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Nutrition as a pharmacological approach to metabolic disorders and ageing

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Nutrition as a pharmacological approach to metabolic disorders and ageing

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Editorial: Nutrition as a pharmacological approach to metabolic disorders and ageing

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Editorial on the Research Topic
Nutrition as a pharmacological approach to metabolic disorders and ageing

Introduction

Modifying lifestyle and dietary habits is widely recognized as a powerful strategy for counteracting diseases associated with metabolic dysfunction (e.g., obesity and ageing) and represents the first line of intervention (Baskin and Karp, 2025). In the classical biochemical view, food-derived molecules are regarded primarily as substrates for energy production. However, accumulating evidence indicates that specific nutrients actively modulate metabolic health, influencing both disease development and prevention (Ruocco et al., 2021). These bioactive components (i.e., amino acids, short-chain fatty acids, and polyphenols) regulate signaling pathways, interact directly with proteins, and shape the gut microbiota (Ryan and Seeley, 2013; Menichetti et al., 2025). Consistently, more than 139,000 dietary compounds have been identified with potential biological activity, and approximately 2,000 are already employed for pharmacological purposes (Menichetti et al., 2025). In other words, owing to their mechanisms of action, nutrients may be closer to drugs than to the simple energy substrates traditionally defined in nutritional biochemistry. Together, these insights emphasize that the qualitative composition of the diet, beyond caloric content, plays a crucial role in determining whole-body metabolism, longevity, and health span (Figure 1).

Ageing and obesity are associated with several chronic diseases, including cardiovascular disorders, type 2 diabetes (T2D), leaky gut, metabolic dysfunction-associated steatotic liver disease (MASLD), and cancer. Both conditions share hallmark features such as systemic inflammation, reduced mitochondrial activity, excess reactive oxygen species (ROS) production, and impaired metabolic flexibility across organs and tissues (Bratic and Larsson, 2013; Muoio, 2014). These common features suggest that both conditions arise from overlapping cellular and molecular pathways regulating metabolic health. Moreover, long-term obesity has been associated with the expression of molecular ageing signatures during young adulthood in both females and males, reinforcing the view that obesity may

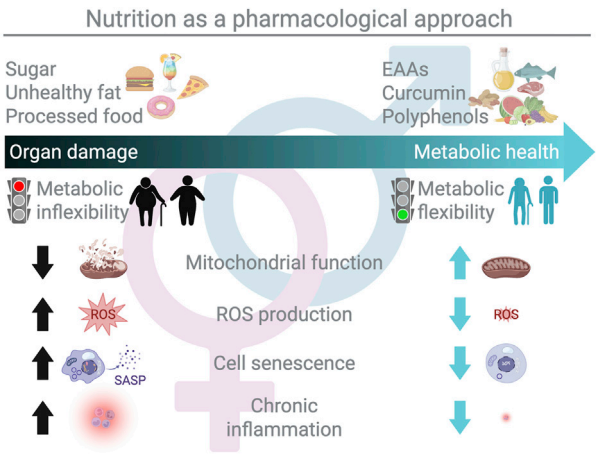

FIGURE 1
Nutrition as a pharmacological approach: from organ damage to health by restoring metabolic flexibility. The figure illustrates nutrients as metabolic modulators that directly influence metabolic flexibility (i.e., the ability to switch efficiently between fuel sources in response to changing metabolic demands) with sex-specific differences. Diets high in sugars, unhealthy fats, and ultra-processed foods (e.g., a Western-style diet) promote mitochondrial dysfunction, increased reactive oxygen species (ROS) production, cellular senescence (e.g., the senescence-associated secretory phenotype, SASP), and chronic inflammation. These processes drive metabolic inflexibility across multiple organs and tissues (adipose tissue, liver, heart, and skeletal muscle), ultimately contributing to organ damage and metabolic diseases (e.g., obesity, ageing, and associated disorders). By contrast, targeted nutrient-based interventions, including essential amino acids (EAAs), curcumin, polyphenols, and other bioactive food-derived compounds, can improve mitochondrial function, lower ROS levels, limit senescence, and attenuate chronic inflammation. Collectively, these effects support metabolic flexibility at the tissue/organ level and improve whole-body metabolic health, reinforcing the concept that appropriately designed nutritional strategies can function as pharmacological-like tools to prevent or reverse metabolic dysfunction.

constitute a state of accelerated biological ageing (Correa-Burrows et al., 2025). Metabolic homeostasis depends on a highly integrated network of signaling pathways, and perturbations in any component of this system can propagate across tissues, ultimately leading to multi-organ dysfunction (Sung et al., 2023). In this context, the chronic consumption of a Western diet rich in processed foods, sugars, and unhealthy fats promotes chronic inflammation in multiple organs (Clemente-Suárez et al., 2023), driving adipose tissue expansion and dysfunction, factors that *The Lancet Commission* identified as key determinants of the clinical manifestations of obesity (Rubino et al., 2025). In turn, the resulting metabolic disturbances impair nutrient utilization (i.e., fatty acids, amino acids, and glucose) and contribute to the development of obesity-related diseases (Sung et al., 2023) (Figure 1).

Beyond calories: nutrients as metabolic modulators

Challenging the notion that "a calorie is just a calorie," this Research Topic examines how nutrient quality shapes metabolic health and contributes to the prevention or mitigation of metabolic diseases. Despite increasing recognition of the therapeutic potential of nutrients, mechanistic understanding remains incomplete and is currently the subject of extensive research. The bibliometric analysis included in this Research Topic confirms the rapid expansion of studies in this area. Notably, citation burst analysis in the field of vitamin D reveals a shift from an early emphasis on correlation-based studies (e.g., associations between vitamin D levels and obesity or the effects of dietary calcium supplementation) toward investigations exploring causal relationships between obesity and vitamin D deficiency, as well as the preventive or therapeutic effects of vitamin D supplementation on metabolic diseases (Song et al., 2024).

Substantial inter- and intra-individual variability in nutrient metabolism, shaped by sex, age, genetics, and physiological state, is critical for understanding metabolic responses to nutritional interventions. Such variability provides key insights into the molecular mechanisms through which dietary restriction (DR) may influence lifespan (Mihaylova et al., 2023). In this context, Ruocco et al. discuss DR, long considered a universal "magic bullet" for longevity. Human studies, including the CALERIE trial, show that moderate DR improves cardiometabolic health and modulates pathways related to proteostasis, DNA repair, mitochondrial function, and inflammation while revealing links between cellular senescence and metabolic health (Kitzman et al., 2016; Aversa et al., 2024). However, DR responses vary markedly, genetic background can outweigh the intervention itself, and sex-specific factors critically shape lifespan outcomes (Di Francesco et al., 2024; Ruocco et al., 2024). Together, these findings underscore the necessity of personalized nutritional interventions that account for biological variability to effectively promote health span (Figure 1).

Nutritional interventions as therapeutic tools against obesity and ageing

Building on previous evidence demonstrating the preventive and therapeutic effects of essential amino acids (EAAs) on obesity and insulin resistance (Ruocco et al., 2020), Spataro et al. (2025) show that EAAs can also counteract obesity-induced chronic gut inflammation. Whereas mice fed a high-fat diet (HFD) develop obesity and T2D, associated with impaired intestinal barrier integrity, replacing dietary protein with EAAs reduces weight gain, ameliorates glucose homeostasis, and protects against gut dysfunction. These beneficial effects are mediated by enhanced mitochondrial activity, stimulation of antioxidant defenses, and reduction of gut inflammation, in line with findings showing that dietary EAAs modulate metabolism by acting directly on peripheral tissues such as muscle, adipose tissue, heart, and liver (D'Antona et al., 2016; Tedesco et al., 2018; Ragni et al., 2023).

Consistent with the central role of mitochondria in metabolic homeostasis, Segala et al. (2025) show that EAA supplementation (named α5 mixture) stimulates mitochondrial function and ameliorates MASLD. In mice fed a Western-type high-fat, high-sugar diet, α5 supplementation reduces hepatic steatosis and fibrosis and alleviates MASLD-associated anxiety-like behaviors (Segala et al., 2025).

Ding et al. (2025) also provide a comprehensive overview of nutrients and natural products as anti-ageing therapeutics. Several compounds, including resveratrol, curcumin, apigenin, epigallocatechin-3-gallate (EGCG), coenzyme Q10, and flavonoids

such as quercetin and kaempferol, enhance mitochondrial function, reduce ROS production, and inhibit pro-ageing pathways. In line with this, Matin et al. (2025) show that ginger supplementation, whose antioxidant activity derives largely from its phenolic compounds, counteracts age-related oxidative stress in the mouse liver, restoring antioxidant capacity in a dose- and ageing-dependent manner. This aligns with the previous work demonstrating that replacing saturated fats with extra-virgin olive oil enriched in polyphenols improves cardiometabolic and hepatic health (Ruocco et al., 2022).

Ageing, as well as obesity, is accompanied by metabolic alterations that drive cellular senescence. Consequently, targeting senescence has emerged as a promising strategy to counteract these processes and improve metabolic health (Childs et al., 2015; Zumerle et al., 2024). Accordingly, He et al. (2025) report that ageing induces senescence in tongue mucosa and causes disruptions to amino acid and carbohydrate metabolism. Carnosine levels decline with age in both mice and humans, suggesting its potential as a biomarker of ageing, whereas carnosine supplementation attenuates oxidative stress-induced senescence and lowers p21 and β-galactosidase expression.

Accumulating evidence indicates that cellular senescence is a significant risk factor for atherosclerosis, a condition closely linked to both ageing and obesity. Tao et al. (2025) show that sodium nitrate supplementation reverses atherosclerotic lesions in apolipoprotein E-knockout mice fed HFD, a classical preclinical model of atherosclerosis, while reducing vascular inflammation and preventing senescence. These effects are mediated through modulation of the miR-34a–FGF21 axis, highlighting how nutrient-derived interventions can exert anti-senescent and vasculo-protective actions.

Together, these findings reinforce the concept that specific nutrient compositions can act as metabolic modulators by restoring mitochondrial function, enhancing antioxidant defenses, reducing senescence, and lowering inflammation across organs and tissues, thereby preventing or reversing metabolic disturbances associated with obesity and ageing (Figure 1).

Nutritional interventions as therapeutic tools against cancer

Obesity and ageing are also closely associated with cancer development, a condition characterized by mitochondrial dysfunction and metabolic reprogramming that drive tumor cells toward aerobic glycolysis (i.e., Warburg effect) and amino acid addiction (Ragni et al., 2022a). Branched chain amino acids (BCAAs) can fuel tumor growth, raising concerns about their supplementation in nutrient-based anti-cancer strategies. In this Research Topic, Zhou et al. (2025) highlight that many tumors undergo BCAA metabolic rewiring, with some cancers overexpressing key catabolic enzymes and others exhibiting elevated BCAA levels that promote mechanistic target of rapamycin activation. Notably, BCAA supplementation improves survival in hepatocellular carcinoma, whereas colorectal cancers display inhibition of BCAA catabolism. However, Ragni et al. demonstrated that an EAA-enriched diet impairs tumor growth in mice by stimulating BCAA catabolism, restoring metabolic flexibility, and promoting apoptosis selectively in cancer cells (Ragni et al., 2022b). These findings underscore the context-dependent nature of amino acid metabolism, which varies according to tumor type and reflects the inherent complexity of amino acid metabolic pathways.

Accordingly, Akinlalu et al. (2025) comprehensively review the cancer-specific and often exclusive metabolic roles of individual amino acids, proposing tailored supplementation or withdrawal strategies, as well as targeting amino acid transporters. They further argue that mapping amino acid metabolic heterogeneity across cancer types may yield diagnostic biomarkers and guide precision nutrition-based therapies. This is confirmed by Liu et al. (2024), who report in a nested case–control study that higher serum taurine correlates with reduced overall cancer risk, elevated S-adenosylmethionine (SAM) with increased gastrointestinal cancer incidence, and cysteine with cancer risk in women. However, Bayesian kernel machine regression revealed that the combined mixture of taurine, SAM, and cysteine was negatively associated with cancer risk, indicating a complex, non-linear interaction among these metabolites.

Nutritional strategies may also support the management of cancer-associated conditions such as cachexia, a disorder characterized by muscle wasting, inflammation, and metabolic dysfunction. Faiad et al. (2025) highlight that early, individualized nutritional interventions, particularly high protein intake and anabolic amino acids (e.g., BCAAs), can help attenuate muscle loss and inflammation, especially when combined with supplements such as arginine, omega-3 fatty acids, glutamine, and β-hydroxy-β-methylbutyrate. However, clinical evidence supporting the use of carnitine, omega-3 fatty acids, or antioxidants such as resveratrol, curcumin, and EGCG when administered individually remains limited and inconclusive.

Conclusion

Building on the concepts summarized in this Research Topic, it is increasingly clear that treating obesity and ageing as whole conditions, rather than as clusters of individual complications, may offer a more effective strategy for improving health span (Garmany et al., 2021; Rubino et al., 2025). Within this framework, nutrient-based interventions, by targeting mitochondrial function, antioxidant and anti-inflammatory pathways, and cellular senescence, represent powerful therapeutic tools, either as alternatives or complements to pharmacological treatments, which, despite their efficacy, are often limited by adverse side effects. This is particularly relevant given the rapid rise of incretin-based drugs, such as GLP-1R agonist (i.e., semaglutide), GLP-1R/GIPR dual agonists (i.e., tirzepatide), or next-generation GLP-1R/GIPR/GlucagonR triple agonists (i.e., retatrutide), which exhibit unprecedented clinical efficacy in metabolic disease management. Notably, evidence that tirzepatide induces a thermogenic-like amino acid signature in brown adipose tissue (i.e., increase in energy expenditure), similar to that observed with nutrient-based interventions such as EAA feeding, highlights a mechanistic convergence between drugs and nutrient modulation of metabolism (Samms et al., 2022; Ruocco et al., 2023). Yet, incretin-based therapies pose nutritional challenges, including lean mass loss, underscoring the need for tailored dietary strategies encompassing adequate protein intake, amino acid support, and resistance training (Ben-Porat et al., 2025). Altogether, these insights emphasize the importance of integrating personalized nutritional approaches with pharmacological treatments to optimize metabolic health and promote longevity.

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Bibliometric analysis of vitamin D and obesity research over the period 2000 to 2023

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Background: Globally, the incidence rates of obesity and its related diseases, such as cardiovascular diseases and type 2 diabetes, are continuously rising, posing a significant public health challenge. Studies have indicated a potential correlation between vitamin D deficiency and obesity. However, a quantitative analysis of the studies related vitamin D and obesity is lacking. This investigation aims to fill this gap by providing a comprehensive bibliometric analysis to uncover the collaborative networks, research hotspots, and evolutionary trends within the field of vitamin D and obesity research.

Methods: This study retrieved literature related to vitamin D and obesity from the Web of Science database spanning from 2000 to 2023. Bibliometric analysis was conducted using tools such as HistCite, VOSviewer, and CiteSpace to excavate multi-dimensional information including countries, institutions, authors, journals, citations, and keywords.

Results: A total of 6,144 records were retrieved, involving 123 countries, 6,726 institutions, and 28,156 authors, published in 1,551 journals. The number of published papers and citations showed a generally increasing trend. The United States led in terms of publication volume and influence, with journals such as Nutrients and Obesity Surgery having the highest publication counts. Nasser M. Al-Daghri was the most prolific and influential author. Keyword clustering revealed that research topics covered metabolic health, nutrition, immunity, and bariatric surgery. Citation burst analysis indicated a shift in research focus from the relationship between dietary calcium and obesity to the preventive effects of vitamin D supplementation on metabolic diseases.

Conclusion: The application of bibliometric methods to analyze the research literature in the fields of obesity and vitamin D has provided a comprehensive understanding of the collaborative networks, key research focus, and evolutionary trends in this field, offering insights for guiding future research directions.

KEYWORDS

vitamin D, obesity, vitamin D deficiency, trends, bibliometric analysis

1 Introduction

Obesity has emerged as a pressing global health concern, affecting diverse age groups and populations. It contributes to chronic conditions such as cardiovascular diseases, type 2 diabetes, and metabolic syndrome (Piché et al., 2020). According to a recent study published in The Lancet, by 2022, more than one billion people in the world are now living with obesity. Since 1990, the prevalence of obesity among adults worldwide has more than doubled, while the rate among children and adolescents (aged 5–19) has quadrupled (Phelps et al., 2024). Obesity has emerged as a major public health concern worldwide, necessitating the implementation of effective preventive and control measures to mitigate its impact on population health. Hence, it is vital to establish extensive research and multifaceted treatment approaches for obesity.

Vitamin D, a fat-soluble vitamin, exists in two primary forms: vitamin D_2 (VD2) and vitamin D_3 (VD3). Human vitamin D primarily originates from skin synthesis (VD3) and dietary intake (VD2 or VD3). To exert biological activity, vitamin D undergoes hydroxylation in the liver to form 25-hydroxyvitamin D (25(OH)D, the circulating form), followed by further hydroxylation in the kidneys to produce 1,25-dihydroxyvitamin D ($1,25(OH)_2D$, the active form) (Kulda, 2012). It plays crucial roles in calcium-phosphate metabolism, immune modulation, cellular growth, differentiation, and apoptosis (Zmijewski, 2019). Additionally, emerging evidence suggests a potential anti-obesity role for vitamin D (Abdullah Thani et al., 2019). Vitamin D deficiency is more prevalent among obese individuals, and its role in the association between obesity and cancer risk has been suggested (Sánchez-Bayona et al., 2022). Evidence indicates that vitamin D may participate in the onset and progression of obesity by influencing fat metabolism, modulating hormone levels, and regulating inflammation and immune responses (Argano et al., 2023). Consequently, understanding the complex relationship between obesity and vitamin D has become a key area of research.

