, *qnrB, oqxA, oqxB, aac(6')-Ib-cr,* and *aac(6')-Ib*	IncFII	(76)
China	2018–2019	2020	unknown	Sputum, pus, ascites, urine, blood	ST11, ST76, ST4854, ST35, ST34	KPC, NDM-1, IMP-4	NA	NA	(77)
Egypt	Nov 2015–Apr 2016	2020	Late-onset sepsis	Blood	ND	OXA-48 and NDM	ND	ND	(16)
India	Jan 2016	2020	Septicemia	Blood	ST23	OXA-232	$bla_{SHV-190}$, bla_{TEM-1B}, $bla_{CTX-M-15}$, bla_{CMY-4}, *aac(6')-Ib-cr, oqxB, oqxA,* and *qnrB1*	IncColKP3 type	(78)
China	Apr- June 2016	2017	NA	NA	ST15	OXA-232	$bla_{CTX-M-15,}bla_{SHV-1}$	IncColE type	(79)

(Continued)

TABLE 1 | Continued

Country	Study timeline	Year of publication	Clinical presentation	Source(s)	Sequence Types (STs)	Carbapenemases identified	Other resistance genes identified	Plasmid type(s) and integrons	References
Spain	2012–2014	2017	Colonizer	NA	ND	OXA-48	ND	ND	(80)
Algeria	Jan 2017–Apr 2017	2019	Colonizer	Rectal swab	ST13, ST45, and ST1878	OXA-48	ND	IncL/M	(81)
India	2013–2016	2021	Septicemia	Blood	ST14	OXA-181, NDM-5	$bla_{CTX-M-15}$, bla_{TEM-1}, bla_{OXA-1}, bla_{OXA-9}, *rmtB*, *aac(6')-Ib)*, *aac(6')-Ib-cr*, *oqxA*, *oqxB*	IncColKP3, IncFII, IncR	(82)
Italy	Apr 2015–Mar 2016	2017	Colonizer	Rectal swab	ST104	VIM-1	bla_{SHV12}, *ant(3'')*, *aph(3'')*, *aacA4*, *qnrA1*, *sul1*, and *dfrA14*.	IncA/C1	(83)
China	Mar 2010–Feb 2011	2014	Respiratory distress syndrome	Trachea cannula	ND	IMP-38	ND	ND	(84)

[a]*ND, Not determined.*
[b]*NA, Not available.*

in *K. pneumoniae* causing neonatal infections (51, 52). It was observed that isolates harboring bla_{NDM-1} are not associated with any particular sequence type and these strains belonged to varied STs (ST15, ST17, ST20, ST29, ST76, ST101, ST234, ST347, ST433, ST476, ST888, ST1043, ST1412, ST1419, ST1224, ST2558, ST4854) (**Table 1**). These isolates also possessed several antibiotic resistance determinants such as *armA, rmtB* (aminoglycoside resistance), *qnrB, qnrS, aac(6')-Ib-cr* (plasmid-mediated fluoroquinolone resistance), $bla_{CTX-M-15}$ (ESBL) (21, 28, 86) along with bla_{NDM-1} gene in different plasmid types such as IncFIIK, IncHIB-M, IncFII, IncFIA, IncFIB, IncF, IncA/C, IncL/M, IncA/C, IncX3, etc. (28, 51).

Isolates harboring bla_{NDM-1} have also caused outbreaks in several healthcare settings. During August 2011–Jan 2012, an outbreak occurred due to *K. pneumoniae* ST1043 in a neonatal unit in Colombia infecting six neonates. As the neonates had no contact with people from countries that reported NDM-1-producing bacteria, the authors proposed that autochthonous clones were acquiring the bla_{NDM} gene (63). Another outbreak around the same time was reported from Nepal by a bla_{NDM-1}-positive *K. pneumoniae* ST15. This outbreak caused high mortality among the neonates. Apart from the outbreak cluster, three smaller genetically close clusters were also identified in this study (65). An outbreak of NDM-5-producing *K. quasipneumoniae* was reported from Nigeria in April 2016. The outbreak occurred when the neonatal ward was overcrowded and less critical neonates often shared cots. bla_{NDM-5} gene was carried on an IncX3 plasmid (66). Five separate studies from different parts of China (Nanjing, Wuhan, Hunan, Jiangsu, Shandong) reported outbreaks of bla_{NDM-1}-possessing *K. pneumoniae* in neonatal units. Strains were isolated from blood or sputum or umbilical secretions of neonates and belonged to different sequence types (68–70, 72, 77). In another study from China, five CRKP isolated from neonates (blood, urine, and catheter tips) possessed bla_{NDM-1} and belonged to ST20 ($n = 4$) and ST888 ($n = 1$), which were susceptible to gentamicin, amikacin, aztreonam, ciprofloxacin, and levofloxacin. NDM-1-producing ST20 strains ($n = 2$) were also isolated from the hospital environment (71). A systematic review from China on carbapenem-resistant Enterobacteriaceae reviewed seventeen studies of neonatal infections among which seven studies were of NDM-1-producing *K. pneumoniae* (87).

OXA-48-positive CRKP causing neonatal infection or intestinal colonization was reported from Algeria, Spain, and Egypt (75, 80, 81). In the study from Algeria, bla_{OXA-48}-carrying *K. pneumoniae* of two different STs (ST13 and ST1878) were found colonizing the gut of the neonates in two maternity wards. Carriage of carbapenem-resistant strains was significantly related to the low-birth-weight of the neonates (81). In the study from Egypt, *K. pneumoniae* was the predominant organism causing LOS but not EOS. Eventually, the mortality was significantly higher in neonates suffering from LOS (16). OXA-232, another OXA-48-like enzyme, was reported from a hypermucoviscous *K. pneumoniae* causing septicemia from India. The gene was carried in a ColKP3 type plasmid (73). Another clonal outbreak of OXA-232-producing *K. pneumoniae* ST15 was reported from a NICU in Shanghai, China (79).

The other carbapenemases such as IMP or VIM have not been reported frequently in neonatal infections. IMP-38-positive CRKP causing respiratory distress syndrome in neonates was reported from China. The nine IMP-38-positive strains were clonal and were isolated from the trachea cannula of neonates. bla_{IMP-38} was a novel allele and differed by a single mutation from IMP-4 which was found in the other wards of the same hospital (84). In another recent study from China, fourteen bla_{IMP-38}-possessing *K. pneumoniae* ST307 were recovered from neonates suffering from sepsis (88). Another variant, IMP-4 has also been reported from China causing neonatal infections (69, 77, 87). VIM-positive CRKP was recovered from the neonates in the USA, Italy, and Egypt (75, 83, 89). In the study from Italy, VIM-1, associated with an IncA/C plasmid, was primarily found in *K. pneumoniae* ST104 recovered from the rectal swabs of neonates admitted in a NICU from 2015 to 2016. The strains were susceptible to fluoroquinolones, amikacin, and colistin (83).

It is noteworthy that earlier studies reported the presence of a single carbapenemase in *K. pneumoniae* but in recent years reports of co-occurrence of multiple carbapenemases are emerging (16, 75, 82). In a recent study from India OXA-181/OXA-232 was concomitantly present with NDM-5 in *K. pneumoniae* causing neonatal septicemia (82). A study from Egypt also reported (previously mentioned) presence of bla_{NDM} and bla_{VIM} in *K. pneumoniae* causing LOS. bla_{KPC} was also present in 96% of these strains (75). Another (previously mentioned) study from Egypt reported the presence of bla_{OXA-48} in 61% strains and co-occurrence of bla_{NDM} and bla_{OXA-48} in 52% strains (16). The presence of multiple carbapenemases and other resistance genes pose additional limitations to the treatment protocols.