Bibliometric analysis is a method that quantifies and visualizes published literature. It involves the analysis of the quantity and quality of literature, publications, and citation information (Agarwal et al., 2016). This method aims to review the development, impact, and trends within scientific research. Combining approaches from informatics, statistics, sociology, and other disciplines, it seeks to quantify and evaluate the impact of research outcomes, authors' contributions, and the dynamic changes within academic domains. Its applications include investigating the dynamics of literature production, assessing journal influences, determining citation patterns, and identifying research themes or future directions, including hot topics (Nicolaisen, 2010). In recent years, the utilization of bibliometric analysis as a scientific research tool has steadily increased in publication volume (Ellegaard and Wallin, 2015). For newcomers in a specific research field, timely and comprehensive systematic reviews offer valuable overviews of knowledge domains and guide effective initiation of research. For experienced and active researchers, systematic reviews aid in keeping abreast of the latest advancements in their field (Chen and Song, 2019). Scholarly analysis using bibliometric methods has explored the role of vitamin D in immunity (Luo et al., 2022), bone metabolism (Malik et al., 2022), infections (He et al., 2022), reproductive health (Lu et al., 2022), and non-alcoholic fatty liver disease (Wang and Chang, 2023). However, the relationship between vitamin D and obesity remains unexplored, which is particularly intriguing given the high prevalence of vitamin D deficiency in obese individuals and its potential implications for metabolic health. This study aims to bridge this gap by conducting a comprehensive bibliometric analysis of literature pertaining to vitamin D and obesity from 2000 to 2022. Utilizing advanced data visualization techniques, we will quantify and map the development and focus of this interdisciplinary research field, shedding light on the current research status and trends. Our findings are expected to not only elucidate the complex interplay between vitamin D and obesity but also provide a solid foundation and valuable insights for guiding future research directions and clinical applications.

2 Materials and methods

2.1 Search strategy in web of science core collection

In this study, we have chosen the Web of Science Core Collection (WoSCC) as our data source. The Web of Science is regarded as the world's largest and most comprehensive collection of information resources, and its standardized data structure and rich citation information make it particularly suitable for bibliometric analysis (Wang et al., 2020). To ensure the precision and relevance of our analysis, we have decided to focus exclusively on English-language articles from the WoSCC. Considering the various English expressions for "vitamin D" and "obesity" or "overweight", we referenced keywords used by relevant researchers to effectively extract literature related to vitamin D and obesity or overweight. Our aim was to accurately encompass research articles on these topics within the WoSCC database. After multiple searches and comparisons, we finalized our search formula as "TS = (("vitamin D" OR "Vit D") AND ("obese" OR "obesity" OR "overweight"))". We restricted publication years from 2000 to 2023, and the document types were "articles" or "reviews". The article language was set as English. Search results were downloaded as "Full

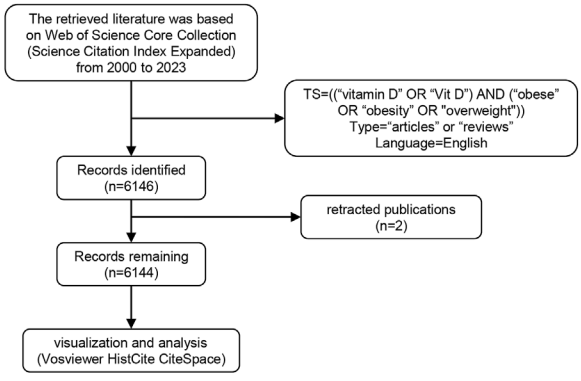

FIGURE 1
Flow chart of document selection and identification.

Record and Cited References" and "Plain Text". The exported content was saved in a txt file for subsequent analysis. Initially, we obtained 6,146 publications, subsequently removing two retracted publications (see Supplementary Table S1). To avoid the potential bias caused by the continuous updating of the database, the search and export of files were all conducted within a single day (5 January 2024) (Figure 1).

2.2 Bibliometric analysis

HistCite (version 12.03.17) is a pivotal tool in bibliometric analysis, specializing in illustrating citation relationships among scholarly works (Garfield et al., 2006). Its functionalities encompass citation network graphs, timeline analyses, and in-depth citation metrics, offering insights into publication impact and research trends within academic domains. We imported the downloaded txt file into HistCite for analysis, yielding data on the quantity of publications per year, active countries, institutions, authors, and core publications, as well as the global total citation scores (TGCS) and local total citation scores (TLCS) for all results. TGCS represents citation counts within the Web of Science, while TLCS indicates citations concentrated within the current publication set. The results were summarized and organized using Excel 2021 (Microsoft). We also used Excel 2021 to visualize the trends in publication volume and the distribution of publications by country, providing a clear and interpretable representation of our results.

VOSviewer (version 1.6.19) is a specialized knowledge mapping tool used extensively in bibliometric analysis (Van Eck and Waltman, 2010). It is particularly adept at creating visual representations, such as network maps, time overlay maps, and density visualization maps, to reveal relationships among scholarly publications. Employing clustering analysis and co-occurrence networks, VOSviewer swiftly displays topic distributions, keyword associations, and collaboration networks, offering a comprehensive understanding of the scientific research landscape (Qiu et al., 2014). This paper primarily utilizes VOSviewer to visualize the countries, institutions, keywords, and authors of articles. To obtain the corresponding views, the literature data is imported into VOSviewer, and the content for visualization is selected. In cases where clustering is not apparent, Pajek (http://mrvar.fdv.uni-lj.si/pajek/) is used for adjustment. Additionally, Scimago Graphica (version 1.0.41) (Hassan-Montero et al., 2022) is employed to depict the publication volume of countries and international cooperation among states.

Citespace (version 6.2.R4) is a robust bibliometric analysis tool primarily focused on visualizing citation networks among scholarly publications, emphasizing the temporal and spatial relationships between works (Chen, 2006). Its key functionalities include identifying critical paths, significant nodes, and literature clustering, aiding in comprehending knowledge structures and research evolution (Wang et al., 2022). In the analysis of the field concerning vitamin D and obesity, CiteSpace primarily conducted burst analysis for references and keywords. This analytical approach helps to uncover research trends, emerging topics, and key turning points in the field, providing valuable insights for researchers and guiding future research directions. The time slice was set from January 2000 to December 2023, with a 2-year interval per slice. Node types were defined as "references" or "keywords", selecting the top 50 levels based on the most cited or frequently occurring criteria within each time slice. Modularized Q and average silhouette were used to assess clustering reliability; where Q > 0.3 and average silhouette >0.5 indicate sufficient clustering structure and convincing clustering results. The criteria selected the top 50 levels based on the most cited or frequently occurring references and keywords within each time slice.

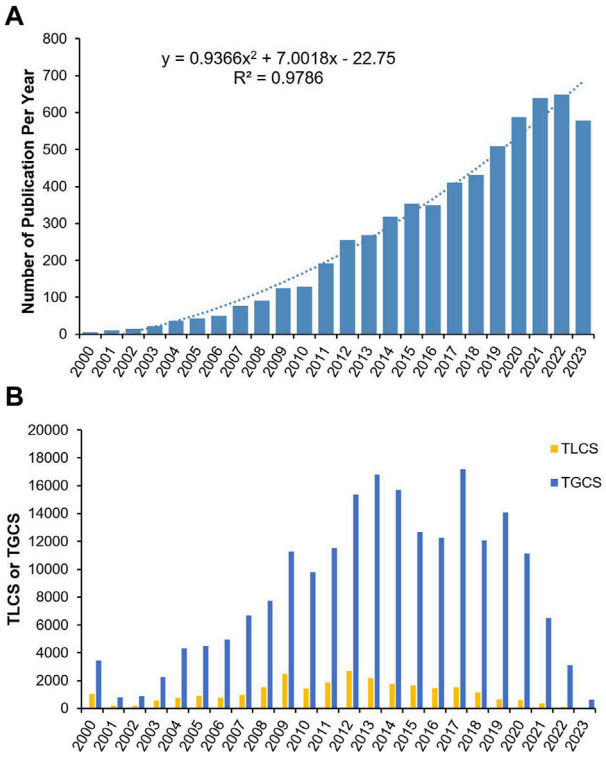

FIGURE 2
Publication outputs and citations on vitamin D and obesity in the past 23 years. **(A)** Global annual production trends. Blue bars represent the number of publications per year. **(B)** Annual TGCS and TLCS of publications.

3 Result

3.1 The global growth trend of publication outputs in vitamin D and obesity research

Between 2000 and 2023, a total of 6,144 publications concerning vitamin D and obesity were retrieved from WoSCC, after excluding two rejected articles. This collection comprises 4,881 original research articles (accounting for 79.44%) and 1,263 review articles (accounting for 20.56%). With the exception of minor fluctuations in 2016, there has been a consistent uptrend in publication numbers from 2000 to 2023, reaching a peak in 2022 with 642 publications. Notably, there was significant growth during the intervals of 2010 to 2012 and 2018 to 2021 (Figure 2A). In general, although the early stage of development featured a modest

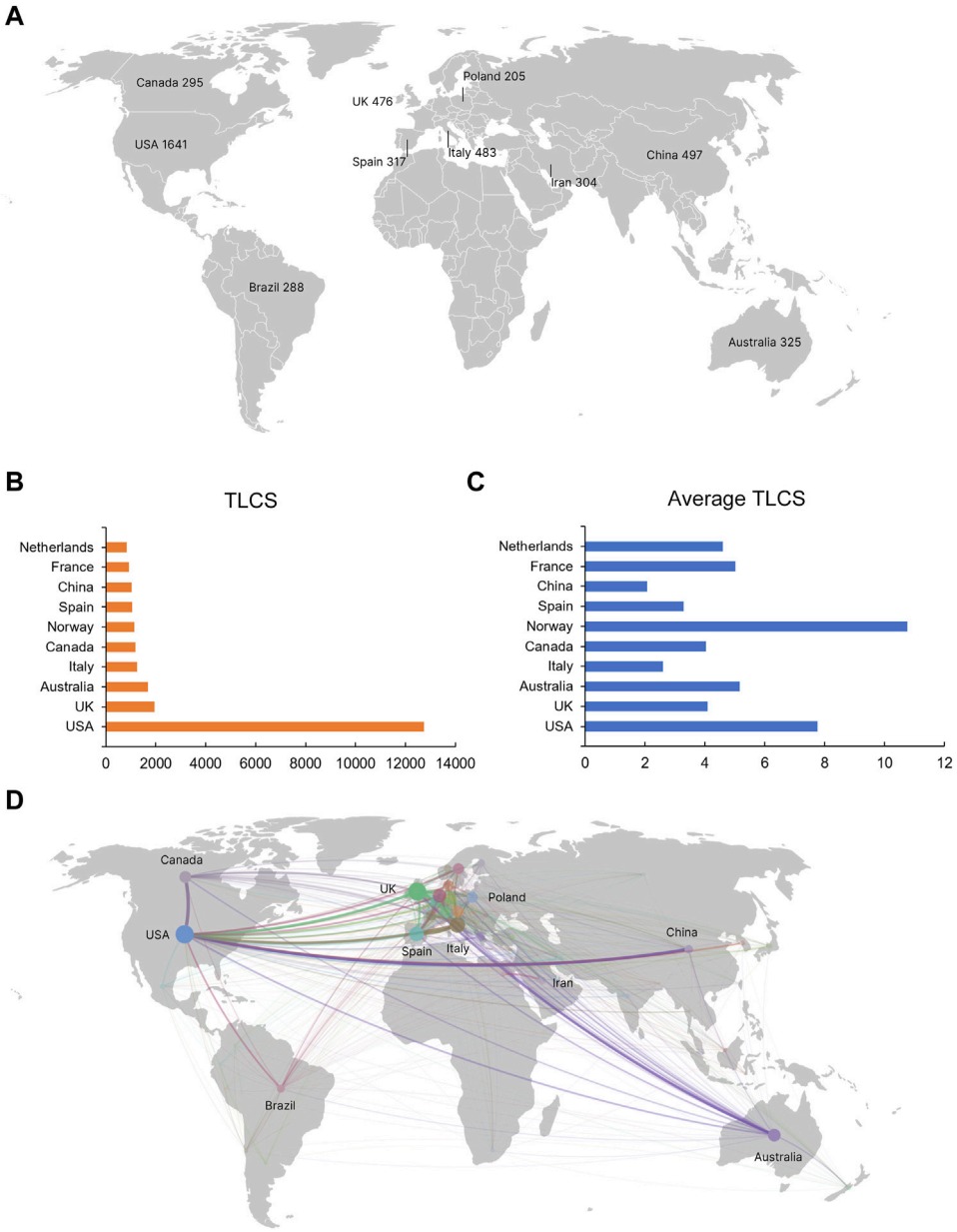

FIGURE 3
The leading country in the field of vitamin D and obesity research. **(A)** Top 10 countries by the number of publications. **(B)** Top ten countries with the highest TLCS. **(C)** Top ten countries with the highest average TLCS. **(D)** International cooperation among States. Each country is represented as a node, and each line represents a co-author relationship. The size of the node is proportional to the strength of the cooperative link.

number of publications, the volume of citations was remarkably high. In the year 2000, a modest sum of six articles were published, of which the study conducted by Wortsman et al. (cited 2,258 times) uncovered a potential link between vitamin D and obesity (Wortsman et al., 2000), stimulating an increase in subsequent research on this topic. Corresponding to the increase of publications, the TGCS and TLCS have been relatively high since 2000. The TGCS demonstrated a progressive increase from 2001, reaching its peak in 2013. Despite notable variations in 2017, the TGCS and TLCS have been relatively stable since 2015, corroborating that research into vitamin D and obesity remains a popular topic to date (Figure 2B).

3.2 Analysis of country contribution and country burst in vitamin D and obesity research

It was found that 123 countries and regions participated in the study of vitamin D and obesity. The five countries that contributed the most publications were the United States (1,641), China (497), Italy (483), the United Kingdom (476), and Australia (325) (Figure 3A) (Table 1). In the TLCS ranking, the United States still ranks first (12,734 citations), followed by the United Kingdom (1,946), followed by Australia (1,675), Italy (1,256) and Canada (1,190) (Figure 3B). Norway,

TABLE 1 Analysis of top ten countries with the highest TLCS.

Rank	Country	Publications	TLCS	TGCS	Average TLCS
1	United States	1,641	12,734	90,369	7.76
2	United Kingdom	476	1,946	23,660	4.09
3	Australia	325	1,675	12,851	5.15
4	Italy	483	1,256	15,239	2.60
5	Canada	295	1,190	11,581	4.03
6	Norway	105	1,131	5,261	10.77
7	Spain	317	1,041	11,809	3.28
8	China	497	1,029	9,448	2.07
9	France	184	923	11,939	5.02
10	Netherlands	182	838	12,590	4.60

TABLE 2 Analysis of top ten Institution with the highest TLCS.

Rank	Institution	Publications	TLCS	TGCS	Average TLCS
1	Boston University	44	1,643	4,800	37.34
2	Harvard University	104	1,353	10,438	13.01
3	Tufts University	45	653	3,244	14.51
4	University of Tennessee	46	645	3,237	14.02
5	University of Tromso	20	506	1,906	25.3
6	University of Minnesota	33	493	1,666	14.94
7	Creighton University	23	482	2,175	20.96
8	Mayo Clinic	54	449	2,449	8.31
9	Brigham and Women's Hospital	71	432	5,310	6.08
10	University of California, Los Angeles	24	420	2,919	17.5

notwithstanding not having the highest publication or TLCS volumes, stood out with the highest average TLCS (Figure 3C), indicating its considerable impact. There were 65 countries with more than ten publications that were included in the co-authorship analysis. The highest total link strength was observed in the United States (total link strength = 866 times) (Figure 3D), In this largest cooperative network led by the United States, the United Kingdom (838), Italy (626), Spain (551), and Netherlands (481) were in key positions. Despite China's considerable contribution to the volume of publications, the nation manifests a total link strength of merely 184, reflecting a comparatively restrained impact within the scope of global research collaborations.

3.3 Active institutes in vitamin D and obesity research

A total of 6,726 institutions contributed to publications in this field. The five institutions of the most contributed publications are: Harvard University (104 publications), University of Tehran Medical Sciences (96), King Saud University (84), Harvard Medical School (78), and Monash University (75) (Table 2). The top 10 productive institutions are mainly distributed in the United States (3 institutions) and Iran (2 institutions). Strikingly, the United States has a staggering 9 of the top 10 institutions in TLCS (Table 2). We further screened 334 institutions with more than 10 papers, excluding 1 institution with no relationship, and the institutional collaboration network is mainly divided into 11 main clusters (Figure 4). The top institutions are located in the upper right cluster, and the University of Copenhagen had the most cooperation with other institutions (total link strength = 304), followed by Medical University of Vienna (260), Poznan University of Medical Sciences (255), University of Padua (249), and Medical University of Graz (242).

3.4 Active authors in vitamin D and obesity research

A total of 28,156 authors contributed to publications in this field, with Nasser M. Al-Daghri from King Saud University being the most prolific, having published 44 papers in this field (Table 3). The

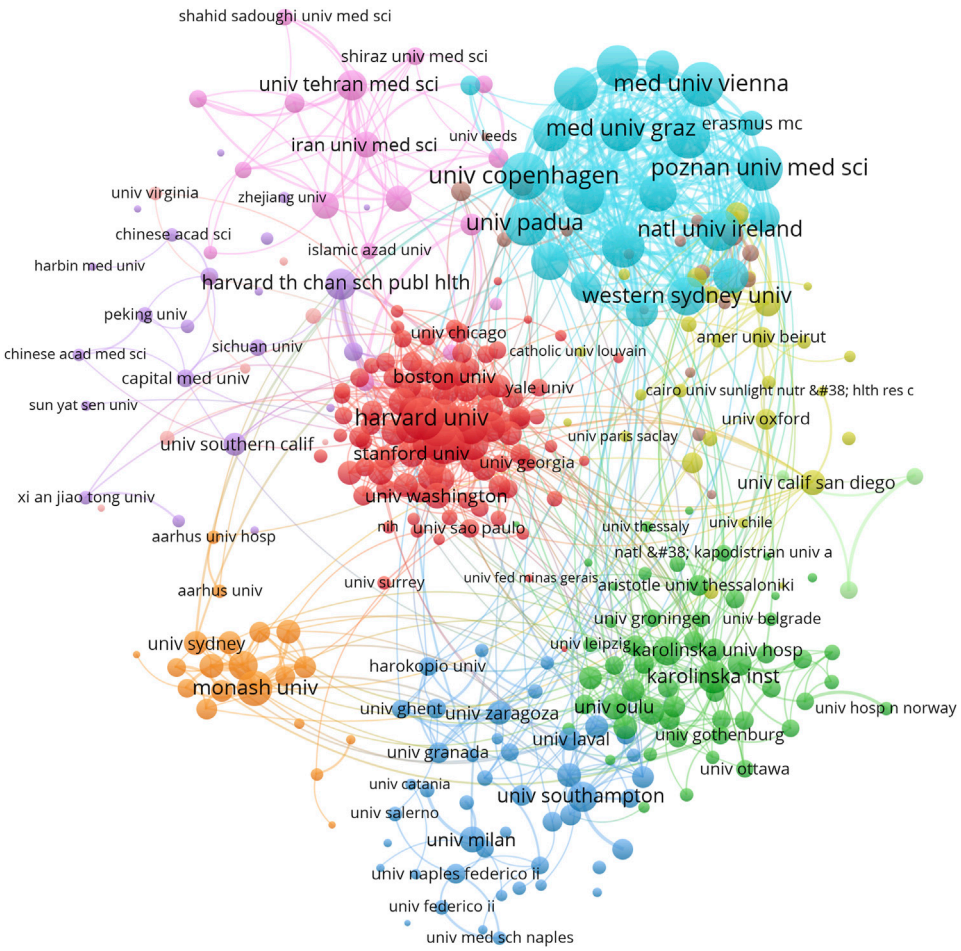

FIGURE 4
Active institutional analysis. Each organization represents a node, the size of the node is proportional to the strength of the cooperative link, each line represents a co-authoring relationship, and the line thickness indicates the strength of the collaborative link.

TABLE 3 Analysis of top 10 productive authors.

Rank	Name	Institutions	Publications	TLCS	TGCS
1	Nasser M. Al-Daghri	King Saud University	44	45	1,140
2	Giovanna Muscogiuri	The Catholic University of America	29	251	1,133
3	Yue Chen	Hunan Normal University	28	296	883
4	Wang Y	Johns Hopkins University	28	76	436
5	Colao A	Università degli Studi di Napoli Federico II	26	112	830
6	Alokail MS	King Saud University	25	1	719
7	Luigi Barrea	Università degli Studi di Napoli Federico II	24	111	729
8	Y Zhang	Xi'an Jiaotong University	24	25	457
9	Omar S Al-Attas	King Saud University	22	1	654
10	Barbora de Courten	Monash University	22	155	664

author with the highest LTCS was Michael F. Holick (1,288) from Boston University (Supplementary Table S2). We further conducted co-authorship analysis on 433 authors with more than 5 publications, resulting in 197 authors after excluding 52 irrelevant authors. These authors were then divided into 13 largest collaborative networks. The highest total link strength was observed for Nasser M. Al-Daghri with a total link strength of 172, followed by Gernot Desoye (163), Peter Damm (162), David

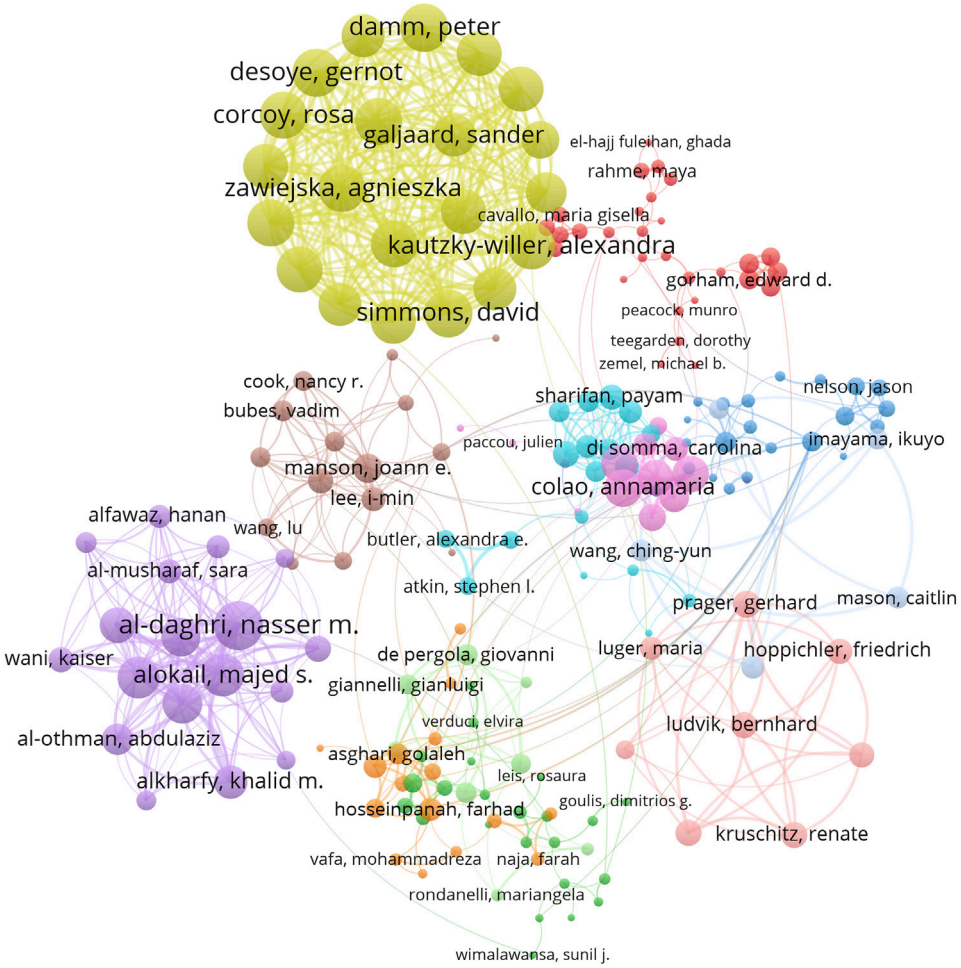

FIGURE 5
Analysis of active author. Each author represents a node, the size of the node is proportional to the strength of the collaboration link, each line represents a co-authoring relationship, and the line thickness indicates the strength of the collaboration link. The color of a node represents the cluster it belongs to.

Simmons (162), and Alexandra Kautzky-Willer (160) (Figure 5). These authors are leading authorities in the collaborative research domain of vitamin D and obesity.

3.5 Core journals in vitamin D and obesity research

Studies on the relationship between vitamin D and obesity have been published in 1,551 journals, and the five journals with the largest number of literature in this field are Nutrients (382 articles), Obesity Surgery (204), PLOS ONE (99), Journal of Clinical Endocrinology and Metabolism (82), and American Journal of Clinical Nutrition (70) (Table 4). These publications account for approximately 20.64% of all the papers on this topic. Although PLOS ONE, Frontiers in Endocrinology, and International Journal of Molecular Sciences have published a large number of articles, these articles have a relatively low TLCS in the local literature on vitamin D and obesity. It is worth noting that although American Journal of Clinical Nutrition (67 articles) ranks only fifth in the number of articles contributed, it is the highest journal in TLCS, with 2,436 TLCS, followed by Obesity Surgery (2,050) and the Journal of Clinical Endocrinology and Metabolism (2,004) (Supplementary Table S3). The top ten journals with the highest TLCS account for approximately 38.18% of the overall TLCS, underscoring their significant influence and demonstrating their pivotal role in the research of vitamin D and obesity. The co-citation analysis encompassed 299 journals each cited over 200 times. The American Journal of Clinical Nutrition (Total Link Strength = 663,815), The Journal of Clinical Endocrinology and Metabolism (540,670), Nutrients (337,032), PLOS ONE (320,538), and The New England Journal of Medicine (308,467) were the most frequently co-cited with other journals (Figure 6).

3.6 Keywords analysis in vitamin D and obesity research

A total of 341 keywords (set as author keywords) were identified as having occurred more than ten times. According to the clustering,

TABLE 4 Analysis of top 10 productive journal.

Rank	Journal	Recs	TLCS	TGCS	Impact factor (2022–2023)	H Index
1	Nutrients	382	239	7,435	5.9	75
2	Obesity Surgery	204	2,050	7,036	2.9	128
3	PLOS ONE	99	0	3,635	3.7	268
4	Journal of Clinical Endocrinology and Metabolism	82	2,004	6,903	5.8	328
5	American Journal of Clinical Nutrition	70	2,436	8,948	7.1	307
6	British Journal of Nutrition	62	398	2,280	3.6	166
7	Frontiers in Endocrinology	61	0	682	5.2	51
8	International Journal of Molecular Sciences	60	21	1,370	5.6	114
9	Journal of Steroid Biochemistry and Molecular Biology	57	507	1,512	4.1	116
10	European Journal of Clinical Nutrition	56	321	1,774	4.7	141

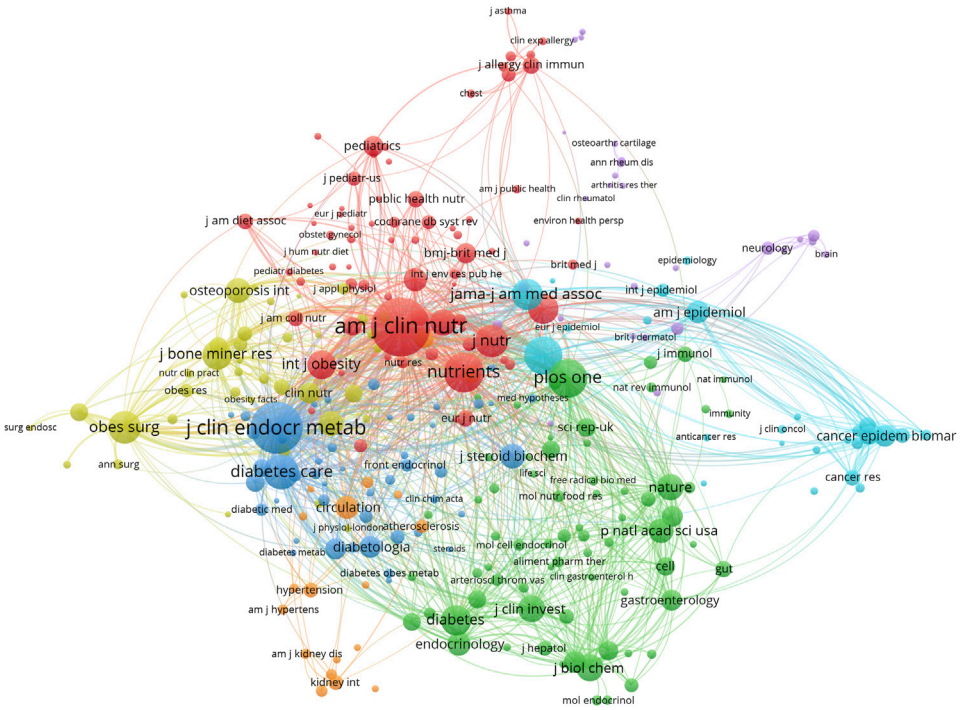

FIGURE 6
Analysis of core journals. Each node represents a journal and each row represents a co-citation relationship. The node size is proportional to the strength of the co-referenced link, and the node color reflects the cluster it belongs to.

the research topics in vitamin D and obesity can be roughly divided into four parts: metabolic health and disorders, nutrition and dietary health, metabolic and immune health, and bariatric surgery and related nutritional issues (Figure 7A). Additionally, on the timeline view, it was found that "stunting", "COVID-19", and "biomarker" are recent research trends (Figure 7B). We also conducted keyword burst detection and extracted 68 keywords with high burst intensity. The top 20 keywords are shown in Figure 8. The evolution of research topics in the field encompassing vitamin D and obesity is outlined by citation bursts, illustrating a shift in focus over the years. In the period from 2000 to 2010, initial studies focused on the association between dietary calcium and morbid obesity, and began to investigate the relationship between blood pressure and obesity. Additionally, the surgical approach of biliopancreatic diversion was explored as a treatment for severe obesity. There was also an increasing focus on issues of obesity related to secondary hyperparathyroidism, as well as studies centered on body weight, parathyroid hormone, and hypovitaminosis D. In the years spanning 2010 to 2017, research focus shifted to the impact of insulin sensitivity and vitamin D insufficiency on the risk of

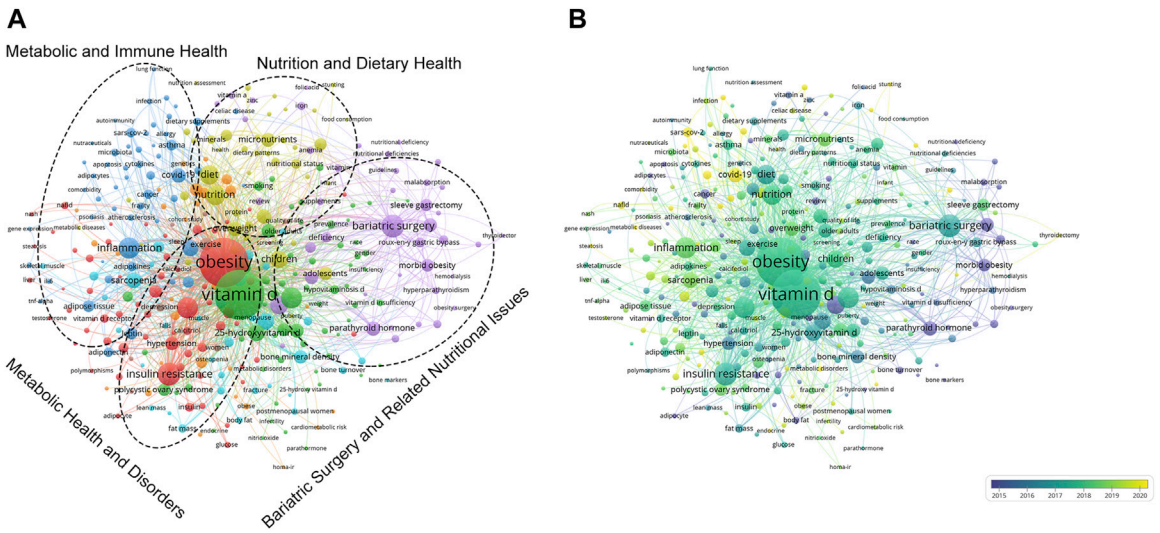

FIGURE 7
Visualization map of keywords. **(A)** Divide keywords into four different clusters by topic. **(B)** Dynamics and trends of the keywords.

Keywords	Year	Strength	Begin	End	2000 - 2023
dietary calcium	2001	36.7	**2001**	2011	
morbid obesity	2002	40.14	**2002**	2011	
blood pressure	2001	18.32	**2002**	2013	
biliopancreatic diversion	2002	15.91	**2002**	2009	
secondary hyperparathyroidism	2004	33.45	**2004**	2011	
body weight	2004	22.08	**2004**	2011	
parathyroid hormone	2000	20.07	**2004**	2013	
hypovitaminosis d	2005	37.88	**2005**	2013	
united states	2000	22.85	**2008**	2013	
insulin sensitivity	2010	24.42	**2010**	2017	
d insufficiency	2005	24	**2010**	2017	
coronary heart disease	2003	16.17	**2010**	2013	
adiposity	2004	19.31	**2012**	2015	
sensitivity	2014	12.76	**2014**	2015	
oxidative stress	2016	17.16	**2018**	2023	
skeletal muscle	2018	14.58	**2018**	2019	
polycystic ovary syndrome	2018	13.7	**2018**	2019	
sleeve gastrectomy	2020	20.25	**2020**	2023	
mortality	2007	20.02	**2020**	2023	
type 2 diabetes mellitus	2010	15.69	**2020**	2021	

FIGURE 8
Top 20 keywords with the strongest citation bursts.

coronary heart disease, and the study of the relationship between vitamin D insufficiency and obesity. Molecular insights gained prominence in the years 2018–2019, as the direction of research gravitated more towards the cellular and molecular levels with the inclusion of oxidative stress, skeletal muscle, and polycystic ovary syndrome, illustrating a shift from macro-level studies to exploring underlying micro-mechanisms. In the current stage from 2020 to 2023, novel therapeutic and pathophysiological research methods emerged, such as the adoption of sleeve gastrectomy in weight reduction surgeries. Simultaneously, the long-term effects of mortality and diabetes mellitus continued to be scrutinized. The research field of vitamin D and obesity has transitioned from early dietary concerns related to obesity to in-depth explorations of treatment strategies, cellular mechanisms, and the long-term impacts on health.

3.7 Analysis of highly cited articles in vitamin D and obesity research

The top 20 publications with the highest TLCS are presented in Table 5. In addition to two reviews, the rest are research articles, most of which focus on the study of obesity and vitamin D levels in the body. Among of them, seven articles are related to the

TABLE 5 Top 20 publications with the highest TLCS.