HYPERVIRULENT *K. pneumoniae* (hvKP)—ANOTHER DIMENSION TO THE PROBLEM

hvKP and cKP: We Beg to Differ

Recently, *K. pneumoniae* has gained a revised and serious global attention due to the emergence of the hypervirulent pathotype. Over the last few decades, the majority of hospital-acquired infections reported globally were due to the classical *K. pneumoniae* (cKP). However, since the mid-1980s, the emergence of hypervirulent *K. pneumoniae* (hvKP) in the clinical context poses a far greater challenge to the clinicians (90). Although, both cKP and hvKP pathotypes have their own global importance, the incidence of infections due to hvKP has been reported at an escalating rate over the last three decades (91). Unlike cKP, hvKP pathotypes are more virulent and have the potential to cause several community-acquired invasive, life-threatening, and unusual infections, such as pyogenic liver abscess, lung abscess, meningitis, endophthalmitis, brain abscess, and necrotizing fasciitis in otherwise healthy adults (92). Initially, hvKP infections were reported primarily from Taiwan and South-East Asia, but several sporadic reports of hvKP have now been observed in other Asian, European, and American countries (25, 91, 93–98). Although hvKP cause community-acquired diseases, some current reports argued that the infiltration of these notorious strains is increasing in the healthcare settings also (99–104). Infections due to hvKP are found to be more complicated due to their ability to metastatically disseminate to other organs or systems and subsequently cause multiple sites of infection (90). This type of dissemination is common for some selected Gram-positive pathogens, such as *S. aureus*, but it is unusual for an enteric Gram-negative bacillus to involve multiple sites of infection (105). In addition, unlike cKP, hvKP pathotypes commonly possess a hypermucoviscous phenotype, produce a robust capsule, synthesize several iron-scavenging siderophore molecules, especially salmochelin and aerobactin, and harbor several chromosomal and large virulence plasmid-encoded factors (91, 106). Due to their enhanced virulence, these *K. pneumoniae* are considered hypervirulent. An overview of the differences between cKP and hvKP strains is depicted in **Table 2**.

hvKP and cKP: Molecular Markers

Recently, the advancement of whole-genome sequencing (WGS) in the clinical context has identified a set of important and unique hypervirulent biomarkers which can accurately differentiate hvKP from cKP, including several capsular serotypes (K1, K2, K5, K20, K54, and K57), mucoviscosity-associated gene A (*magA*), regulator of mucoid phenotype A (*rmpA*) genes, biosynthetic genes responsible for the production of siderophore salmochelin (*iro* cluster), aerobactin producing biosynthetic genes (*iuc* cluster), virulence plasmid-encoded gene with unknown function (*peg-344*), and the virulence plasmid-encoded heavy metal resistance genes for tellurite (*ter* cluster) and silver (*silS*) (78, 91, 106). Due to presence of these factors, hvKP strains are more resistant to macrophage- or neutrophil-mediated phagocytosis & the complement-mediated bactericidal activity of human serum, they exhibit enormous biofilm-forming capability, and also display enhanced lethality in the *in vivo* mouse model compared to cKP (107). In the following section, we discuss briefly the several hvKP-specific biomarkers detected till date which are responsible for the increased virulence and severe clinical expression.

(a) Capsular Serotypes

Capsule is the most established virulence factor of *K. pneumoniae*. Both cKP and hvKP possess chromosomally-encoded capsular polysaccharide (K antigens) genes, known as the *cps* cluster (108). Till date, around 78 capsular serotypes (K1–K78) have been reported in *K. pneumoniae* (33). However, majority of reports have shown the strong association of K1 and/or K2 serotypes with hvKP strains (109, 110). Recent studies, especially from China, reported the occurrence of K2 serotypes in at least 70% and 42% of hvKP strains (93, 111) and K1 in 24% (93). Why are the increased incidences of virulence associated typically with K1 and K2 serotypes? There are several explanations for this. One study suggested that strains with K1 and/or K2 serotypes have better survival probably because of the induction of slightly greater amount of reactive oxygen species released by neutrophils than other serotypes (112). In addition, several studies have argued that the strains of the K1

TABLE 2 | Characteristic features of classical *K. pneumoniae* (cKP) and hypervirulent *K. pneumoniae* (hvKP) strains.

Parameter	Characteristic(s) for pathotype	
	cKP	**hvKP**
Primary site of acquisition	Nosocomial	Community acquired[a]
Population(s) at risk	Immunocompromised individuals	Often otherwise healthy individuals
Liver abscess	Usually do not occur[b]	Often occur in the absence of biliary disease
Ability of metastatic spread	None	Frequent
Number of sites of infection	Usually single	Often multiple
Unusual infections	None	Often encountered
Geographical distribution	Worldwide	Mostly Asia-Pacific Rim
Capsule type(s)	K1-K78	Mostly hypercapsule K1 or K2[c]
Siderophores	Mostly enterobactin and yersiniabactin	All four siderophores but specifically salmochelin and aerobactin
magA and c-*rmpA*	Usually do not occur	Frequently occur
Virulence plasmid-encoded factors (p-*rmpA*, *rmpA2*, *iroBCDN*, and *iucABCDiutA*)	Usually do not occur[d]	Predominantly occur

[a] *Recently an escalating number of hvKP infections are emerging in the healthcare settings.*
[b] *cKP strains can also cause hepatic abscess but unlike hvKP, cKP mediated liver abscess occur in the presence of biliary disease.*
[c] *Apart from the frequently encountered K1 or K2 serotype, several other capsular type, such as K5, K20, K54, and K57 have also been detected in hvKp strains.*
[d] *During the course of evolution, cKP strains are also increasingly reported to acquire hvKP virulence plasmid.*

and K2 serotypes are significantly more resistant to phagocytosis and subsequently intracellular killing by macrophages and neutrophils than other serotypes (113, 114). Moreover, others have suggested reduced uptake of K1 and K2 serotypes by the innate immune cells probably due to the presence of a significant amount of sialic acid on their surfaces, which may mimic the host cell and allow them to easily evade the immune response (115). Apart from the K1 and K2 serotypes, recent studies revealed the occurrence of other serotypes, such as K5, K20, K54, and K57 in hvKP strains causing various invasive infections (91). However, significant reports of immune evasion are still scarce for these capsular types.

(b) Major Players in Hypercapsule Production: rmpA and magA

The capsule is found in both cKP and hvKP, but the hvKP strains produce an increased amount of capsular polysaccharide compared to that of cKP. This robust capsule synthesis in hvKP strains is chiefly mediated by the *rmpA* and *magA* genes. In hvKP strains, a total of three *rmpA* genes are generally found, of which two are large virulence plasmid-encoded (p-*rmpA* and p-*rmpA2*) and one is chromosomally-encoded (c-*rmpA*) (116). Several reports suggested that the *rmpA* genes along with the regulation of capsule synthesis A and B genes (*rcsAB*) can induce the transcription of entire *cps* operon, resulting in hypercapsule production (106). In separate studies, Hsu et al. and Li et al. revealed that about 55–100% hvKP strains express at least one copy of *rmpA* or *rmpA2* (116, 117). In the absence of *rmpA* or *rmpA2,* hypercapsule biosynthesis can be triggered alone by the chromosomally-encoded *magA* gene which was isolated from hypermucoviscous liver abscess-causing *K. pneumoniae* (118, 119). Subsequent bioinformatics and genetic experiments determined that *magA* is a K1-specific factor and encodes a polymerase gene termed *wzy* in the *cps* operon (118, 120, 121).