Rank	First author	Journal	Year	TLCS	TGCS
1	J Wortsman	The American Journal of Clinical Nutrition	2000	1,042	2,258
2	Shamik J Parikh	The Journal of Clinical Endocrinology and Metabolism	2004	266	479
3	Andjela T Drincic	Obesity	2012	256	426
4	Marieke B Snijder	The Journal of Clinical Endocrinology and Metabolism	2005	231	523
5	Sonia Arunabh	The Journal of Clinical Endocrinology and Metabolism	2003	228	470
6	Susan Cheng	Diabetes	2010	204	370
7	C P Earthman	The International Journal of Obesity	2012	195	305
8	Ramin Alemzadeh	Clinical and Experimental	2008	167	317
9	Armin Zittermann	The American Journal of Clinical Nutrition	2009	141	412
10	Miriam Blum	International Journal of Endocrinology and Metabolism	2008	139	296
11	Simon Vanlint	Nutrients	2013	134	250
12	Cherlyn Ding	The British Journal of Nutrition	2012	131	226
13	M F McCarty	Medical Hypotheses	2003	123	216
14	L Wamberg	The International Journal of Obesity	2013	115	166
15	Jared P Reis	Pediatrics	2009	114	267
16	Christy B Turer	Pediatrics	2013	114	198
17	Anthony M Belenchia	The American Journal of Clinical Nutrition	2013	109	210
18	Kari E Wong	Endocrinology and Metabolism	2009	105	194
19	Kari E Wong	The Journal of Biological Chemistry	2011	103	140
20	Carmen J Narvaez	Endocrinology	2009	102	177

relationship between obesity and vitamin D levels, four articles are about the treatment of obesity with vitamin D supplement, four articles are about the role of vitamin D in adipose tissue, and three articles are about vitamin D receptor (VDR) research. The most cited article is the one published by Jacobo Wortsman et al. in the American Journal of Clinical Nutrition (Wortsman et al., 2000), which found that the bioavailability of vitamin D_3 in obese individuals is reduced, possibly due to its deposition in adipose tissue, leading to a higher prevalence of vitamin D deficiency in obese individuals. The article also suggests that the vitamin D supplement dose for obese individuals may need to be larger (Wortsman et al., 2000). This article has laid the foundation for the study of vitamin D and obesity, and serves as a pioneer in this research field.

3.8 Analysis of publications in vitamin D and obesity research

We conducted a citation burst detection from 2000 to 2023, identifying the top 20 references with the strongest citation bursts as shown in Figure 9. Most of the publications are research articles, accompanied by 5 reviews. From 2004 to 2009, influential articles by Parikh et al., Snijder et al., and Alemzadeh et al. predominantly discussed the association between vitamin D levels and obesity (Parikh et al., 2004; Snijder et al., 2005; Alemzadeh et al., 2008), as well as the impact of dietary calcium supplementation on obesity. Building upon these studies, work by Wang, T. J. et al. and Cheng et al. suggested links between vitamin D and cardiovascular diseases as well as metabolic disorders (Wang et al., 2008; Cheng et al., 2010). Besides, investigations by Bischoff-Ferrari et al. and Ross et al. provided insights on optimal serum levels of vitamin D and recommended dietary intakes (Bischoff-Ferrari et al., 2006; Ross et al., 2011). Between 2010 and 2016, the causal relationship between obesity and vitamin D deficiency was further investigated by Drincic et al., Pereira-Santos et al., and Vimaleswaran et al. (Drincic et al., 2012; Vimaleswaran et al., 2013; Pereira-Santos et al., 2015). From 2017 to 2020, Cruz-Jentoft et al. discussed the significance of vitamin D among the elderly and patients with sarcopenia (Cruz-Jentoft et al., 2019). Research by Manson et al. and Pittas et al. explored the potential roles of vitamin D supplementation in the prevention of cancer and type 2 diabetes, respectively (Manson et al., 2019; Pittas et al., 2019).

3.9 The evolving trends of research on vitamin D and obesity

In 2000, Wortsman et al. uncovered lower baseline 25(OH)D levels in obese individuals, hinting at a possible link between obesity and vitamin D (Wortsman et al., 2000). Subsequently, in 2001, Shi et al. reported the impact of $1,25(OH)_2D$ on calcium ion signaling in

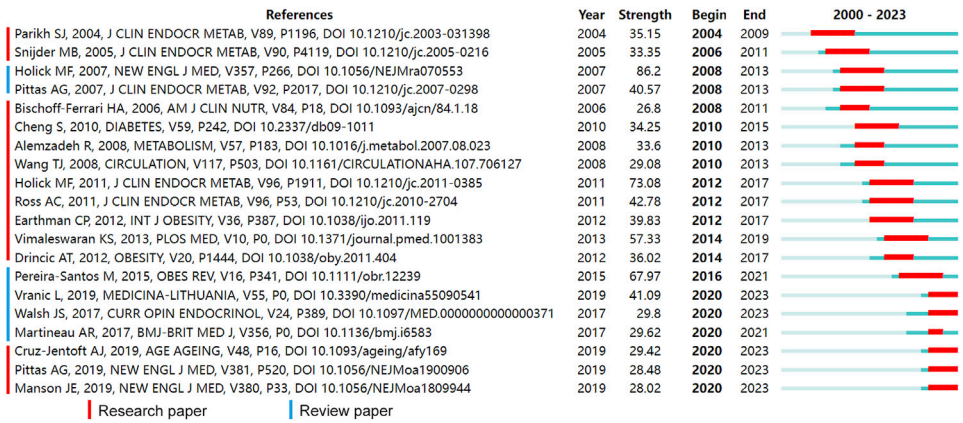

FIGURE 9
Top 20 references with the strongest citation bursts.

adipocytes (Shi et al., 2001), which governs their lipogenesis and lipolysis. Furthermore, the nongenomic pathway mediated by VDR may represent a crucial target for the development of obesity treatment interventions. However, the precise mechanisms remained elusive for years, sparking a surge in interest in understanding the relationship between vitamin D and obesity, leading to an expanding body of research. Findings from Arunabh et al., Parikh et al., and Snijder et al. further supported the negative correlation between vitamin D levels and body weight across different demographic groups (Arunabh et al., 2003; Parikh et al., 2004; Snijder et al., 2005). In 2006, Hyppönen and Power suggested that vitamin D status might impact glucose metabolism, potentially contingent on body size, paving new paths for investigation (Hyppönen and Power, 2006). By 2008, Sneve et al. noted that supplementing cholecalciferol in individuals did not significantly reduce weight among overweight or obese subjects (Sneve et al., 2008). This study strongly suggested a limited association between vitamin D supplementation and weight reduction but hinted at a potential preventive role. In the same year, multiple studies unveiled a close link between vitamin D deficiency, abnormalities in glucose metabolism, and obesity, particularly in children and adolescents (Alemzadeh et al., 2008; Goldner et al., 2008). This raised awareness regarding vitamin D's potential role in metabolic diseases. In 2009, several studies delved into VDR functionality within adipose tissue and its impact on energy metabolism and inflammation, shedding further light on the connection between vitamin D and obesity (Narvaez et al., 2009; Wong et al., 2009; Zittermann et al., 2009). From 2010 to 2013, researchers further explored the intricate relationship between vitamin D and obesity alongside related metabolic diseases (Cheng et al., 2010). This encompassed its effects on visceral obesity, inflammation, insulin resistance, and offered new perspectives on correcting vitamin D deficiency in the treatment of obesity and its associated metabolic abnormalities. Since 2014, vitamin D deficiency has been observed more frequently in obese individuals, suggesting a connection between vitamin D levels and obesity (Pereira-Santos et al., 2015; Walsh et al., 2016). However, the causal relationship remains unclear. Vitamin D supplementation appears to impact obesity-related factors such as blood pressure, glucose levels, and insulin resistance (Mousa et al., 2017). Mechanistically, vitamin D is implicated in regulating fat synthesis, adipocyte differentiation, and energy expenditure (Roth et al., 2018). Obese individuals often exhibit lower serum vitamin D levels, potentially due to factors like vitamin D storage in adipose tissue and impaired hepatic hydroxylation processes (Roizen et al., 2019). In summary, while vitamin D deficiency may contribute to obesity, and *vice versa*, the intricate mechanisms underlying this relationship warrant further investigation.

4 Discussion

This study conducted a bibliometric analysis to clarify the collaborative networks, research trends, and hot topics in the field of vitamin D and obesity. The findings revealed that this field of study has garnered extensive global attention, with a total of 6,144 records retrieved, involving 123 countries, 6,726 institutions, and 28,156 authors, published in 1,551 journals. From the research results, while the initial publications in the field of vitamin D and obesity were not substantial, a pivotal study published in 2000, titled "Decreased bioavailability of vitamin D in obesity" (Wortsman et al., 2000) established a potential correlation between vitamin D deficiency and obesity. This study became an early key literature in the field and laid the foundation for subsequent research. Over the past 23 years, the annual publication volume of related literature has continued to increase, reaching a peak of 648 articles in 2022, indicating the sustained attention and importance of this field. This trend suggests that vitamin D and obesity research holds enduring academic value and social impact, and it will continue to be an area of significant academic importance in the future.

In terms of national output, the United States, China, Italy, the United Kingdom, and Australia are the top five countries in terms of publication volume in the field of vitamin D and obesity research, indicating their significant research contribution in this area. Their leading position in publication volume highlights their important influence in this field. Although Norway does not have the highest number of publications or TLCS, its average TLCS value is among

the highest, indicating that Norway's research in this field has significant academic influence. The United Kingdom exhibits a high intensity in the research collaboration network and has close collaboration with the United States, showing its advantage in scientific collaboration. However, although China has a large number of publications, its total link strength in the global collaboration network is relatively low, primarily collaborating with the United States, while its collaboration links with other countries are relatively weak, indicating that China's global collaborative influence in the field of vitamin D and obesity needs to be further enhanced.

The study also found that among the institutions with the highest publication volume in the field of vitamin D and obesity research, the United States accounts for three of them: Harvard University, Harvard Medical School, and Brigham and Women's Hospital, indicating the significant research advantage of American institutions in this field. In addition, universities such as Copenhagen University, Medical University of Vienna, Medical University of Poznan, University of Padua, and Medical University of Graz have shown outstanding performance in research collaboration. Moreover, in the institutional collaboration network, research institutions in Europe and the United States have formed two significant clusters, indicating that American and European institutions are at the forefront of research in the field of vitamin D and obesity. Nasser M. Al-Daghri from King Saud University is the author with the highest number of publications in this field, reflecting his significant influence in this area. His research focuses mainly on the relationship between vitamin D deficiency and cardiometabolic health, as well as the connection between vitamin D and metabolic health, making significant contributions to these fields. Michael F. Holick from Boston University is the author with the highest TLCS, and "Decreased bioavailability of vitamin D in obesity" is his representative work, for which he is the corresponding author. The TLCS of the authors related to this article are all relatively high, which shows the influence of this article in this field.

Research on vitamin D and obesity is mainly published in journals such as "Nutrients", "Obesity Surgery", "PLOS ONE", "Journal of Clinical Endocrinology and Metabolology", and "The American Journal of Clinical Nutrition". These journals cover a wide range of research directions including nutrition, endocrinology, epidemiology, and surgical treatment, providing a comprehensive perspective and in-depth analysis for the field of vitamin D and obesity. Among them, "The American Journal of Clinical Nutrition" holds a central position in this study with the highest collaboration relationships and total link strength, indicating its high professional reference value. It focuses mainly on research in the fields of nutrition and dietetics, publishing the latest studies on nutrition, nutrition and disease, and energy metabolism.

Through keyword cluster analysis, the research topics on vitamin D and obesity can be roughly divided into four parts: metabolic health and disorders, nutrition and dietary health, metabolic and immune health, and bariatric surgery and related nutritional issues, indicating the central role of metabolism and nutrition in vitamin D and obesity research. From the keyword bursts, it can be seen that early research focused on the association between "dietary calcium" and "morbid obesity", as well as the physiological processes centered around "blood pressure" and "parathyroid hormone". Subsequently, the research focus shifted to "insulin sensitivity", with an increasing emphasis on cellular and molecular levels, covering areas such as "oxidative stress", "skeletal muscle", and "polycystic ovary syndrome", as well as the adoption of "pathophysiological" research methods. This shift marks a transition from observing macroscopic phenomena to delving into underlying microscopic mechanisms. Meanwhile, "type 2 diabetes", a common complication of obesity, has continued to receive widespread research attention.

According to citation bursts, early research on vitamin D primarily focused on the correlation between vitamin D levels and obesity, as well as the impact of dietary calcium supplementation on obesity. Over time, mid-term research began to delve deeper into the causal relationship between obesity and vitamin D deficiency, and studied the role of vitamin D in preventing acute respiratory infections and enhancing immune system function. In the later stages, the research scope expanded further, focusing on the importance of vitamin D in the elderly and patients with muscle wasting syndrome, as well as the potential role of vitamin D supplementation in preventing cancer and type 2 diabetes. Overall, vitamin D research has shifted from basic associative studies to more applied prevention and treatment strategies.

In summary, the field of vitamin D and obesity research continues to attract attention and has become a hot research area. The establishment of research collaboration networks, the enhancement of scientific research strength, and diverse research directions have provided a solid foundation for the development of this field. Future research should continue to deepen our understanding of the relationship between vitamin D and obesity, and explore effective prevention and intervention strategies.

5 Limitations

Our study has several limitations. Firstly, our focus on vitamin D and obesity means that our analysis is based on the Web of Science Core Collection and includes only English articles and reviews related to this topic, which may result in selection bias. Secondly, the results from VOSviewer and CiteSpace are based on machine algorithms, which may introduce algorithmic bias. Lastly, the assessment of research progress relies primarily on the HistCite tool, which may not have captured all potential advancements and trends, thus there is a certain degree of informational bias.

6 Conclusion

The regulatory role of vitamin D in adipocyte differentiation, fat storage, and metabolism is pivotal in understanding its connection to obesity. Vitamin D exerts multifaceted regulation within the human body, impacting intricate mechanisms such as insulin resistance, immune response, and inflammatory pathways, which are crucial in studying the relationship between vitamin D and obesity. The influence of vitamin D supplementation on obesity and its associated metabolic disorders continues to be a significant area of investigation within the scientific community. Employing bibliometric analysis to survey the scholarly literature can

provide researchers with valuable insights into the collaborative networks, key research focal points, and emerging trends within this field of study.

Author contributions

XS: Visualization, Methodology, Data curation, Writing–review and editing, Writing–original draft. SQ: Writing–review and editing, Writing–original draft, Visualization, Methodology, Data curation. SC: Data curation, Writing–review and editing, Writing–original draft. CZ: Software, Investigation, Conceptualization, Writing–review and editing, Writing–original draft. LL: Writing–review and editing, Writing–original draft, Supervision. ZS: Writing–review and editing, Writing–original draft, Supervision, Project administration, Funding acquisition.

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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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Supplementary material

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The heat of longevity: sex differences in lifespan and body temperature

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Dietary restriction (DR) has long been recognized as a powerful intervention for extending lifespan and improving metabolic health across species. In laboratory animals, DR—typically a 30%–40% reduction in caloric intake—delays aging and enhances mitochondrial function, oxidative defense, and anti-inflammatory pathways. In humans, findings from the CALERIE™ trial confirm DR's potential benefits, with a 25% caloric reduction over 2 years resulting in reduced visceral fat, improved cardiometabolic health, and favorable gene expression changes linked to proteostasis, DNA repair, and inflammation. However, recent research in genetically diverse mouse populations reveals that the impact of DR on lifespan is substantially modulated by genetic background, underscoring the importance of individual variability. Additionally, emerging evidence challenges previous assumptions that lower body temperature universally benefits lifespan extension, with data indicating complex relationships between thermoregulation, sex, and longevity. These findings underscore the need for nuanced approaches to DR in both research and potential therapeutic applications, with considerations for genetic and sex-specific factors to maximize healthspan and lifespan outcomes.

KEYWORDS

dietary restriction, lifespan, genetic variability, sex differences, thermoregulation, mitochondrial biogenesis, ageing markers, age-related disease

Introduction

Laboratory mice and rats are typically maintained on *ad libitum* (AL) feeding regimen, allowing free access to food and water. In contrast, dietary restriction (DR)—a reduction of caloric intake by 30%–40% from the average AL intake starting around sexual maturity—has been shown to significantly extend lifespan, making it a widely recognized geroprotective intervention (Green et al., 2022). Since McCay et al. (1935) first reported DR's lifespan-extending effects in rats, DR has become a standard model for longevity research, emphasizing reduced caloric intake rather than nutrient restriction. While restricting single nutrients alone does not reproduce the lifespan extension of DR, limiting proteins, branched-chain amino acids, or specific other amino acids (e.g., methionine, isoleucine, threonine, or tryptophan) without reducing total calories has independently shown positive effects on lifespan in rodents [reviewed by Mihaylova et al. (2023)].

Despite DR's efficacy, adherence to such regimens remains challenging, prompting investigations into the physiological and molecular mechanisms mediating its effects. Proposed mechanisms include enhanced mitochondrial biogenesis (Nisoli et al., 2005), improved oxidative stress defenses, adaptive anti-inflammatory responses, and reduced

cellular senescence, although a complete mechanistic picture remains elusive (Green et al., 2022). A complicating factor in DR studies is the substantial difference in feeding patterns between DR and AL groups: DR-fed mice consume their daily ration within ~2 h, leading to prolonged fasting phases (Acosta-Rodríguez et al., 2017). Recent findings indicate that this prolonged fasting itself is critical for DR's metabolic and geroprotective effects, as similar benefits can be achieved with a prolonged fasting period alone, even without calorie reduction (Pak et al., 2021). This distinct feeding behavior, however, complicates comparisons between DR- and AL-fed animals, as metabolic and physiological responses vary with fasting duration. Further research by Pak and colleagues reveals that DR-associated improvements in insulin sensitivity, adiposity, metabolite profiles, and tissue-specific regulation of mechanistic target of rapamycin 1 (mTORC1) activity are heavily influenced by fasting duration and tissue context, rather than by DR alone (Pak et al., 2024). Thus, fasting duration appears critical to understanding DR's physiological impacts, suggesting that temporal factors (e.g., time-restricted feeding) may be as crucial as caloric reduction in DR regimens.