(c) The Iron Scavengers: Aerobactin and Salmochelin

Iron, a crucial and limiting metal, essential for bacterial growth and plays a pivotal role in the progression of bacterial infection, especially in the case of *K. pneumoniae*. However, this essential metal is not readily available in the host during the infection because of the non-specific immune response exhibited by the host where the host efficiently sequesters this metal ion (Fe^{3+}) with several iron-binding molecules, such as transferrin and lactoferrin, eventually restricting the growth of many opportunistic pathogens (122). Therefore, to acquire iron from such an iron-poor environment, *K. pneumoniae* secrete several small proteins with high iron-scavenging ability, called siderophores (123). Molecular epidemiological studies have shown that hvKP strains produce all the siderophores (enterobactin, yersiniabactin, salmochelin, and aerobactin) compared to cKP which produce only enterobactin and yersiniabactin (124). Apart from producing all siderophores, hvKP strains are also capable of synthesizing quantitatively more siderophores than cKP (125, 126). Among the four siderophores, the activity of enterobactin and yersiniabactin is greatly hindered by host molecule lipocalin-2 and transferrin, respectively (127, 128). Therefore, *K. pneumoniae* with only enterobactin and/or yersiniabactin are unlikely to cause systemic infection in immunocompetent individuals (128). In contrast, the functionality of both salmochelin and aerobactin cannot be inhibited by these host proteins. Several studies have shown that salmochelin is more prevalent in hvKP strains, sometimes more than 90% of pyogenic liver abscess-causing hvKP strains possess this scavenger (128, 129). Aerobactin, a citrate-hydroxamate siderophore, is also rarely detected in cKP (124). Studies revealed its presence in 93–100% of

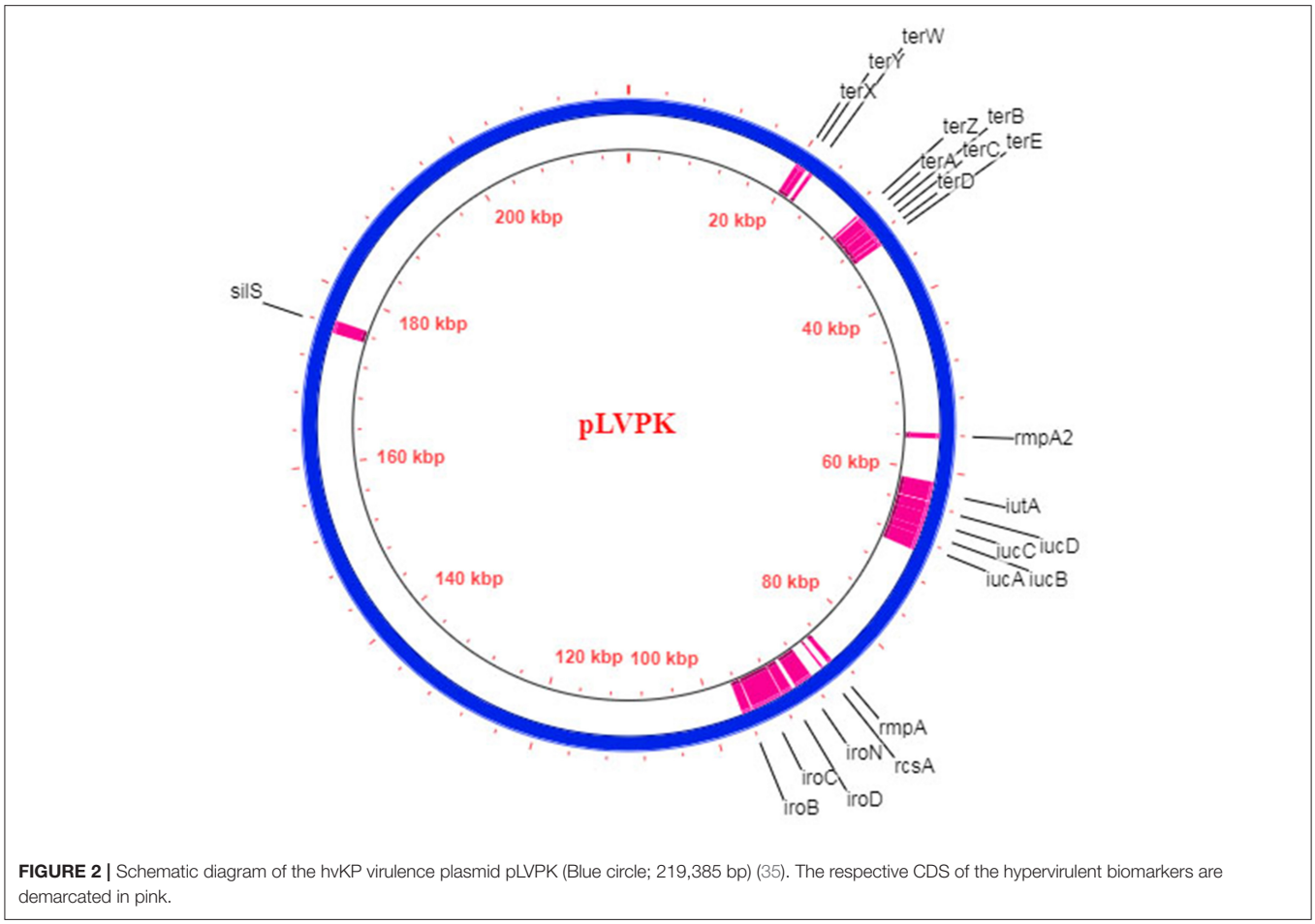


FIGURE 2 | Schematic diagram of the hvKP virulence plasmid pLVPK (Blue circle; 219,385 bp) (35). The respective CDS of the hypervirulent biomarkers are demarcated in pink.

hvKP strains (130). Immense genome-based analysis in the molecular epidemiologic studies confirmed that in most cases *iro* gene cluster for salmochelin and *iuc* gene cluster for aerobactin specifically reside on the large virulence plasmid of hvKP (131).

(d) Plasmids That Matter: pLVPK

WGS analysis of initially identified hvKP strains revealed the presence of the unique ∼220 kb large virulence plasmids pK2044 (224,152bp) and pLVPK (219,385bp) (34, 35). All the best described genetic markers which confer the hypervirulent phenotype are located on these plasmids, including complete biosynthetic gene cluster of aerobactin (*iucABCDiutA*) and salmochelin (*iroBCDN*), regulators of hypercapsule production (*rmpA*, *rmpA2*, and *rcsA*), resistance genes for tellurite (*terZABCDE* and *terWXY*) and silver (*silS*) (**Figure 2**). Recently, bioinformatics studies revealed that all the hvKP strains possessed either pK2044-like or pLVPK-like plasmid. Struve et al. showed that all 30 studied community-acquired liver abscess-causing hvKP strains harbored pLVPK-like plasmids (131). Similarly, another study revealed the presence of pK2044-like plasmids in 94 hvKP strains (132). The lateral gene transfer in bacteria is largely mediated by plasmids and as the hypervirulent biomarkers mostly reside in the non-conjugative virulence plasmids, it is quite likely that hvKP strains acquire drug-resistant plasmids from the cKP strains due to their conjugative nature and this molecular incidence is now increasingly evident in recent studies.

(e) hvKP and Sequence Type 23 (ST23): In Search of an Association

Several studies showed that the genes conferring hypervirulence are widely distributed across diverse STs (133) but some selected STs, such as ST23, ST65, and ST86 are found to be predominantly associated with hvKP (134). Recently, core genome multi-locus sequence typing (CG-MLST) and/or WGS revealed that strains of clonal group 23 (CG23) are strongly associated with K1 capsular type causing severe and invasive disease which occur in typical hvKP infection (131). ST23 was frequently associated with hvKP strains especially in the Asia-Pacific Rim. Studies from China and South Korea show that majority of the hvKP strains belonging to ST23 possessed K1 capsular type (134, 135). Recently, a study from India in 2020 also described a case of community-acquired neonatal septicemia caused by a carbapenem-resistant ST23 hvKP strain (78). Although the reasons behind the association of the hvKP and/or K1 capsular type with ST23 are still uncertain, it is hypothesized that the CG23 probably has a discrete genetic

TABLE 3 | Epidemiology of some carbapenem-resistant hypervirulent *K. pneumoniae* (CR-hvKP) strains.

Patient population	Country	Sequence types (STs)	Capsule type(s)	Hypervirulent markers	Associated carbapenemase	Ambler class	Year	References
Neonates	India	ST2318	ND[a]	*rmpA, rmpA2,* aerobactin, and salmochelin	NA[b,#]	NA	2016	(136)
	Russia	ND	ND	*rmpA*	NA[#]	NA	2017	(137)
	Russia	ND	ND	*rmpA,* and aerobactin	NA[#]	NA	2018	(5)
	India	ST23	K1	*magA, rmpA, rmpA2, iucABCDiutA,* and *iroBCDEN*	$bla_{OXA-232}$	Class D	2020	(78)
	Sudan	ND	K2	*magA* and *rmpA*	bla_{NDM} and $bla_{OXA-48-like}$	Class B and D	2020	(138)
	India	ST5235	ND	*rmpA* and *rmpA2*	bla_{NDM} and $bla_{OXA-48-like}$	Class B and D	2021	(139)
Adults	China	ST11, ST25, and ST65	K2 and non-typeable	*rmpA, iucABCDiutA*, and *iro*	bla_{KPC-2}	Class A	2015	(140)
	China	ST23 and ST1797	K1	*magA* and *rmpA*	bla_{KPC-2}	Class A	2015	(141)
	China	ST11	K1	*magA*, *rmpA*, *rmpA2*, *iro*, and *iucABCDiutA*	bla_{KPC-2}	Class A	2016	(142)
	China	Unknown	K1	*rmpA* and *iucABCDiutA*	bla_{KPC-2}	Class A	2017	(143)
	China	ST11, ST268, ST65, ST692, and ST595	K2, K20, and non-typeable	*rmpA* and *iucABCDiutA*	bla_{KPC-2}	Class A	2017	(144)
	China	ST11	K47	*iucABCDiutA* and *rmpA*	bla_{KPC-2}	Class A	2017	(145)
	China	ST15	KL112[c]	*rmpA2* and *iucABCDiutA*	$bla_{OXA-232}$	Class D	2018	(146)
	Iran	ST23	K1	*rmpA, magA,* and aerobactin	bla_{VIM-2}	Class B	2018	(147)
	China	ST86	K2	*rmpA, iucABCD,* and *iroBCDN*	bla_{NDM-1} and bla_{KPC-2}	Class A and B	2018	(148)
	Canada	ST86	K2	*rmpA, rmpA2, iucABCD,* and *iroBCDN*	bla_{KPC-2}	Class A	2019	(149)
	Japan	ST23	K1	*rmpA, rmpA2* (with frameshift), *iucABCD,* and *iroBCDN*	bla_{IMP-6}	Class B	2019	(150)
	Singapore	ST23, ST65, and ST86	K1 and K2	*rmpA, rmpA2, iuc,* and *iro*	bla_{KPC-2}	Class A	2019	(151)
	Singapore	ST23, ST65, ST86, ST420, and ST893	K1, K2, and K20	*rmpA, rmpA2, iuc,* and *iro*	bla_{KPC-2}	Class A	2020	(152)
	France	ST86	K2	*rmpA, rmpA2, iucABCDiutA,* and *iroBCDEN*	bla_{OXA-48}	Class D	2020	(153)
	China	ST1764	K64	*rmpA, rmpA2,* and *iroN*	bla_{SIM}	Class B	2020	(154)

[a]*ND, Not determined.*
[b]*NA, Not available.*
[#]*ESBL-producing hvKP strains.*
[c]*KL means K Locus: associated cps cluster type.*

infrastructure that confers the hypervirulence. However, in-depth research will be required to fulfill the existing knowledge gaps.

THE NEXT GENERATION SUPER-BUG: CARBAPENEM-RESISTANT hvKP (CR-hvKP)

It is debatable whether the association of virulence and antibiotic resistance is deleterious or helpful for the microbe (90, 124). However, recent reports on *K. pneumoniae* that are both antimicrobial-resistant and virulent have surely put an end to this debate. *K. pneumoniae* are extremely capable of receiving and incorporating DNA segments from other bacteria, mostly via large plasmids (26). Carbapenem resistance genes, as discussed earlier can easily spread across species via these mobile genetic elements. Their presence in hvKP has challenged the health system and the emerging CR-hvKP pathotypes are now being considered as "the next generation super-bug." The epidemiological data of some CR-hvKP strains is given in **Table 3** and the worldwide distribution of CR-hvKP strains is depicted in **Figure 3**.

From the standpoint of the microbe, the convergence of resistance and virulence is beneficial particularly under antibiotic pressure. Exchange of genes can happen in two ways, hvKP strains can acquire the antibiotic-resistant plasmids from the XDR *K. pneumoniae* strains (155) or the XDR cKP strains can acquire hvKP-specific virulence plasmids (145). The first option seems more plausible given the fact that majority of pLVPK-like plasmids are non-conjugative, lacking the transfer (*tra*) genes (131, 134). On the other hand, carbapenem resistance genes are primarily found on conjugative plasmids. However, some recent in-depth investigations on hvKP revealed that due to the immense antibiotic selection pressure and an extraordinary ability of *K. pneumoniae* to acquire highly antibiotic-resistance- and hypervirulence-encoding genetic determinants, new hybrid and/or conjugative plasmids with both carbapenem-resistant and hypervirulent markers have emerged in the *K. pneumoniae* strains as a result of convergent evolution. This molecular phenomenon has eventually led to the emergence of new and perilous variants of CR-hvKP strains (156, 157).

Since 2015, multiple epidemiological studies from China showed the prevalence of KPC-2-producing CR-hvKP strains in the clinical settings causing several infections in otherwise healthy individuals, including UTI, pneumonia, septicemia, bacteremia, abdominal infections, septic arthritis, catheter-associated bacteremia, and ventilator-associated pneumonia (140–142, 144). Majority of the reported bla_{KPC-2}-harboring CR-hvKP strains from China belonged to ST11. Although ST11 clone of *K. pneumoniae* is an internationally categorized high-risk clone known to harbor bla_{KPC-2} as the major carbapenemase gene, these recent studies have confirmed its association with the hypervirulence attributes.

Typical to hvKP, pLVPK-like virulence plasmid pVir-CR-hvKP4 was identified. However, compared to the pLVPK, pVir-CR-hvKP4 had a 41.231 bp deletion. The subsequent plasmid-curing experiment suggested that this modified pLVPK-like plasmid was responsible for enhanced virulence phenotype both *in vitro* and *in vivo* (145). Although majority of the CR-hvKP reports were initially detected with bla_{KPC-2}, recently the occurrence of other classes of carbapenemases (Class B -MBL-type and Class D—OXA-48-like) have also been detected in hvKP strains. For example, a recent study from China showed the emergence of an ST86 CR-hvKP K2 strain that co-harbored two different classes of clinically important carbapenemase-producing genes bla_{NDM-1} and bla_{KPC-2}. This strain was also