Recent studies suggest that DR may also attenuate cellular senescence markers in humans (Aversa et al., 2024). The Comprehensive Assessment of Long-term Effects of Reducing Intake of Energy (CALERIE™) trial has demonstrated that a 25% reduction in caloric intake over 2 years in young and middle-aged individuals without obesity led to sustained weight loss, reduced visceral fat, with modest muscle loss, and improvements in cardiometabolic health and blood pressure, all without compromising quality of life (Shen et al., 2021; Kraus et al., 2019). Through linear mixed-effect models, researchers observed DR-associated shifts in gene expression related to proteostasis, circadian rhythm, DNA repair, mitochondrial function, apoptosis, and inflammation (Das et al., 2023). Collectively, these findings indicate that DR may help reduce age-related risk factors also in humans.

Genetics matters more than diet for lifespan

In a recent issue of Nature, Di Francesco and colleagues investigated the effects of DR and intermittent fasting (IF) on health and lifespan using a genetically diverse cohort of 960 female mice (Di Francesco et al., 2024). The study explored two levels of DR (20% and 40%) and IF (1 or 2 days of fasting per week). While all interventions extended lifespan, only DR increased the mortality doubling time, and the ability to maintain body weight during handling emerged as the strongest predictor of lifespan. Other predictors included changes in immune cells, red blood cell distribution width, and retention of adipose tissue later in life. Lifespan was heritable (h^2 = 0.24), with genetic background having a greater influence than dietary interventions. Previous research has highlighted physiological adaptations to DR in both rodents and humans—such as improved glucose homeostasis, lower energy expenditure, decreased body temperature, and preserved metabolic flexibility—as potential mechanisms for lifespan extension (Redman and Ravussin, 2011; Guijas et al., 2020). However, Di Francesco and colleagues found no significant associations between lifespan and fasting glucose, energy expenditure, or metabolic flexibility. Surprisingly, higher body temperature, contrary to expectations, was moderately linked to increased lifespan. These findings suggest that while dietary restriction induces significant metabolic changes, their relevance to lifespan extension may be limited, indicating the need to explore alternative biomarkers of aging.

The exclusive use of female mice in this study raises critical questions about the generalizability of the findings. Although it is common practice to use female mice to avoid the aggressive behavior often observed in cohoused male mice, especially in resource-limited experimental groups like those undergoing DR (Behnke and Sewell, 1994), this approach neglects potential sex-specific responses to dietary interventions. Sexual dimorphism is a well-known factor influencing lifespan and metabolic traits, with numerous studies reporting distinct physiological responses between males and females (Austad and Fischer, 2016). For instance, females generally have a longer lifespan than males across species, and hormonal differences, particularly the protective effects of estrogen, play a significant role in modulating metabolic pathways and stress resilience (Austad, 2006; Kane et al., 2018). Studies have also shown that DR and IF can elicit divergent effects on males and females, including differences in body weight regulation, fat distribution, and insulin sensitivity (Redman and Ravussin, 2011).

More specifically, Sanchez-Alavez et al. (2011) demonstrated that differences in core body temperature (Tc) contribute to the sex-specific longevity observed in C57BL76J mice. In young females, Tc was influenced by the estrous cycle but was overall higher than in males; this difference became more pronounced in older mice, where age eliminated the estrous-related variations. Interestingly, while Tc homeostasis is centrally regulated by the sexually dimorphic hypothalamic preoptic area, these differences were dependent on the gonads. These results may explain, at least in part, the linkage observed by Di Francesco et al. (2024) between higher body temperature and increased lifespan, challenging the previous observations that body temperature is beneficial for lifespan extension (Conti et al., 2006; Zhao et al., 2022).

It highlights that the reliance on single-sex cohorts—whether male or female—limits the ability to fully understand the biological complexity underlying responses to dietary restriction and related interventions. Incorporating both sexes to capture the influence of sexual dimorphism on lifespan and healthspan outcomes will improve the translational relevance of these findings to human populations, where sex differences in aging are well documented.

Conclusion

This commentary underscores the complexity of DR and its interplay with genetic and sex-specific factors in shaping lifespan. Di Francesco et al. (2024) study highlights that while DR extends lifespan, genetic diversity exerts an even greater influence. Their findings on the role of body temperature challenge conventional views, suggesting that higher, rather than lower, body temperature could be advantageous for lifespan. Moving forward, incorporating both sexes in future DR studies is essential to capture the full scope of biological variability in lifespan and healthspan, with significant implications for translational studies targeting human aging. Additionally, this work emphasizes that temporal aspects—such as fasting duration—must be considered in DR regimens, as they may prove as impactful as calorie reduction itself. These insights

pave the way for more personalized dietary interventions that account for individual genetic and biological variability, optimizing healthspan and potentially longevity.

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

CR: Data curation, Formal Analysis, Writing–review and editing. MR: Data curation, Formal Analysis, Writing–review and editing. EN: Conceptualization, Data curation, Formal Analysis, Supervision, Writing–original draft, Writing–review and editing.

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Interplay of serum taurine, S-adenosylmethionine, and cysteine levels in cancer risk: a prospective study

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Background: Amino acids are known to play critical roles in cancer metabolism and progression. Among them, taurine, S-adenosylmethionine (SAM), and cysteine have garnered particular attention due to their interconnected metabolic pathways. This study sought to explore the associations between serum levels of these amino acids and cancer risk within Chinese adults.

Methods: A nested case-control study was conducted within the China H-Type Hypertension Registry Study cohort, comprising 1,391 cancer cases and 1,391 matched controls. Serum concentrations of taurine, SAM, and cysteine were quantified, and their associations with cancer risk were evaluated using conditional logistic regression and Bayesian Kernel Machine Regression (BKMR) models.

Results: A total of 1,391 pairs of participants were included in this study. Their average age was 69.3 years ± 7.77 years, and 56% were male. Higher serum taurine levels were associated with a reduced risk of overall cancer. In contrast, elevated serum SAM levels were linked to an increased risk of digestive cancers. The BKMR model identified complex interactions among these amino acids and showed a significant overall negative association between the combined effect of taurine, SAM, and cysteine and cancer risk.

Conclusion: Serum taurine levels may offer protective benefits against cancer, particularly for digestive cancers, while its metabolites do not have such significant benefits. The intricate interactions among taurine, SAM, and cysteine underscore the need for a comprehensive approach to understanding their roles in the metabolic processes that drive tumorigenesis.

Clinical Trial Registration: https://www.chictr.org.cn/showproj.html?proj=28262, identifier ChiCTR1800017274.

KEYWORDS

taurine, S-adenosylmethionine, cysteine, cancer, cohort

Introduction

Cancer is a leading cause of morbidity and mortality worldwide, characterized by the uncontrolled proliferation and spread of abnormal cells. Extensive research on cancer incidence and its associated mortality reveals significant public health implications (Siegel et al., 2023). As the global population continues to grow and age, it is anticipated that cancer incidence will increase, further highlighting the urgent need for effective prevention, early detection, and treatment strategies (Mistry et al., 2011). A critical aspect of cancer biology is the metabolic reprogramming that enables cancer cells to sustain their rapid growth and survival. Among the various metabolic pathways involved, non-essential amino acid metabolism is particularly crucial for tumor progression. For example, glutamine, serine, and arginine, though non-essential under normal conditions, are vital for supporting the high proliferation rates of cancer cells (Zhang et al., 2017; Liu T. et al., 2022; Liu et al., 2023). Dysregulations in glucose, fatty acid, and amino acid metabolism pathways are hallmarks of cancer, underscoring the complex relationship between metabolic shifts and tumorigenesis. Gaining a deeper understanding of these metabolic alterations can identify potential therapeutic targets and strategies for combating the disease.

Taurine, a sulfur-containing amino acid, is unique in that it exists in free form in many tissues, particularly in the nervous system, retina, and muscle, rather than being incorporated into proteins. It serves diverse physiological functions, such as acting as a neurotransmitter, regulating calcium homeostasis, and maintaining cell membrane stability (Ping et al., 2023). From a health perspective, taurine is well-known for its antioxidant properties, protecting cells from damage by neutralizing free radicals. It also plays a key role in bile acid conjugation in the liver, thereby aiding in fat digestion and absorption. Beyond these functions, taurine has been linked to cardiovascular health, with evidence suggesting its potential in lowering blood pressure and reducing inflammation (Ridlon et al., 2016). Recent studies have begun to explore taurine's role in cancer biology. For instance, taurine has been shown to enhance the antitumor efficacy of certain therapies by promoting $CD8^+$ T cell function (Ping et al., 2023). However, other studies suggest that taurine metabolism may be involved in cancer progression, indicating that taurine might play a dual role in cancer, depending on the context (Cai et al., 2017). These findings highlight the need for further research to delineate taurine's specific role and therapeutic potential in cancer.

Taurine, S-adenosylmethionine (SAM), and cysteine are intricately connected through amino acid metabolism. SAM, a universal methyl donor, is involved in numerous biochemical reactions, including the transsulfuration pathway, which leads to cysteine synthesis. Cysteine, in turn, can be converted into several important compounds, including taurine (Brosnan and Brosnan, 2006). This metabolic interrelationship suggests that these amino acids collectively influence various physiological processes. Therefore, analyzing the impact of taurine on cancer risk without accounting for SAM and cysteine levels could result in incomplete or misleading conclusions. Given their complex interactions, a comprehensive analytical approach is essential. In our study, we utilized the Bayesian Kernel Machine Regression (BKMR) model, a nonparametric regression method based on Bayesian statistics, to prospectively evaluate the combined effects of serum taurine and its related amino acids on cancer risk.

Materials and methods

Study populations

The participants in this nested case-control research were derived from the China H-Type Hypertension Registry Study (CHHRS; URL: https://www.chictr.org.cn/showproj.html?proj=28262; unique identifier: ChiCTR1800017274). The CHHRS was a real-world observational registry study primarily conducted in Rongcheng city, Shandong Province, China. Running from July 2018 to July 2021, it involved 87,492 participants. Eligible individuals were adults aged 18 and above who were diagnosed with hypertension (defined as SBP ≥ 140 mmHg and/or DBP ≥ 90 mmHg) and had no history of cancer at the time of screening. This study encompassed two main phases: recruitment and a three-year observational follow-up. Recruitment was conducted based on the following inclusion and exclusion criteria. The inclusion criteria were: individuals aged over 18 years, local permanent residents who could cooperate with the required examinations and follow-up, and those who agreed to the study design and signed the informed consent form. The exclusion criteria included: individuals with psychological or nervous system impairments that prevented them from demonstrating informed consent, those unable to comply with follow-up according to the study protocol or planning to relocate in the near future, pregnant individuals, and those in poor health conditions that rendered them unable to cooperate with the investigation and subsequent studies. Follow-ups were organized every 3 months to gather measurement data. These sessions documented parameters such as blood pressure, medication use, and outcomes like cardiovascular disease, cancer, and all-cause mortality.

Outcome ascertainment

Between 2018 and 2021, cancer cases were identified based on specific clinical criteria. These criteria included surgical records, imaging outcomes, serum tumor markers, and confirmed histopathology data from hospitals treating cancer patients. In the absence of pathological data, two oncologists reviewed potential cases. Both oncologists were required to reach a consensus on the cancer diagnosis, using the International Classification of Diseases, 10th Revision (ICD-10) for coding.

Nested case-control study design

During the CHHRS follow-up in Rongcheng District, 1,419 new cancer cases were identified among 87,492 participants. An equal number of controls (1,419) were selected from participants who remained cancer-free and alive during the follow-up period (2018–2021). These controls were matched with the cases at a 1: 1 ratio based on age, gender, and residency. After excluding individuals without complete data on taurine, SAM, and cysteine,

as well as unmatched participants, the final analysis included 1,391 incident cancer cases and their corresponding controls, as detailed in Supplementary Figure 1. The Institute of Biomedicine, Anhui Medical University in Hefei, China, approved both the CHHRS and the present study. All participants provided written informed consent prior to their participation.

Exposure and covariates

Data on participants' socioeconomic status, lifestyle habits, medical history, and family medical background were collected through a standardized survey. Height, weight, waist, and hip circumference were measured by trained medical personnel. Venous blood samples were obtained after an overnight fast and collected in EDTA-containing vacuum tubes. Serum concentrations of taurine, SAM, and cysteine were measured using liquid chromatography with tandem quadrupole mass spectrometry (LC-MS/MS) at the Beijing DIAN Medical Laboratory. The specific procedure involved the following steps: first, the samples were prepared by appropriate extraction and purification methods to isolate the target metabolites. Then, the LC-MS/MS system was calibrated with standard solutions of known concentrations of taurine, SAM, and cysteine to ensure accurate quantification. The chromatographic separation was achieved using a specific column and mobile phase conditions optimized for the separation of these metabolites. Mass spectrometry detection was performed in the tandem quadrupole mode, with specific mass-to-charge ratios set for each metabolite to identify and quantify them precisely. Biochemical indicators, including alanine aminotransferase (ALT), triglycerides (TG), total cholesterol (TC), albumin (ALB), high-density lipoprotein cholesterol (HDL-C), fasting blood glucose (FBG), uric acid (UA), and creatinine, were assessed at the Shenzhen Tailored Medical Laboratory using automated clinical analyzers (Beckman Coulter).

Statistical analysis

Normally distributed variables were presented as mean ± standard deviation (SD), skewed variables as median (interquartile range), and categorical variables as counts and percentages (n, %). Comparisons between cases and controls were conducted using paired Student's t-tests, nonparametric Kruskal-Wallis tests, or chi-square tests, depending on the distribution of the variables. The dose-response relationships between serum levels of taurine, SAM, and cysteine and the risk of overall, digestive, and non-digestive cancers were evaluated using restricted cubic spline regression (RCS). Conditional logistic regression models were employed to estimate odds ratios (ORs) for cancer incidence associated with continuous and quartile-based serum concentrations of taurine, SAM, and cysteine. The boundaries of the quartiles were population-specific. For example, in the cases of digestive and non-digestive system cancers, we calculated the population-specific quartiles. The models were adjusted for potential confounders, including body mass index (BMI), smoking status, alcohol consumption, antihypertensive drug use, systolic blood pressure SBP, FBG, TC, TG, UA, creatinine, ALT, ALB, and family history of cancer.

To minimize potential reverse causation bias arising from the relatively short follow-up period, the population was categorized based on the median follow-up duration. Further stratified analyses were conducted to evaluate potential interaction effects between serum taurine, SAM, and cysteine levels and cancer risk, with participants categorized into subgroups according to age (median), sex, BMI, and smoking and drinking status. In addition, we also verified the efficacy of different variables in predicting cancer risks through machine learning algorithms and ranked their importance. The models involved include Decision Tree, Lasso Regression, Random Forest, and eXtreme Gradient Boosting (XGboost). Although this is a nested case-control study, we noticed that there were still some variables with differences between the two groups. Therefore, we also conducted propensity score matching (PSM) to verify the relationship between taurine, SAM, and cysteine and cancer risk.

Given the interrelationships among serum taurine, SAM, and cysteine, it is critical to assess whether taurine's effect on cancer risk remains consistent when considering these metabolites as a combined mixture. To this end, Bayesian Kernel Machine Regression (BKMR) was employed to evaluate the associations between serum taurine, SAM, and cysteine concentrations and cancer risk using the BKMR package in R (Wei et al., 2023). Four parallel Markov Chain Monte Carlo (MCMC) chains were initiated with unique random seeds, each containing 10,000 iterations, with the "family" argument set to "binomial" due to the binary nature of the outcome. Convergence of the BKMR model was confirmed by examining trace, autocorrelation, density, and Gelman-Rubin diagnostic plots. BKMR, which integrates Bayesian statistics with kernel regression, offers the advantage of addressing non-linearities and interactions among predictors, making it superior to traditional logistic regression that assumes linearity and requires explicit interaction terms. Statistical significance was set at a p-value of less than 0.05 for all tests. All analyses were conducted using SAS software (version 9.4) and R (version 4.2.0, https://www.r-project.org).

Results

Baseline characteristics

In our research, we examined 1,391 cancer instances, of which 543 were related to the digestive system and 848 were not. The baseline characteristics of the cancer subjects and their corresponding controls are detailed in Supplementary Tables 1, 2. Compared with controls, individuals with cancer displayed reduced ALB and taurine levels, exhibited a higher tendency to smoke, and had an increased incidence of prior coronary heart disease (CHD) and stroke events. It is noteworthy that a higher proportion of cancer patients utilized antihypertensive medications. However, there were no significant disparities in characteristics like age, gender, BMI, blood pressure, ALT, TG, TC, UA, HDL-C, FBG, creatinine, marital status (being married), educational attainment (at least high school), alcohol consumption habits, history of CKD and dyslipidemia, and familial cancer history between the two groups (all p-values > 0.05). The relationships among serum taurine, SAM, and cysteine are illustrated in Supplementary Figure 2. There exists a negative

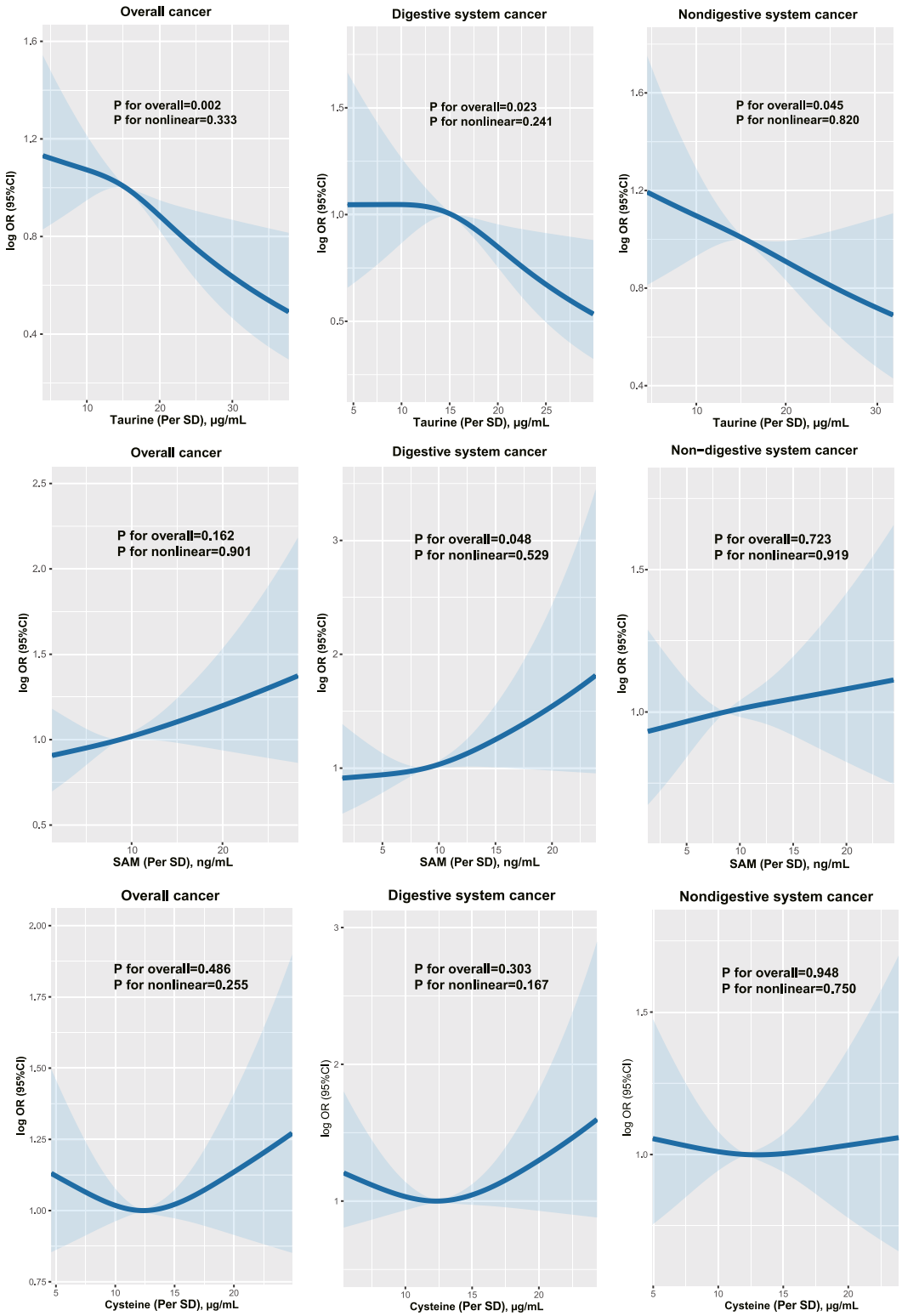

FIGURE 1
The dose-response associations of taurine, SAM and cysteine with overall, digestive and non-digestive system cancer risk.

correlation between serum taurine and SAM, and a positive correlation between serum taurine and cysteine. Additionally, serum cysteine is positively correlated with SAM. The correlation coefficients are −0.26 between serum taurine and SAM, 0.15 between serum taurine and cysteine, and 0.24 between serum cysteine and SAM.

TABLE 1 The association of taurine, SAM, and cysteine with overall cancer risk.

	Cases/controls (ratio 1:1)	Crude model		Adjusted model	
		OR (95%CI)	*p*-value	OR (95%CI)	*p*-value
Taurine (per SD)	1,391/1,391	0.86 (0.78, 0.93)	<0.001	0.84 (0.75, 0.91)	<0.001
Quartiles of taurine (μg/mL)[a]					
Q1	368/327	Ref.		Ref.	
Q2	350/344	0.89 (0.71, 1.11)	0.287	0.91 (0.71, 1.17)	0.497
Q3	341/357	0.83 (0.66, 1.03)	0.090	0.80 (0.61, 1)	0.048
Q4	332/363	0.78 (0.62, 0.98)	0.034	0.72 (0.53, 0.92)	0.007
P for trend		0.028		0.002	
SAM (per SD)	1,391/1,391	1.09 (1.00, 1.19)	0.051	1.07 (0.96, 1.19)	0.208
Quartiles of SAM (ng/mL)[b]					
Q1	351/383	Ref.		Ref.	
Q2	350/333	1.15 (0.94, 1.42)	0.411	1.20 (0.95, 1.51)	0.125
Q3	330/352	1.05 (0.84, 1.30)	0.123	1.14 (0.89, 1.46)	0.300
Q4	360/323	1.26 (1.00, 1.59)	0.050	1.27 (0.97, 1.67)	0.085
P for trend		0.107		0.124	
Cysteine (per SD)	1,391/1,391				
Quartiles of cysteine (μg/mL)[c]		1.03 (0.93, 1.13)	0.580	1.01 (0.90, 1.13)	0.747
Q1	355/340	Ref.		Ref.	
Q2	335/362	0.88 (0.70, 1.10)	0.265	0.85 (0.67, 1.09)	0.220
Q3	349/346	0.97 (0.75, 1.26)	0.831	0.97 (0.71, 1.31)	0.936
Q4	352/343	0.99 (0.76, 1.31)	0.963	0.95 (0.70, 1.30)	0.786
P for trend		0.647		0.392	

Note: Models were adjusted for taurine, S-adenosylmethionine, body mass index, smoking status, alcohol drinking, systolic blood pressure, triglycerides, cholesterol, uric acid, fasting blood glucose, high-density lipoprotein cholesterol, creatinine, albumin, alanine aminotransferase, cysteine, sleep quality, antihypertensive drug usage, and family history of cancer.
[a]Quartile for taurine: 0 < Q1<11.72 μg/mL ≤ Q2<15.34 μg/mL ≤ Q3<19.14 μg/mL ≤ Q4.
[b]Quartile for SAM: 0 < Q1<5.72 μg/mL ≤ Q2<8.69 μg/mL ≤ Q3<11.89 μg/mL ≤ Q4.
[c]Quartile for cysteine: 0 < Q1<8.40 μg/mL ≤ Q2<12.26 μg/mL ≤ Q3<15.84 μg/mL ≤ Q4.

Association of serum taurine, SAM, and cysteine with the risk of incident cancer

Figure 1 shows the dose-response associations of serum taurine (per SD), SAM (per SD), and cysteine (per SD) with the incidence of overall, digestive system, and non-digestive system cancers. Serum taurine is negatively associated with the risk of overall, digestive system, and non-digestive system cancers. Serum SAM is positively associated with the risk of digestive system cancers. Serum cysteine is not significantly associated with the risk of cancer incidence. The associations of serum taurine, SAM, and cysteine levels with cancer risk are presented in Tables 1, 2. After adjusting for other covariates, a continuous increase in serum taurine levels (per SD) was associated with a 16% reduction in the overall risk of cancer (OR = 0.84, 95% CI = 0.75–0.91), a 17% reduction in the risk of digestive system cancers (OR = 0.83, 95% CI = 0.70–0.96), and a 15% reduction in the risk of non-digestive system cancers (OR = 0.85, 95% CI = 0.75–0.96). However, when taurine levels were categorized into quartiles, the highest quartile (Q4) compared to the lowest quartile (Q1) showed a decreased risk of overall cancer.

To avoid reverse causation and eliminate the impact of relatively short follow-up time in this study, we further grouped the subjects based on the median follow-up time (Supplementary Table 3). It is worth noting that the impacts of serum taurine and SAM on cancer incidence became evident only in the subgroup observed beyond the median follow-up time. Specifically, we observed that serum taurine (per SD increase) and its quartiles (Q4 vs. Q1) were associated with a decreased overall cancer risk, with odds ratios (95% confidence intervals) of 0.81 (0.71, 0.92) and 0.67 (0.47, 0.97), respectively. In contrast, serum SAM (Q3 vs. Q1) was associated with an increased overall cancer risk, with an OR (95% CI) of 1.51 (1.07, 2.13). In addition, different machine learning models also confirm the relationship between taurine and cancer risk. In the Decision Tree, Random Forest, and XGBoost, taurine ranks first in terms of importance (Supplementary Figure 3). Meanwhile, we also carried out a PSM analysis. The matched cohort contained data of

TABLE 2 The association of taurine, SAM, and cysteine with the digestive and non-digestive system cancer risk.

	Digestive system cancer		Non-digestive system cancer	
	Cases/controls	OR (95%CI)	Cases/controls	OR (95%CI)
Taurine (per SD)	543/543	0.83 (0.70, 0.96)	848/848	0.85 (0.75, 0.96)
Quartiles of taurine[a]				
Q1	143/128	Ref.	224/199	Ref.
Q2	140/132	0.97 (0.64, 1.40)	210/215	0.85 (0.62, 1.17)
Q3	127/144	0.79 (0.50, 1.17)	216/210	0.84 (0.61, 1.17)
Q4	133/139	0.80 (0.52, 1.26)	198/224	0.72 (0.52, 1.00)
P for trend	0.061		0.072	
SAM (per SD)	543/543	1.21 (1.01, 1.44)	848/848	1.02 (0.90, 1.16)
Quartiles of SAM[b]				
Q1	133/150	Ref.	216/232	Ref.
Q2	137/131	1.02 (0.70, 1.50)	214/204	1.26 (0.93, 1.70)
Q3	130/138	1.20 (0.80, 1.80)	204/211	1.15 (0.83, 1.58)
Q4	143/124	1.33 (0.85, 2.08)	214/201	1.19 (0.84, 1.70)
P for trend	0.561		0.271	
Cysteine (per SD)[c]	543/543	1.02 (0.84, 1.22)	848/848	1.01 (0.88, 1.17)
Quartiles of cysteine[c]				
Q1	136/134	Ref.	219/207	Ref.
Q2	136/137	0.94 (0.60, 1.43)	201/222	0.80 (0.58, 1.11)
Q3	132/140	0.97 (0.59, 1.60)	214/208	0.98 (0.66, 1.43)
Q4	139/132	0.94 (0.55, 1.61)	214/211	1.02 (0.69, 1.49)
P for trend	0.794		0.531	

Note: All the OR, values were the adjusted OR, values. Models were adjusted for taurine, S-adenosylmethionine, body mass index, smoking status, alcohol drinking, systolic blood pressure, triglycerides, cholesterol, uric acid, fasting blood glucose, high-density lipoprotein cholesterol, creatinine, albumin, alanine aminotransferase, cysteine, sleep quality, antihypertensive drug usage, and family history of cancer.

[a]The cutoffs of taurine in the digestive system were 11.74, 15.19, and 18.90.
The cutoffs of taurine in the non digestive system were 11.74, 15.19, and 18.90.

[b]The cutoffs of SAM, in the digestive system were 5.68, 8.82, and 11.94.
The cutoffs of SAM, in the non digestive system were 5.74, 8.62, and 11.86.

[c]The cutoffs of cysteine in the digestive system were 8.51, 12.47, and 15.92.
The cutoffs of cysteine in the nondigestive system were 8.33, 12.15, and 15.83.

1,021 pairs of participants, and the baseline characteristics are shown in Supplementary Table 4. Conditional logistic regression indicated that for each standard deviation increase in taurine, the cancer risk decreased by 17% (95% CI: 10%–28%) (Supplementary Table 5).

In our subgroup analysis (Figure 2), we noted a consistent protective effect of serum taurine against cancer risk in several demographic groups: males, individuals aged under 69 (median), current/former smokers, current/former drinkers, and those classified as obese. Notably, the protective influence of serum taurine against cancer was significantly modified by smoking habits (interaction P value < 0.05). For female and obese populations, there were discernible positive associations between serum SAM levels and heightened cancer risk. Furthermore, elevated levels of serum cysteine corresponded to a higher cancer risk among female subjects and among those who are current or former drinkers. The impact of serum cysteine on cancer risk was found to be significantly influenced by sex (interaction P value < 0.05).

Analyzing micronutrient mixtures using BKMR

To comprehensively account for the intercorrelations among taurine, SAM, and cysteine, as well as their associations with incident cancer, we employed BKMR methodology. Supplementary Figure 4 illustrates the exposure-response relationship between a singular variable and the outcome, while holding the levels of the other two variables constant at their respective median values. The primary objective of this analysis

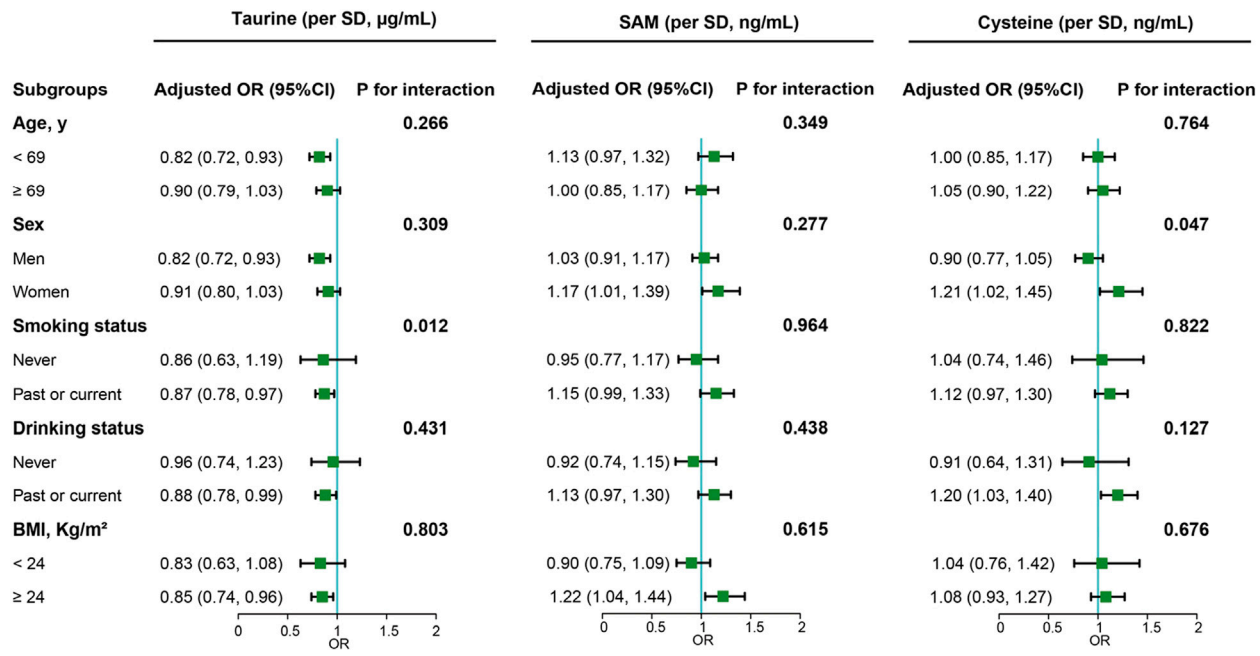

FIGURE 2
Subgroup analyses of the associations of taurine, SAM and cysteine with overall cancer risk. Note: Models were adjusted for taurine, S-adenosylmethionine, body mass index, smoking status, alcohol drinking, systolic blood pressure, triglycerides, cholesterol, uric acid, fasting blood glucose, high-density lipoprotein cholesterol, creatinine, albumin, alanine aminotransferase, cysteine, sleep quality, antihypertensive drug usage, and family history of cancer.

is to investigate the nonlinear correlation between the exposure variable and the outcome. After controlling for the levels of SAM and cysteine, there is a negative correlation between serum taurine and the risk of overall cancer incidence. Figure 3 illustrates the bivariate dose-response functions of taurine, SAM, and cysteine concerning the risk of incident cancer. Within each panel, the principal variable of interest is designated at the uppermost section of the figure. Meanwhile, the second predictor, denoted on the side of the figure, is held constant at the 10th, 50th, and 90th percentiles, while the remaining predictor is fixed at its median value. These curves depict the overall cancer risk across continuous values of the primary exposure at the specified percentiles of the secondary exposure. This analysis unveils that the relationships between SAM and cysteine with overall cancer risk may be contingent upon the levels of taurine. Conversely, the inverse associations observed between taurine and cancer risk remain unaffected by the levels of SAM and cysteine. Figure 4 demonstrates the association between the overall effect of the mixture (taurine, SAM, and cysteine) and overall cancer risk. When the three micronutrients are held constant at different percentiles, they can be compared to when they are set at their respective medians. This comparison reveals a significant negative association between the combined effect of taurine, SAM, and cysteine and the overall cancer risk.

Discussion

In this large-scale population-based case-control study nested within CHHRS, we identified a robust correlation between serum levels of taurine, SAM, and cysteine. Notably, taurine demonstrated a protective effect, decreasing the overall risk of cancer incidence. This effect was observed for both digestive and non-digestive system cancers. In contrast, elevated SAM levels were associated with a heightened risk for gastrointestinal cancers. Subgroup analyses revealed that increased serum cysteine concentrations were associated with a higher incidence of cancers, particularly in women and the obese population. Within the context of the BKMR model, the oncogenic effects of serum cysteine and SAM were found to be influenced by taurine concentrations. Intriguingly, increased combined levels of these three micronutrients were concomitantly linked to a reduced overall cancer risk.