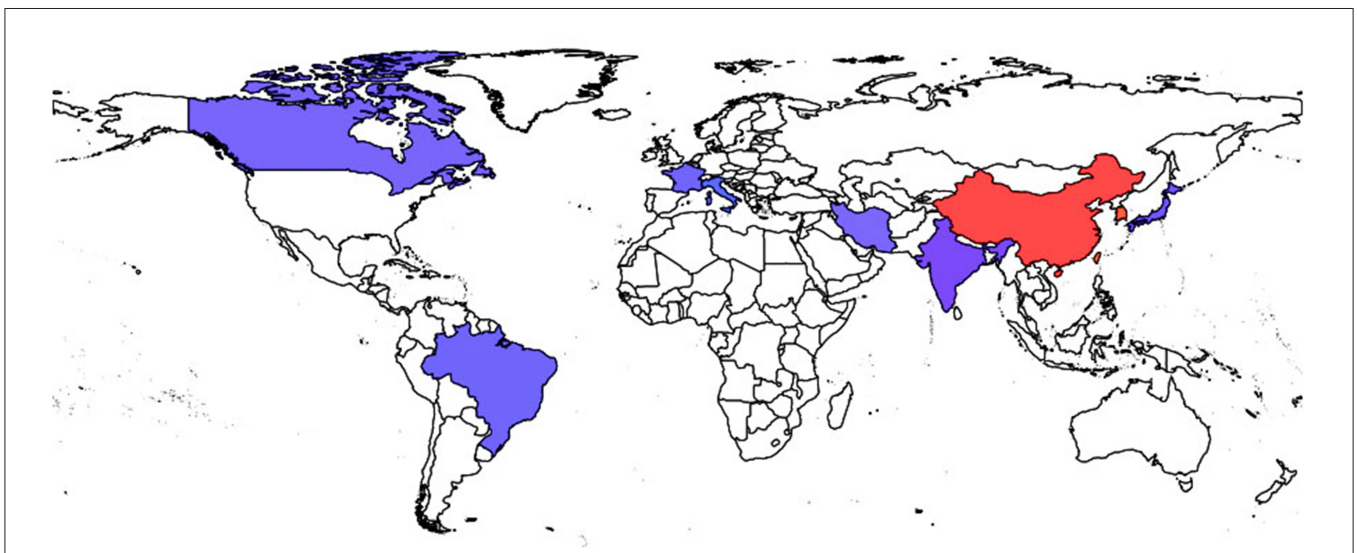


FIGURE 3 | Worldwide spread of CR-hvKP strains. Endemic spread of CR-hvKP strains were reported from the countries indicated in red while the blue colored regions represent the sporadic occurrence of CR-hvKP strains.

responsible for significant mouse lethality (148), refuting the fact that association of resistance and virulence is deleterious for the microbe.

hvKP/CR-hvKP AND THE NEWBORN

Till date, majority of the cases of hvKP and/or CR-hvKP infections have been reported in adults and reports regarding hvKP infection in cases of neonatal sepsis are just a handful (**Table 3**). When resistance and virulence meet to cause infection in a vulnerable population, the outcome is fatal, as was evident from most neonatal studies (5, 137, 139). Previous studies have concluded that the risk of development of severe forms of neonatal infection can be associated with virulence factors (5). In 2016, a study from India reported the occurrence of an ESBL-producing hvKP ST2318 strain causing neonatal sepsis. The strain was isolated from the blood of a very low-birth-weight neonate suffering from hepatosplenomegaly with severe thrombocytopenia, coagulopathy, and metabolic acidosis. Cefoperazone-sulbactam and amikacin were administered as an empirical treatment which was later changed to meropenem with amikacin after receiving the clinical microbiology report. The neonate was discharged after proper medical supervision. Genome analysis revealed that the strain harbored *rmpA*, *rmpA2*, and several siderophores, including aerobactin, enterobactin, yersiniabactin, and salmochelin (136). Khaertynov et al. in 2017 reported another fatal case of pyogenic neonatal meningitis caused by an ESBL-producing hvKP. The strain was isolated from the blood and cerebrospinal fluid of a 12-day-old neonate exhibiting several clinical symptoms, such as pallor, loss of appetite, fever, seizures, and a bulging anterior fontanelle. In addition, low Pediatric Glasgow Coma Scale (PGCS) score indicated that the neonate suffered from severe brain injury. Though the newborn was initially treated with ampicillin and amikacin it was changed to meropenem and later cefoperazone due to unsatisfactory clinical outcome and isolation of ESBL-producing *K. pneumoniae* from the infection sites. Apart from antibiotic treatment, IgM-enriched intravenous immunoglobulin and infusion therapy were also given to the neonate but the neonate succumbed to the infection. Laboratory-based characterization revealed that the strain exhibited hypermucoviscous phenotype and also harbored *rmpA* gene (137). The following year another report from Kazan, Russia revealed the occurrence of ESBL-producing neonatal septicemic hvKP strains in the clinical settings with fatal outcome, harboring *rmpA* and aerobactin biosynthetic genes. In this study, two groups of neonates were registered, infected by *K. pneumoniae*. The first group comprised of 10 neonates with culture-positive sepsis and the second group consisted of neonates with UTI. All the 10 septicemic neonates received a comprehensive treatment, including antibiotics, intravenous immunoglobulins, and infusion therapy. However, three neonates died despite the therapy, of which two suffered from purulent meningitis and one from necrotizing enterocolitis. The most grievous forms of sepsis in neonates, were correlated with meningitis (5). The first report of CR-hvKP was in 2020 from our laboratory showing the occurrence of a unique community-acquired neonatal septicemic case caused by a hypermucoviscous CR-hvKP ST23 K1 strain. The neonate was low-birth-weight, delivered at home in a rural area and was given cow milk since birth. During admission, the baby displayed several clinical signs and symptoms of sepsis. As an empirical treatment, piperacillin/tazobactam and netilmicin were given to the neonate. The baby was hospitalized for 22 days before taking a high-risk discharge. The strain displayed all hvKP-associated features, including hypermucoviscous phenotype, *magA*, *rmpA*, *rmpA2*, *rcsAB*, *iroBCDEN*, and *iucABCDiutA*. In addition, the strain also harbored $bla_{\mathrm{OXA-232}}$ as the candidate carbapenemase gene. Molecular typing and WGS analysis revealed that the studied strain belonged to an internationally high-risk clone, ST23 and possessed a pLVPK-like virulence plasmid sequence in the studied genome. Moreover, comparative genomic analysis with the other hvKP ST23 reported genomes showed >99.5% of inter-genomic resemblance (78). The virulence determinants such as *magA*, *rmpA*, *rmpA2*, *iroN*, *iucA*, and *iutA* were not transmitted along with the carbapenem resistance gene, $bla_{\mathrm{OXA-232}}$, in conjugation experiments. However, the possibility of such transmission is worrisome. The same year another study from Khartoum, Sudan revealed that about 16.7% CR-hvKP strains were recovered from the blood of septicemic neonates and adults. Molecular characterization suggested that among the recovered CR-hvKP strains, eight were positive for $bla_{\mathrm{OXA-48-like}}$ gene and two for bla_{NDM}. Moreover, the strains belonged to K2 capsular serotype and also harbored *rmpA* and *magA* (138). In 2021, a study from India showed the occurrence of nine XDR hvKP ST5235 strains causing sepsis in neonates. All the neonates in this study were empirically treated with piperacillin/tazobactam and amikacin followed by imipenem/meropenem and vancomycin. However, 100% neonatal mortality was recorded in this study even after the treatment with polymyxin B. Molecular characterization showed that the strains harbored *rmpA*/*rmpA2* in various combinations. Although all strains showed resistance against carbapenems in phenotypic tests, $bla_{\mathrm{OXA-48-like}}$ gene and bla_{NDM} were detected only in three strains (139).

Clearly, more and more reports of hvKP and CR-hvKP in cases of neonatal sepsis are being published. Clinical laboratories do not evaluate *K. pneumoniae* on the basis of their hypervirulent features and thus such strains are underreported. In neonates, the combined effect of an immature immune system, use of several invasive devices, the involvement of extensively drug-resistant cKP, and the emergence of hvKP/CR-hvKP is worrisome. Comprehensive analysis of cKP and hvKP, their differences, and also the distinguishing biomarkers for hvKP will probably help the clinicians in future to take a more strain-specific therapeutic approach.

THE POSSIBLE ACQUISITION OF cKP AND hvKP IN NEONATES

Just like the other members of the Enterobacteriaceae family, *K. pneumoniae* is also found in the normal human microbiota

(158). Although the acquisition of *K. pneumoniae* appears to be obligatory for the infection, this scenario is not obvious always (159). The time of pathogenic exposure, bacterial inoculum size, immune status of the individual, and the virulence potential of the causative microorganism collectively manifest the clinical expression of an infection (3). At birth, the neonates do not possess their endogenous microbiota which is naturally acquired through the perinatal transfer of the maternal flora and from environmental sources. Due to the lack of an established microbiota, immaturely developed gut barrier, and the high permeability of the mucosa in the GI tract, neonates get infected easily, particularly low-birth-weight and premature neonates requiring prolonged hospitalization (160). The probable modes of infiltration of *K. pneumoniae* in the neonates are largely accomplished by *in-utero* acquisition, acquisition from maternal flora, and postnatal acquisition from the hospital or community (3).

(a) *K. pneumoniae* Acquisition From Maternal Flora

In-utero infection in neonatal septicemic cases is largely reported to be a result of chorioamnionitis (161). This is an acute inflammation of fetal membranes, probably due to the bacterial infiltration and this clinical condition is often found to be caused by two specific genital mycoplasmas, such as *Ureaplasma parvum* and *Ureaplasma urealyticum* (3). The vaginal microenvironment also provides a suitable ground for the colonization of pathogenic bacteria. Ascending movement of these microorganisms followed by the premature rupture of amniotic membrane results in the contamination of amniotic fluid. The inhalation or swallowing of the infected amniotic fluid by the neonate can subsequently lead to the colonization of pathogenic *K. pneumonia*e in their gut which can lead to intrapartum sepsis (162–164). However, reports regarding *in-utero* infection due to *K. pneumoniae* are extremely scarce. Two exclusive studies confirmed the occurrence of *K. pneumoniae* in intrauterine infection. In the first study in 2005, Sheikh et al. reported a unique case of acute chorioamnionitis and acute villitis due to *K. pneumoniae* infection in a 40-year-old woman at 18 weeks of gestation (165). Torabi et al. also reported a case of severe chorioamnionitis with umbilical cord and chorionic plate vasculitis due to *K. pneumoniae* in a woman who had suffered from fetal expiration at 20 weeks of gestation (166).

Acquisition can also frequently happen during the process of vaginal delivery, the neonates might get infected by the pathogens residing in the birth canal (167). Besides, the neonates can also acquire maternal flora through the contaminated hands, from the skin during the kangaroo care, and/or from the infected breast milk. Recent studies have revealed the incidence of acquisition of ESBL- or carbapenemase-producing *K. pneumoniae* from maternal flora to the neonates. For example, a study from Italy in 2017, reported a case of mother to child transmission of KPC-3-producing CRKP at birth (168). In 2018, Herindrainy et al. reported that the acquisition of about 24% ($n = 20$) of ESBL-producing *K. pneumoniae* detected in the neonates were from the maternal gut flora (169).

(b) Nosocomial Acquisition of *K. pneumoniae*

Till date, the majority of neonatal septicemic cases from LMICs are reported to be caused by hospital-associated drug-resistant classical *K. pneumoniae* strains which we have already discussed earlier. In several studies, we also have noticed the prevalence of hospital-acquired *K. pneumoniae* and/or CRKP infections in the neonates. The MDR or XDR nature of the *K. pneumoniae* strains offer them an immanent selective advantage by which they can easily persist in the flora of hospitalized patients as well as in the nosocomial environment (170). In a study from India, Das et al. revealed that among the studied bacterial strains, *K. pneumoniae* was detected as the predominant microorganism colonizing the neonatal gut. Neonates with longer stay in the NICU and those with prolonged feeding through an enteral tube were predisposed toward such colonization. Moreover, molecular typing showed that about 50% of the *K. pneumoniae* strains isolated from the blood were genotypically identical with their gut counterparts, reflecting the possible association between gut colonization and neonatal sepsis (162).

(c) Acquisition of *K. pneumoniae* From the Community

Till date, the majority of community-acquired *K. pneumoniae* have been reported to cause infections in healthy adult individuals. But the reports regarding community-acquired *K. pneumoniae* infections in neonates are very limited. In a study, Waters et al. showed that about 11.6% of *K. pneumoniae* strains caused community-acquired neonatal sepsis in the LMICs (171). In another study, Khatri et al. reported a case of community-acquired pyelonephritis caused by a KPC-2-producing *K. pneumoniae* (172). Recently, in a study we reported a unique case of CR-hvKP, causing sepsis in a neonate who was delivered at home and was given cow milk after birth (also discussed earlier). In that study, we hypothesized that the consumption of cow milk after birth was probably a cause of community-acquired hvKP infection (78). Due to various factors like low female literacy rate, unavailability of clinicians and other healthcare workers, the inadequate number of hospitals and/or well-equipped healthcare facilities, improper transport system, and most importantly inequality in the society, some of the neonates in the LMICs are still delivered either at home or at resource-poor primary care facilities where they might acquire infections from the environmental sources.

DETECTION OF CRKP AND hvKP: SEEK AND YOU SHALL FIND

The initial detection of carbapenemase-producing organisms in a lab is carried out by the trusted disk diffusion method which uses a carbapenem disk placed on a lawn of sample organism (173, 174). If there is a zone of inhibition, the diameter is measured and according to the cut-off mark delineated by CLSI (175) or EUCAST (176), the organism is declared resistant, or intermediate, or susceptible. Automated machines such as VITEK® 2 from bioMérieux and Phoenix™ from BD are also frequently used in setups that can afford them. Phenotypic

detection methods (though some are still not cost-effective) may be considered in resource-poor settings. A suitable test should be chosen based upon the sensitivity, specificity, turn-around time, and most importantly, cost of the test. Some phenotypic methods suitable for detection of carbapenemase-producing organisms are presented here in brief, exhaustive reading on these methods are available elsewhere (177). For easy detection of KPC and MBLs, combined disk test was developed. This test uses a disk of meropenem and another disk of a meropenem supplemented with an inhibitor (EDTA/ aminophenyl boronic acid/ dipicholinic acid). If the difference between the zone of inhibition of the two disks is >5 mm, a positive result is indicated (178). These tests are now commercially available (179, 180). mCIM uses a simple method and has high sensitivity and specificity. In this test, a meropenem disk is incubated with 1 μl loopful bacterial culture in Tryptic soy broth for 4 h. After that the disk is placed on a plate spread with a susceptible isolate. If the initial sample is carbapenem-producer then the disk is inactivated and zone of inhibition will be absent on the plate. Both these tests yield results after an overnight incubation (181). On the other hand, CarbaNP test uses the property of a carbapenemase-producer to hydrolyze imipenem within 2 h (182). This hydrolysis produces an acid which lowers the pH and triggers phenol red to change its color from red to yellow. There are several variants of this test. These tests detect all carbapenemases but has low sensitivity for OXA-48-like carbapenemases. The modified CarbaNP test uses a different buffer (0.02% cetyltrimethylammonium bromide lysis buffer) of higher (pH 7.8 instead of pH 7.5) and has higher sensitivity than CarbaNP test. For *K. pneumoniae* the modified test is beneficial as mucoid cells tend to give false negative results in the original test. To differentiate between classes of carbapenemase, CarbaNP test II is used which discriminates based on the use of inhibitors tazobactam (for KPC) or EDTA (for MBLs) (177).

Colorimetric methods to detect carbapenemases directly from blood without blood culture are cost-effective and less time consuming (3–4 h). A study evaluated two variations of CarbaNP, CarbaNPT-direct which uses Triton X-100 as enzymatic extractor and Blue-carba which uses bromothymol blue instead of phenol red, for detection of carbapenemases directly from blood samples (183). The study reported high sensitivity for MBLs and KPC by colorimetric assays but less sensitivity for detection of GES and OXA-48 carbapenemases. A new method with increased sensitivity is the CarbaLux test which uses a fluorescent carbapenem substrate and substrate with cloxacillin to detect not only all carbapenems but also carbapenem-hydrolyzing AmpC enzymes from a bacterial culture (184). Another highly sensitive and specific method is lateral flow immunoassay which detects widely spread carbapenemases NDM, KPC, OXA-48-like, IMP, VIM on a single strip within 15 min from a bacterial culture (185, 186). These require minimal infrastructure.