To the best of our understanding, this represents the first population-based prospective study examining the impact of serum taurine, SAM, and cysteine on the risk of incident cancers. Nonetheless, several studies partially corroborate our results:

Our findings align with the observation that serum taurine is inversely associated with the incidence of overall, digestive and non-digestive cancers. This association is evident in males under 69 years, smokers, drinkers, and the obese participants. Several epidemiological studies have highlighted a potential link between taurine consumption and a reduced risk of certain cancers. For instance, research has shown that populations with high seafood consumption, a primary source of taurine, have a lower prevalence of certain conditions, including cancer (Murakami, 2015). Moreover, a study focusing on urinary biomarkers identified elevated taurine levels in the urine of bladder cancer patients, suggesting its potential as a biomarker for non-muscle invasive bladder cancer (Srivastava et al., 2010). Furthermore, taurine has been proposed as a novel tumor marker for enhanced detection of

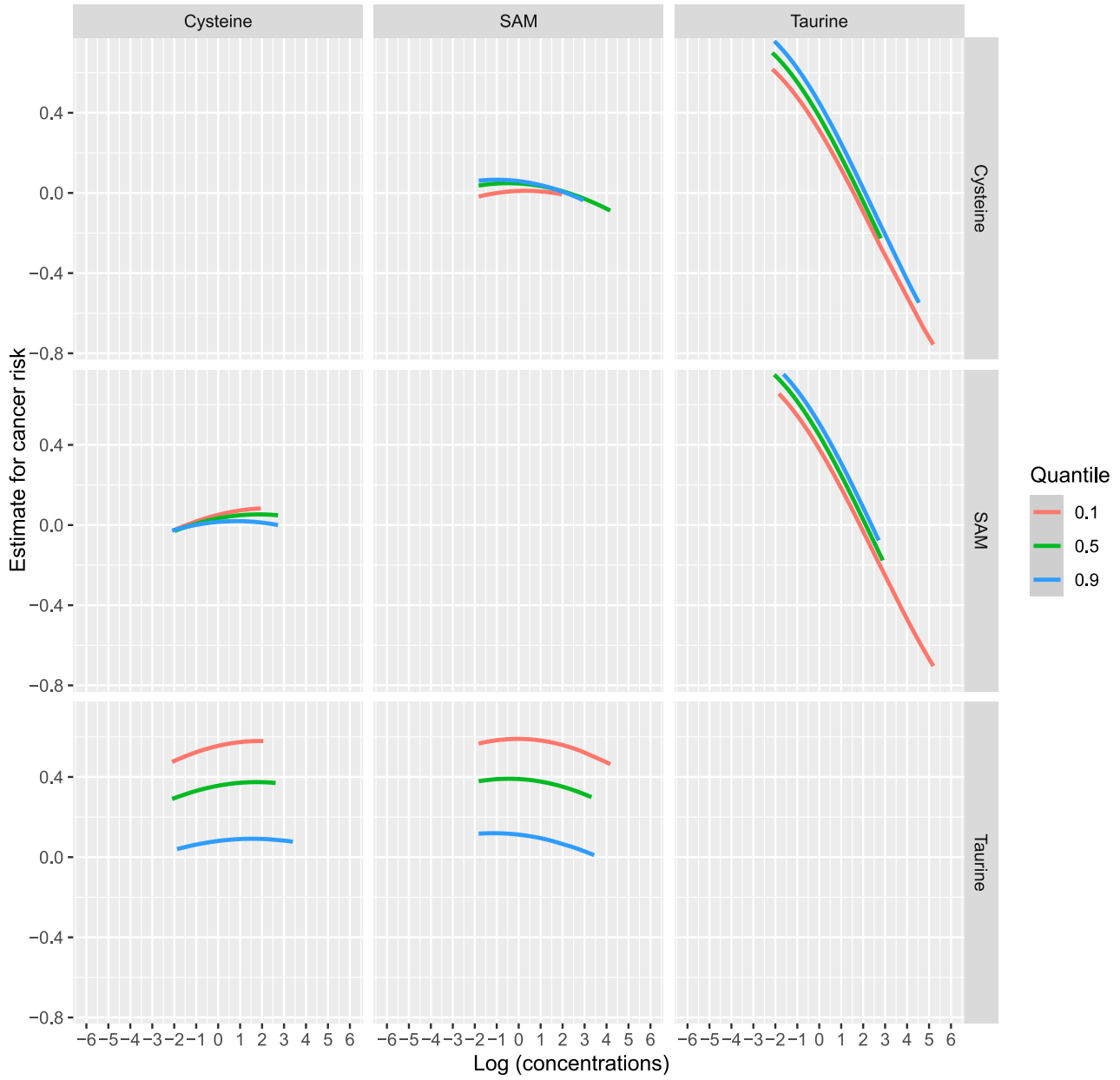

FIGURE 3
Bivariate exposure dose-response curves for taurine, SAM, and cysteine. Note: Dose-response curves for one variable at the 10th, 50th, and 90th percentile levels of the other variable (the remaining two variables were all fixed at the median) versus outcome. The *x*-axis represents the logarithmic values of the concentrations of taurine, SAM, and cysteine, and the *y*-axis represents the estimate for cancer risk.

breast cancer, emphasizing its significance in cancer diagnostics (El et al., 2011). The antioxidant properties of taurine, which play a pivotal role in neutralizing oxidative stress—a key factor in tumorigenesis—further underscore its potential therapeutic value (Jong et al., 2021).

Our findings reveal that taurine demonstrates a protective effect against cancer risk, with this effect varying between different smoking populations. Specifically, while no significant protective effect of taurine was observed in non-smokers, a pronounced protective effect was evident in smokers. This interaction can be attributed to the oxidative stress induced by smoking. Smoking introduces a myriad of reactive oxygen species (ROS) and other harmful agents, leading to oxidative stress. Taurine, known for its antioxidative properties, may mitigate some oxidative damage induced by smoking. Research has shown that taurine can modify monocyte and endothelial dysfunction in smokers, potentially mitigating the vascular damages caused by smoking-related oxidative stress (Shorey-Kendrick et al., 2024). Thus, the pronounced protective effect of taurine in smokers might be due to its enhanced antioxidative action countering the elevated oxidative stress in this group.

We found that elevated serum SAM was linked to a higher incidence of digestive cancers. Additionally, it showed a positive association with overall cancer incidence in females and individuals

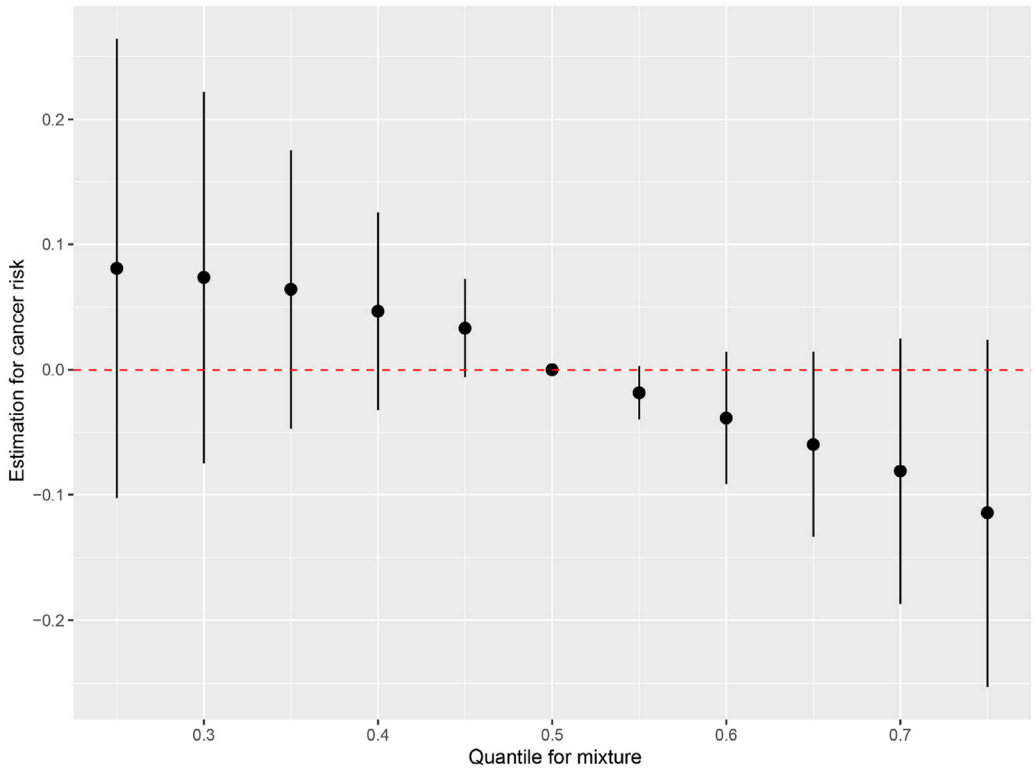

FIGURE 4
The association between the overall effect of the mixture and cancer risk. Note: The change in the estimated outcome when the three compounds were fixed at different percentiles simultaneously compared to when they were fixed at the median shows that the overall effect is negatively correlated with the outcome.

with a BMI over 24 kg/m^2. Dysregulation of SAM metabolism can lead to aberrant methylation patterns, which have been implicated in the initiation and progression of various cancers. While the direct epidemiological evidence linking SAM to cancer risk remains limited, several studies have highlighted the importance of methylation processes in cancer development and progression (Garcia-Martinez et al., 2021). For instance, hypermethylation of tumor suppressor genes or hypomethylation of oncogenes can drive carcinogenesis, and SAM, as a primary methyl donor, plays a central role in these processes (Roadmap et al., 2015). Alterations in SAM levels can also affect substrates' availability for polyamine synthesis, a pathway known to influence cancer cell growth and proliferation (Casero et al., 2018).

Although we observed no direct link between serum cysteine and overall cancer incidence, there was a significant risk in subgroups of women and drinkers. Elevated cysteine levels have been associated with an increased risk of specific cancers. An epidemiological study has observed a positive correlation between cysteine levels and breast cancer risk (Lin et al., 2010). Another study highlighted that the oxidized form of the OGG1-S326C polymorphic variant, which has a cysteine substitution at position 326, is associated with an increased risk of cancer, as observed in several molecular epidemiological studies (Soliman et al., 2020).

Our research has revealed a gender-specific variation in the pro-cancer effect of serum cysteine. Specifically, while no significant effect was observed in males, a pronounced risk of cancer was evident in females. This gender-specific interaction may be rooted in the differential metabolic pathways and hormonal environments between males and females. For instance, estrogen, a predominant female hormone, has been shown to influence various metabolic processes, potentially modulating the effects of cysteine and other metabolites (Yu et al., 2022). Additionally, a study has indicated that oxidative stress markers, including those related to cysteine metabolism, can vary with smoking habits and are influenced by sex (Oda et al., 2022). Thus, the heightened risk observed in females may stem from unique metabolic and hormonal interactions that are less pronounced or absent in males.

While traditional regression provides an initial understanding of individual associations, it may not adequately capture the complex interactions and combined effects of these nutrients on cancer risk. Therefore, we employed BKMR, a more advanced analytical method, to assess the collective impact on tumorigenesis and potential interactions among these three micronutrients. Specifically, the relationship between SAM, cysteine, and cancer risk is influenced by taurine levels. Yet, taurine's protective effect against cancer remains steady regardless of SAM and cysteine concentrations. SAM is a primary methyl donor in cellular reactions. Aberrant methylation patterns, especially in DNA, can lead to the silencing of tumor suppressor genes or activation of oncogenes, thereby promoting tumorigenesis (Mattei et al., 2022). The presence of taurine might influence both SAM's availability and its methylation potential, each of which could modulate its impact on cancer risk (Pepe et al., 2007; Li and Ye, 2020). Cysteine is a precursor to the antioxidant glutathione. An imbalance in the redox state can lead to oxidative stress, a known contributor to cancer initiation and

progression (Chen et al., 2020). Taurine, with its antioxidative properties, might synergize or antagonize with cysteine, influencing the overall redox balance and, consequently, cancer risk (Gulcin, 2020). Our findings indicated a negative association between serum taurine levels and cancer risk, while serum SAM and cysteine levels showed a positive association with cancer susceptibility. Given the intertwined relationships among these biomolecules, their combined influence on cancer risk is of paramount interest. To elucidate this collective impact, we utilized the BKMR model. This model uniquely facilitates the assessment of the overall effect of a mixture on an outcome, capturing the nuanced interactions among multiple components. Our results suggested a potential trend that the combined effect of these three factors might be associated with a certain degree of protection against cancer as their concentrations increase. However, it should be noted that while the BKMR model was applied in our study to explore the relationships among these factors, the evidence for a clear and strong synergistic amplification of the combined protective effect is not yet conclusive. The BKMR model does provide a useful framework to analyze the complex relationships and conditional effects among multiple exposures, which contributes to a more comprehensive understanding of the possible interactions involved in cancer etiology, although further research is needed to more precisely define and validate these relationships (Devick et al., 2022).

The intricate mechanisms behind taurine's tumor-suppressive effects, and the tumor-promoting roles of SAM and cysteine, are subjects of ongoing research. Taurine, which is known for its antioxidative properties, may potentially play a role in counteracting oxidative stress, which is an aspect that has been associated with cancer initiation (Jakaria et al., 2019), but also modulate cellular inflammation, further hindering tumor progression (Li et al., 2022). SAM, central to methylation processes, can influence gene expression. Aberrant methylation, especially of tumor suppressor genes, can drive carcinogenesis (Chi et al., 2023), and SAM might also affect epigenetic modifications, further amplifying cancer susceptibility (Herceg, 2016). Cysteine, when imbalanced, can disrupt redox homeostasis, leading to the production of ROS and interacting with cellular pathways to promote tumorigenesis (Liu S. et al., 2022). Additionally, cysteine's role in modulating certain proteases has implications for both cancer progression and therapeutic outcomes (Jakoš et al., 2019). These insights emphasize the multifaceted roles of amino acids in oncology and the need for continued research in this domain.

The primary advantage of this study lies in its novel insight into the potential tumor-inhibiting properties of taurine and the tumor-enhancing roles of SAM and cysteine within Chinese adults. Furthermore, the prospective design minimizes recall bias and is optimally tailored for analyzing time-to-event data. However, the present study has certain limitations worth mentioning. First, we assessed serum taurine, SAM, and cysteine levels only at the outset. Dynamic evaluations might have provided a clearer perspective on the evolving associations between these amino acids and cancer susceptibility. Second, although we employed the BKMR model to handle the complex interactions between taurine and its metabolites with the aim of enhancing the robustness of the results, the limited number of cancer cases and the relatively short observation period have impeded an in-depth analysis of cancer subtypes. This emphasizes the need for a larger sample size to conduct further validation and draw more reliable conclusions. Third, our study primarily addressed the prevalence and outcomes of H-type hypertension in China, centering on hypertensive individuals. The generalizability of our findings to normotensive populations is yet to be ascertained, although we did account for blood pressure variations in our multivariate analysis, minimizing its potential impact. Fourth, while we identified associations of taurine, SAM, and cysteine with cancer risk, the causative implications of elevated amino acid levels, or their significance in the synthesis of cancer-associated compounds, require deeper exploration. Lastly, given that our results are based on a nested case-control design, they underscore the need for further research through comprehensive cohort studies and randomized trials.

Conclusion

Our findings establish the prospective role of taurine in cancer prevention and highlight the combined effects of taurine, SAM, and cysteine levels on cancer incidence in Chinese adults. Should our results be corroborated in future investigations, they might offer a novel, effective, and safe avenue for cancer prevention.

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 humans were approved by Beijing shijitan hospital capital medical university. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

CL: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing–original draft. TL: Conceptualization, Data curation, Investigation, Project administration, Supervision, Validation, Writing–review and editing. YW: Formal Analysis, Resources, Software, Validation, Writing–review and editing. JS: Formal Analysis, Investigation, Methodology, Project administration, Supervision, Writing–original draft. LD: Formal Analysis, Funding acquisition, Software, Visualization, Writing–review and editing. MS: Data curation, Software, Supervision, Validation, Writing–review and editing. HS: Funding acquisition, Supervision, Visualization, Writing–review and editing.

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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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The author(s) declare that no Generative AI was used in the creation of this 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/fphar.2024.1507125/full#supplementary-material

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Muscle loss in cancer cachexia: what is the basis for nutritional support?

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Cancer cachexia (CC) is characterized by significant skeletal muscle wasting, and contributes to diminished quality of life, while being associated with poorer response to treatment and with reduced survival. Chronic inflammation plays a central role in driving CC progression, within a complex interplay favoring catabolism. Although cachexia cannot be fully reversed by conventional nutritional support, nutritional intervention shows promise for the prevention and treatment of the syndrome. Of special interest are nutrients with antioxidant and anti-inflammatory potential and those that activate pathways involved in muscle mass synthesis and/or in the inhibition of muscle wasting. Extensive research has been carried out on novel nutritional supplements' power to mitigate CC impact, while the mechanisms through which some nutrients or bioactive compounds exert beneficial effects on muscle mass are still not totally clear. Here, we discuss the most studied supplements and nutritional strategies for dealing with muscle loss in CC.

KEYWORDS

nutritional supplementation, cancer cachexia, muscle wasting, chronic inflammation, protein synthesis

1 Introduction

Cancer cachexia (CC) is characterized by progressive functional debilitation and significant skeletal muscle mass loss, often accompanied by fat mass wasting. The decrease in muscle mass contributes to diminished quality of life, increased fatigue and morbidity, and is associated with poorer responses to oncological therapy (von Haehling and Anker, 2014). Cachexia related muscle loss frequently provokes, the early discontinuation of treatment, the necessity of chemotherapeutic drug dose adjustment, and is robustly linked with worsened prognosis and decreased survival (Mattox, 2017).