Several phenotypic tests and commercial kits are available for the detection of CRKP strains (179, 180), such tests and/or kits are yet unavailable for the hvKP strains. The clinical microbiology laboratories are thus, not equipped to distinguish cKP from hvKP during routine diagnosis. Since the hvKP strains are often found to harbor hypercapsule, the hypermucoviscosity appearance of the colonies on the agar plate can be determined by a string test in which a viscous string is generated (>5 mm) when a colony is stretched by an inoculation loop (90). However, the strain discrimination based on only the hypermucoviscous appearance of the colonies and/or the positive string test can be misleading because studies have argued that hypermucoviscosity and hypervirulence are two different phenomena (91, 187). It is noteworthy that several *rmpA* and/or *rmpA2*-positive non-hypermucoviscous strains and also *rmpA* and/or *rmpA2*-negative hypermucoviscous strains were detected in causing invasive syndrome (103, 188, 189). hvKP strains produce quantitatively more siderophores than cKP, and the siderophore assay can be an option for detection. However, as of date, the conclusive discrimination of hvKP is carried out by genotypic methods, such as via specifically amplifying several hvKP-associated genes (*magA*, *rmpA*, *rmpA2*, *iroB*, *iroN*, *iucA*, and *iutA*) using polymerase chain reaction (PCR). These hypervirulent biomarkers hold the promise of a sensitive and specific diagnostic test in future. Since, the methodology is largely genotype-based, it is restricted to the research laboratories and may not be readily available in the healthcare settings of LMICs. Research is on-going for the development of a cost-effective commercial test which will not only help the clinical laboratories but can also be used in surveillance and research studies (189). In some resource-poor settings where the basic blood culture facilities are still unavailable or not properly standardized, the detection of either CRKP or hvKP is far from sight. Moreover, the major hindrance in detecting hvKP is probably the lack of awareness of such strains and the need to detect them.

TREATMENT: IS THERE A SILVER LINING?

Treatment of neonatal sepsis is a great challenge, the signs of the disease are non-specific, the pathogens are numerous and drug-resistant, the diagnosis has limitations and the patient is vulnerable. Antibiotics remain the primary treatment with supportive respiratory and circulatory treatments along with treatment for metabolic derangements. In contrast to the well-established supportive treatments, the protocols for antimicrobial treatments are often found to be inadequate and region-specific. The choice of antibiotics largely depends on the etiology of the prevailing pathogens, their antimicrobial susceptibility profiles, the age of onset of sepsis, and the microbiology laboratory support available (3). The protocol is often specific for a given unit and greatly varies between and within countries. In this review, we discuss the limitations of the currently available antimicrobial therapy particularly in the LMICs in the current situation of drug resistance.

EMPIRICAL ANTIBIOTIC THERAPY: ARE THE GUIDELINES STILL ADEQUATE FOR NEONATES?

In general, the treatment of neonatal sepsis can be broadly divided into suspected or empirical therapy and known

or definitive therapy. In clinical microbiology, it is always recommended to obtain the blood cultures for ascertaining bacterial sepsis before the initiation of antimicrobial therapy. However, culture results often require at least 48–72 h to be reported. Considering the non-specific clinical manifestations of neonatal sepsis, the initial antimicrobial therapy should not be unnecessarily delayed for the culture report in case of severely ill and high-risk neonates. In most cases the treatment should start within 1 h of decision to treat (190). This initial antibiotic therapy which is implemented before obtaining the blood culture report is defined as the empirical therapy. Empirical antibiotic therapy should be guided by the prevalent spectrum of pathogenic bacterial strains and their resistance profiles commonly detected in the given NICU or in the community settings. Microbiology support is varied and insufficient in resource-poor settings and treatment is largely empirical in such situations.

Antibiotic resistance has limited the therapeutic options in neonates and this problem is compounded in resource-limited heath infrastructure in LMICs. CRKP strains in neonatal units have challenged the healthcare settings, as these strains not only produce broad-spectrum carbapenemases but also harbor a repertoire of other plasmid-mediated resistance determinants that confer resistance to almost all clinically important antimicrobial classes, including third- and/or fourth-generation cephalosporins, cephamycins, aztreonam, aminoglycosides, and fluoroquinolones (67). Under these circumstances, the WHO treatment guidelines for neonates were found to be ineffective for the LMICs.

In response to the antibiotic resistance crisis, recently, the WHO has revised the treatment guidelines and launched a global action plan on antimicrobial resistance. The aim of this global campaign is to reduce the spread of AMR through optimizing the use of antibiotics, and also to reduce the adverse events and overall costs. The WHO Essential Medicine List (EML) Working Group adopted a tool, AWaRe. This tool classifies the antibiotics into three groups: *A*ccess, *Wa*tch, and *Re*serve (191). The Access group generally includes narrow-spectrum antimicrobials against a wide range of commonly encountered susceptible pathogens. The WHO EML enlisted 19 antibiotics recommended as first or second choice of empiric treatment options for clinical infections. These antibiotics are affordable, greatly assured, and are generally available at all times. The Watch group contains broader spectrum antimicrobial classes that have higher resistance potential and includes most of the highest priority agents among critically important antimicrobials. Among the 110 Watch group antibiotics, WHO EML enlisted 11 antibiotics as first or second choice of empiric treatment options. However, these antibiotics are recommended only for specific and limited indications. In addition, the Reserve group consists of antibiotics and antimicrobial classes for the treatment of MDR infections. Antibiotics in this group should be considered as "last resort" and also should be highly specific for patients and settings when all other antibiotics have failed. Till date, 22 antimicrobials have been classified as reserve group. In the recent global action plan, WHO enlisted seven reserve group antibiotics in the EML (191).

For both early and late onset neonatal sepsis, the most commonly recommended and used antibiotics, as per the WHO guidelines, is a β-lactam antibiotic (most commonly ampicillin) combined with an aminoglycoside (most commonly gentamicin) (191). Recently, in 2019, the use of AwaRe group of antibiotics was assessed in a pediatric survey across 56 countries (192). The study revealed that among the Access group of antibiotics, gentamicin and ampicillin were commonly given to the septicemic neonates in most of the countries, including Africa, America, Eastern Mediterranean, Europe, and South-East Asia. However, in the Western Pacific region, amoxicillin and β-lactamase inhibitor were used as empiric treatment for neonates. In addition, the study also showed that among the Watch group of antibiotics, globally meropenem and/or cefotaxime were prescribed to the neonates suffering from bacterial sepsis. Moreover, in South-East Asia, among the Reserve group of antibiotics, colistin was given to the critically ill neonates (192).

Due to the emergence and spread of CRKP strains in the healthcare settings, the treatment options for neonates are narrowing. Added to this, are the challenges in sepsis diagnosis. This alarming scenario demands new antimicrobials or additional alternatives for the treatment of CRKP-infected septicemic neonates. Although currently there is a scarcity of new antimicrobials globally, combination therapy is an alternative. Such therapies can expand the spectrum of antibiotic coverage and synergism between the antibiotics with enhanced killing effect. In addition, new alternatives have also been suggested for CRKP infections though all may not be suitable for neonates. For example, avibactam (a non-β-lactam β-lactamase inhibitor) showed high effectivity against KPC- and OXA-48-producing CRKP strains when combined with ceftazidime (193). Similarly, other studies revealed that vaborbactam (a boronic acid derivative) when combined with meropenem can exert bactericidal activity against the KPC-2-producing CRKP strains (194). Current studies have also argued that plazomicin (a novel semisynthetic aminoglycoside) showed impressive activity against most of the CRKP strains (195). The use of polymyxins (including both colistin and polymyxin B) and fosfomycin have also been documented in several case studies for the treatment of CRKP infections in the neonates (196, 197). In the current AwaRe classification, WHO has enlisted these antibiotics in the reserve group for which significant clinical data are still missing (191).

In response to the critical priority pathogens, recently WHO has published a third review of the clinical antibacterial pipeline where they enlisted a total of 8 new antibiotics that gained market authorization since July 2017 (198). Of them, two β-lactam and β-lactamase inhibitor combinations (vaborbactam + meropenem and relebactam + imipenem), one aminoglycoside (plazomicin), and one tetracycline (eravacycline) were found to be active against the CRE. Although all of these newly launched antibiotics are active against the Class A and Class D carbapenemases but found ineffective against the MBLs. To combat the MBL-producing CRE, WHO has also enlisted several other antimicrobial agents that are now in clinical trials (Phase 1–3), such as cefiderocol (Phase 3), taniborbactam + cefepime (Phase 3), BOS-288 (Phase 2), zidebactam + cefepime (Phase 1), and nacubactam + meropenem (Phase 1) (198).

All of these currently developed antibiotics and/or antibiotic combinations are active against the CRE but the clinical data regarding their effectiveness against neonatal septicemia are yet to come. Although several clinical trials are ongoing for neonatal sepsis, additional large-scale clinical trials, strict infection control measures, and antimicrobial stewardship programmes should be undertaken in future to treat infections caused by drug-resistant and virulent *K. pneumoniae* to arrest their further dissemination.

ALTERNATIVE COMBAT APPROACHES

The gut of a term neonate having normal-body-weight is mainly comprised of *Bifidobacterium spp., Bacteroides, Clostridium spp.* and *Lactobacillus spp.* (in minor proportion) (160, 199, 200) and serves key role in providing nutrients, providing defense against gut colonization by pathogens, and development of immunity of the neonate (160). Whereas, in preterm and/or low-birth-weight neonates, intestinal flora lacks colonization by the above mentioned favorable microorganisms (199). Preterm and/or low-birth-weight neonates are often colonized by Gram-negative bacilli, some of which can be opportunistic pathogens and may trigger an inflammatory response which plays the key role in the initiation of the necrotizing enterocolitis and sepsis (160, 200). If the neonates are given supplements of probiotics (consisting of bacteria which forms the healthy gut flora of term neonates) with human breast milk, it helps in reduction of inflammation (200) as well as translocation of pathogenic bacteria (160). The use of *Bifidobacterium spp.* and *Lactobacillus spp,* in different probiotic doses has shown significant decrease in necrotizing enterocolitis and neonatal death (199–201). Despite pre-existing skepticism that probiotics may increase sepsis instead of decreasing it (200, 202), systemic reviews and meta analyses of randomized control trials of large sample sizes have shown that probiotics indeed play a beneficial role in decreasing the rate of LOS in preterm low-birth-weight neonates (203–205). The bacterial strains used in the randomized control trials were mainly *Bifidobacterium bifidum, B. lactis, B. breve, B. infantis, Lactobacillus acidophilus, L. rhamnosus, L. reuteri. Streptococcus thermophilus* in varied doses (203). As probiotics not only provide a low cost, non-invasive, safer way to replenish the preterm neonatal gut with natural gut flora of a healthy term neonate, but also provides defense against LOS, it can be considered for routine use in LMICs where the load of LOS is overwhelming.

Preterm neonates of <32 weeks of gestational age lack maternal transplacental immunoglobulin; transfer of which, from mother to fetus, occurs only after 32 weeks of gestation. In the search for suitable alternative therapy for sepsis in preterm neonates, studies were conducted by administering intravenous immunoglobulin, granulocytes, granulocyte colony stimulating factor and granulocyte-macrophage colony stimulating factor, and pentoxifylline to neonates. The results were not promising except for pentoxifylline which caused decrease in all-cause mortality (206, 207). However, an monoclonal antibody (mAb)—Pagibaximab has shown some positive result (208). Recently, broadly reactive mAbs were raised against the capsular antigens of CRKP and those mAbs showed positive result in efficient killing of *K. pneumoniae* in mice model (209). Although, the efficacy of mAbs can be properly assessed only after clinical trials on neonates, but it shows some ray of hope in an otherwise grim situation.

CONCLUSION

Antibiotic resistance in Gram-negative bacteria has clearly exposed that the World Health Organization (WHO) guidelines for the management of neonatal sepsis, which is currently ampicillin plus gentamicin, is in dire need of modification. The major burden of neonatal sepsis is borne by the LMICs where antibiotic resistance is high and microbiology laboratory support is inadequate. The number of documented neonatal infections caused by CRKP and hvKP are just the tip of the iceberg. Most cases are not recorded as the microbiology laboratories in resource-limited countries lack infrastructure and capability. This is probably likelier for hvKP, as these strains are not specifically detected in clinical microbiology laboratories. This lack of information is thus translated into treatment protocols. Studies have shown that the most severe forms of neonatal sepsis with an unfavorable outcome were due to virulent strains of *K. pneumoniae* (5). The clinician is however unaware of the hvKP phenotype. Some carbapenem resistance genes such as bla_{KPC} and bla_{OXA-48} are also difficult to recognize by routine disc diffusion tests. The presence of these genes may not be detectable by such tests as their capability to hydrolyze carbapenems are variable.

A recurring premise about carbapenemases is their ability to hydrolyze most β-lactam antibiotics, emergence of variants and their promiscuous nature. Plasmids are numerous and have facilitated intra and interspecies transmission of these genes particularly under antibiotic pressure. Globalization has encouraged their spread and most genes have within a few years of identification crossed boundaries and invaded new terrains. Clonal spread is uncommon but not unknown. The evolution of variants of enzymes that are more efficient or have better stability have also made them difficult to contain. Plasmids carrying carbapenem-resistant genes also carry other resistant genes in a bid to make a panel of antibiotics ineffective. With new carbapenem resistance genes being identified and new variants evolving, the problem of CRKP and hvKP that we recognize presently is incomplete.

Another overlooked aspect of *K. pneumoniae* is that it commonly resides in the human gut. The rate of colonization of *K. pneumoniae* increases drastically in hospitalized patients with invasive devices, antibiotic exposure, and prolonged stay. Needless to say, that this would also happen with premature, low-birth-weight neonates with a pristine gut. *K. pneumoniae* that colonize the gut in the hospital are resistant to antibiotics and the gut provides an environment apt for the exchange of resistance genes. Studies have shown that such exchange of resistance genes frequently happen in the gut (210). Further, bacteria from the gut can translocate and cause sepsis in neonates. This can happen in neonates who have an immature immune

system, lower levels of mucus and gastric acid production. The bacteria evade the gut barrier and cause sepsis. Our study on the neonatal flora showed that *K. pneumoniae* was not only the predominant organism that colonized the neonatal gut, but also the most common organism isolated from the neonatal blood specimens (162). The exchange of carbapenem resistance genes in the gut and subsequent translocation can further complicate the situation.

It is difficult to change things overnight and the economic implications of these changes may also be a constraint on health systems that are poorly funded. The COVID-19 situation has further exposed the fragility of health systems around the world and newborns have also been affected. Measures that can reduce infection rates can also reduce CRKP and hvKP. These include surveillance systems to recognize changes in etiology and drug resistance profile, improved laboratories for better and timely detection of pathogenic strains, setting empirical treatment guidelines based on the profiles and proper education of mothers and healthcare workers regarding sanitation. We have come a long way from the days when we understood microbes through the lens of a microscope. We now seem to have an unprecedented power over them by being able to understand their genetic make-up to the last nucleotide. Whole genome sequencing has opened up newer avenues to understand the bacterial genomes. This enormous amount of information generated can be honed to create new cures and products. The challenge is big but there is light at the end of the tunnel.

AUTHOR CONTRIBUTIONS

SB: conception, design, wrote the introduction, and discussion. SMu and SMi: performed literature search, wrote the initial draft, and prepared all the tables and figures. SB and SD: critically revised the manuscript. All authors read and approved the final manuscript.

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