Cancer cachexia definition and staging are still controversial, which makes it difficult to compare the existing data and to determine its prevalence (Wiegert et al., 2020). Within the most widely accepted consensus (Fearon et al., 2011), the syndrome is divided into three progressive stages based on clinical and biochemical parameters: pre-cachexia, cachexia, and refractory cachexia (RCa), the latter being characterized by non-responsiveness to anticancer therapies and an expected survival of up to 3 months, solely (Fearon et al., 2011). Wiegert et al. (2020) compared three diagnostic criteria for cachexia in 1,384 patients with incurable cancer under palliative care. According to their findings, 17.3% of the patients were cachectic, 20.8% were in pre-cachexia, 53.3% were in the RCa stage, and 8.2% were

non-cachectic by Viagno et al.'s classification. Applying Blum et al.'s criteria, 53.9% of the patients were classified as cachectic, 12.3% as pre-cachectic, 26.1 as RCa, and 9.7% as non-cachectic. In contrast, Wallengren et al.'s criteria identified 13.8% of the patients as cachectic and 86.2% as non-cachectic. Cachexia was found to be most prevalent among patients with gastrointestinal tract tumors (Wiegert et al., 2020). On the other hand, a study by Orellana López et al. (2023), which determined cachexia prevalence using the miniCASCO tool in a cohort of cancer patients in Chile, found that 27.5% presented cachexia. Within this group, 45.45% could be classified to be in the stage of pre-cachexia, and 36.36% in RCa, and 18.18%, cachexia. Both studies underscore the importance of CC classification to clinical practice and its potential to guide treatment decisions effectively, particularly at the early stages of the syndrome (Wiegert et al., 2020; Orellana López et al., 2023).

Despite the traditional definition that cachexia cannot be fully reversed by conventional nutritional support (Fearon et al., 2011), multimodal interventions at the onset of the syndrome appear to allow some gains. An anabolic window is observed in patients with cancer cachexia (CC), particularly at the earlier stages of the disease or during periods of clinical stability. These phases are characterized by improved symptom control, optimized pain management, enhanced nutritional intake, and better physical performance (Prado et al., 2013). Later along the progression of the disease, many factors lead to possible anabolic resistance, such as tumor site and stage, anticancer treatment side-effects, life expectancy, nutritional status and dietary intake, and inflammation (Engelen et al., 2016).

The difficulties in cachexia management arise from the fact that the disease is *per se*, multifactorial in nature, and is driven by a combination of inflammation, disrupted metabolic processes, and negative energy and protein balance (Fearon et al., 2011). Chronic inflammation plays a central role in CC onset and progression, promoting tumor-host interaction mediated by pro-inflammatory cytokines (Donohoe et al., 2011). Tumor Necrosis Factor-alpha (TNF-α), also known as cachectin, is one of the key cytokines involved in CC (Fearon et al., 2012). It promotes protein degradation by activating the intracellular NF-κB pathway, which induces the expression of genes associated with the process of proteolysis, including Muscle RING-Finger protein-1 (MuRF1) and atrogin-1 (Armstrong et al., 2020). Interleukin (IL)-6 is another crucial cytokine in cachexia (Fearon et al., 2012). It activates STAT3, which then translocates to the nucleus, promoting the expression of genes involved in protein degradation and inflammation, hence exacerbating tissue loss (Martin et al., 2023; Fonseca et al., 2020). Furthermore, myostatin and activin A, members of the TGF-β superfamily, act as negative regulators of muscle mass by activating the SMAD2/3 pathway, which represses muscle differentiation and regeneration (Fearon et al., 2012). FoxO transcription factors also contribute to muscle atrophy, by up regulating ubiquitin ligases and promoting autophagy (Martin et al., 2023). TWEAK, another cytokine reported to be augmented in CC, exacerbates muscle wasting by inducing proteolytic enzymes and promoting inflammation (Armstrong et al., 2020; Martin et al., 2023). Another highly relevant catabolic factor associated with CC is Growth Differentiation Factor 15 (GDF15), a cytokine released in response to several stress signals. Augmented circulating levels of GDF15 may lead to weight loss and anorexia in patients with CC and are associated with reduced survival (Lerner et al., 2015; Suzuki et al., 2021). To our knowledge, no published studies have established a correlation among nutritional supplementation, GDF15 levels, and muscle mass regulation.

The pathophysiology of CC involves a complex interplay between anabolic and catabolic pathways, with a pronounced shift towards protein degradation in detriment of synthesis. In anabolic states, protein synthesis exceeds degradation resulting in muscle protein gain, while the opposite leads to catabolism, resulting in muscle mass loss (Stipanuk, 2008). The Mammalian Target of the Rapamycin (mTOR) pathway, regulated through the IGF-1/PI3K/AKT cascade, has a central role in promoting muscle protein synthesis (Yoshida and Delafontaine, 2020). However, in CC, mTOR signaling is often inhibited due to increased levels of proinflammatory cytokines, such as TNF-α (Fonseca et al., 2020).

Muscle loss in CC is predominantly mediated by two main pathways: the Ubiquitin-proteasome system (UPS) and the Autophagy-lysosome pathway (ALP) (Martin et al., 2023; Aversa et al., 2016). The UPS is responsible for degrading damaged or malfunctioning intracellular proteins. This process begins with tagging the target by adding ubiquitin chains, a process mediated by MuRF1 and atrogin-1, also known as Muscle Atrophy F-box (MAFbx) (Foletta et al., 2011). These ubiquitinated proteins are then recognized and directed to the proteasome, a proteolytic complex that degrades the tagged proteins into smaller peptides (Foletta et al., 2011). In CC, there is increased expression of E3 ligases, what facilitates the ubiquitination of muscle proteins, leading to enhanced protein degradation and consequent muscle mass loss (Martin et al., 2023). Simultaneously, ALP, which generally maintains cellular homeostasis by degrading damaged organelles, is pathologically increased in CC, contributing to muscle atrophy through the degradation of both fiber proteins and organelles (Sandri, 2013). Catabolic factors derived from the tumor, such as proteolysis-inducing factor (PIF), directly activate the UPS, further exacerbating muscle proteolysis (Fearon et al., 2012).

Fearon et al. (2012) state that the impossibility of reversal of the cachectic state by conventional nutritional support is a marking feature of CC. Nevertheless, extensive research has been carried out on the potential of novel nutritional supplements to mitigate the syndrome's deleterious impact, and understanding the balance between protein synthesis and degradation pathways is mandatory in this scenario. There is no standardized treatment for CC. Various nutritional intervention strategies have shown promise in preventing and treating this syndrome, with some nutrients or bioactive compounds demonstrating beneficial effects, whether alone or in combination. The mechanisms through which these interventions exert their effects on muscle mass are still uncovered, yet they offer a potential therapeutic avenue. One postulated mechanism by which nutrients can mitigate cachexia malnutrition is interfering with systemic inflammation. Overall, this review aims to explore the most studied nutritional supplements and strategies for treating and/or preventing muscle loss in CC.

1.1 Protein intake and turnover

Although recognized as the primary anabolic stimulus in skeletal muscle metabolism, studies investigating the effects of increased

total protein intake in cancer patients are relatively recent. As revised by Prado and colleagues (Prado et al., 2020), current guidelines for adequate protein intake in oncologic patients fail to address factors such as body composition and muscle depletion. The recommended protein intake for cancer patients ranges from 1 to 1.5 g/kg (Muscaritoli et al., 2021), which appears to be insufficient. Colorectal cancer patients submitted to a high-protein diet at the pre-cachexia stage presented a reduction in subclinical inflammation, one of the main drivers of muscle wasting in cachexia, as well as an improvement in the nutritional status and in appetite (Ziętarska et al., 2017). Also, a systematic review (Capitão et al., 2022) comprising different types of cancer associated with sarcopenia (head and neck, lung, esophageal cancer) reported a minimum amount of protein of 1.4 g/kg to ensure muscle mass maintenance in this population, with lower amounts being associated with muscle loss along the treatment.

It is also important to consider the debate on higher protein intake and its association with tumor growth, since protein synthesis in the tumor, muscle, and immune cells involves the same signaling pathways (Butler et al., 2021; Li et al., 2007). A study with a model of cachectic colon tumor-bearing rats undergoing chemotherapy (Boutière et al., 2023) demonstrated that trends in tumor growth and response to chemotherapy remained unaltered despite enhanced protein dietary intake. Moreover, a modest improvement in nutritional status was observed in animals submitted to a high-protein diet, with an increase in relative fat-free mass. However, in neither of these studies, differences were reported regarding protein metabolism in skeletal muscle.

Most recently, a randomized clinical trial reported the effects of the total daily protein intake on patients with stage II-IV colorectal cancer submitted to chemotherapy (Ford et al., 2024). At baseline, most patients had lower protein ingestion than recommended by the guidelines, and individuals who managed to increase their protein ingestion favored the maintenance of muscle mass, physical function, and anabolism. One of the goals of the study was to assess the response to a diet containing 2.0 g/kg/day of protein versus another, in which 1.0 g/kg/day of protein was consumed, but only 35,3% of the patients in the 2.0 g/kg/day achieved the recommended protein intake. Despite not having succeeded in increasing protein ingestion to the proposed values, the study recognizes that individualized nutritional counselling had a promising effect on protein intake and muscle mass maintenance. Even though CC was an exclusion criterion in this pilot study, it represents the first attempt to demonstrate the effect of nutritional support alone on muscle loss prevention in patients with cancer, as well as providing an evidence-based optimal protein dose for this population, considering features such as acceptance, feasibility and efficacy of the diet regimen.

Along with increasing diet protein intake, the use of specific proteins and amino acids has also been the target of many studies. Among the strategies examined, we chose to address those with clearer evidence of beneficial impact.

1.2 Whey protein supplementation

The high concentration of easily digestible essential amino acids in whey protein (WP) renders it ideal as an effective way to add up proteins in the patient's diet. WP has thus become a suitable choice for providing protein support for cancer patients, as it offers greater nutritional value and faster absorption compared to alternative dietary protein sources (Ramani et al., 2024).

WP is particularly rich in the branched-chain amino acids valine, leucine, and isoleucine, which play a crucial role in tissue growth and repair (Brestenský et al., 2015). Additionally, whey protein is a valuable source of cysteine and methionine, which are glutathione precursors and a key component for enhancing immune function (Mir Khan and Selamoglu, 2020). The whey protein subfractions most studied for their ability to disrupt tumor pathways include α-lactalbumin, bovine serum albumin, and lactoferrin (Teixeira et al., 2019). *In vitro* and *in vivo* research has demonstrated their anticancer properties, including decreased tumor occurrence, growth suppression, and improved antioxidant activity. Furthermore, it can potentially improve conventional cancer treatment efficacy and reduce side effects (Teixeira et al., 2019). In addition, WP has been shown to enhance immune function, improve nutritional status, and promote overall health, making it a beneficial strategy for cachectic patients (Zhao et al., 2022). Nonetheless, changes in muscle metabolism, such as a lower anabolic potential, are common in advanced CC (Prado et al., 2013), where protein synthesis remains impaired even when sufficient protein is consumed. Therefore, nutritional strategies should focus on both the increase of muscle protein synthesis and decrease of anabolic resistance (Van de Worp et al., 2020; Antoun and Raynard, 2018).

In a clinical trial involving cachectic patients undergoing chemotherapy and receiving nutritional supplementation, adding WP to the 3-month treatment regimen significantly improved body composition, muscle strength, and body weight, while reducing chemotherapy toxicity (Cereda et al., 2019).

Several studies indicate that WP supplementation, which is rich in leucine and other essential amino acids, can stimulate protein synthesis more effectively than other protein sources. This effect is attributed to the rapid digestion of WP, which leads to a swift increase in plasma amino acid levels, particularly essential amino acids, potentially mitigating muscle loss in cachectic patients (Cereda et al., 2019; Dangin et al., 2002; Deutz et al., 2011a; Dillon et al., 2007).

Therefore, WP may benefit patients with cancer-associated cachexia by improving muscle synthesis and immune function and reducing inflammation. While WP supplementation shows promise in improving clinical outcomes and quality of life, further studies are needed to define the optimal dosage and evaluate long-term effects.

1.3 Branched-chain amino acid (BCAA) supplementation

The amino acids known as BCAA, leucine, isoleucine, and valine, are essential amino acids that play an important role in muscle metabolism. BCAAs have been studied in CC in regard to eventual potential to mitigate muscle mass loss (Ananieva et al., 2016). They interfere with the activation of catabolic pathways, such as the ubiquitin-proteasome system, which are exacerbated in cachexia (Setiawan et al., 2023). BCAA supplementation has been

associated with improvement in muscle strength and function, modulation of inflammation, and improvement in energy metabolism, contributing to better quality of life (Storck et al., 2020; Gala et al., 2020).

The benefits of BCAA supplementation, including improved post-chemotherapy recovery - characterized by weight gain and increased energy levels - have been broadly examined (Deutz et al., 2011b; Nojiri et al., 2017; Katagiri et al., 2020; Hachiya et al., 2020). It has also been suggested that BCAAs may increase mitochondrial biogenesis (Valerio et al., 2011), potentially benefiting skeletal muscle energy metabolism (Borack and Volpi, 2016; van Dijk et al., 2015). On the other hand, recent studies have reported that BCAA supplementation may be detrimental to cancer patients, as potentially, tumor cells can consume these amino acids and thus remain alive even within anaerobic environments (Ananieva and Wilkinson, 2018; Ericksen et al., 2019; Lei et al., 2020; Lieu et al., 2020; Peng et al., 2020; Taherizadeh et al., 2021; Tang et al., 2010; Wang et al., 2018). Although limited by the absence of a control group and different types of tumors, two studies showed improvement in patient strength and quality of life (Van der Meij et al., 2019; Zanetti et al., 2020).

BCAAs act as metabolic regulators, influencing not only protein synthesis but also lipid and glucose metabolism (Zhang et al., 2017). Most circulating BCAAs are reincorporated into proteins, functioning as building blocks for muscle synthesis and providing nitrogen for the biosynthesis of nucleotides and nonessential amino acids (Jung et al., 2021). Supplementation in cachectic patients should be carefully monitored, as excessive intake of BCAAs may lead to an imbalance in the availability and metabolism of other essential amino acids (Storck et al., 2020), while the potential effect on tumor progression should be monitored.

1.3.1 Leucine

Leucine is an essential amino acid from the BCAA group. It is known to be an agonist of the mTOR pathway, playing an important role as an anabolic mediator in protein metabolism (Ananieva et al., 2016; Tian et al., 2019). Studies have shown that leucine may play a role in immune function by activating T cells (Ananieva et al., 2016) and regulating the immune response through mTOR signaling, which is crucial in regulating pro- and anti-inflammatory cytokines (Thomson et al., 2009; Soares et al., 2020; Van der Ende et al., 2018).

A leucine-rich diet has shown promising effects in preserving serum insulin concentration along cancer progression, thus promoting muscle protein synthesis and attenuating anabolic resistance without impacting tumor growth in animal models (Cruz et al., 2019). Studies performed by Salomão et al. (2010) and Viana et al. (2016) reinforce these observations, having demonstrated that leucine supplementation can be an effective strategy to improve protein metabolism in cancer patients (Salomão et al., 2010; Viana et al., 2016).

Research in tumor-bearing animals (Cruz et al., 2019; Viana et al., 2016; Gomes-Marcondes et al., 2003; Peters et al., 2011; Xia et al., 2017) and studies with older sarcopenic individuals (Rasmussen et al., 2016; Dickinson et al., 2013) suggest that leucine is a safe and efficient supplement. It positively affects protein metabolism, muscle mass gain, protein and caloric intake, and effectively modulates the inflammatory process.

Although research has shown that leucine can reduce muscle degradation and improve protein metabolism and the inflammatory profile during cancer progression, few studies have evaluated its direct effects upon tumor growth, vascularization, and proliferation. More research is thus needed to understand its impact in the context of human cancer.

1.3.2 β-hydroxy-β-methylbutyrate (HMB) supplementation

HMB is a metabolite derived from leucine metabolism. It has therapeutic potential in CC due to its effects in stimulating protein synthesis and in inhibiting muscle degradation. HMB was initially employed to improve wound healing (Williams et al., 2002), as this metabolite shows significant capacity to enhance muscle protein accretion and to induce collagen renewal (Zanchi et al., 2011).

The anabolic potential of HMB supplementation has been explored for the treatment of muscle loss in cancer, with results pointing out to its capacity to improve long-term outcomes (Prado et al., 2022). Tumor-bearing models show that HMB supplementation attenuates body weight and muscle mass loss at a dose similar to that adopted for human beings. Furthermore, HMB presents antitumor, anti-inflammatory, and anti-cachectic effects (Nunes et al., 2008; Zaira et al., 2011) Other studies showed increased survival time and promotion of favorable metabolic changes (Caperuto et al., 2007) and a significantly larger fiber cross-sectional area after HMB supplementation (Hao et al., 2011). Therefore, HMB supplementation may be a good option for complementary cancer therapy.

A more recent systematic review provided evidence that HMB supplementation benefits muscle mass and function in cancer patients. Additionally, the supplement has been demonstrated to be safe and tolerable, with no adverse effects (Prado et al., 2022). Studies suggest that HMB may effectively mitigate proteolysis associated with cachexia because of its capacity to reduce the activity of the ubiquitin-proteasome system, the main pathway for protein degradation and caspase activity (Prado et al., 2022). Furthermore, HMB stimulates proteogenesis through direct action on the mTOR pathway, the canonical protein synthesis pathway. HMB can also promote the activation of satellite cells in skeletal muscle and potentially enhance the tissue's regenerative capacity, directly affecting cell proliferation and differentiation (Chodkowska et al., 2018; Yamada et al., 2022).

Nutrients and exercise activate the mTOR pathway in muscle, supporting muscle anabolism. In contrast, in cancer cells, genetic mutations can lead to chronic hyperactivation of the mTOR pathway, fueling tumor growth and bypassing the standard regulatory inhibitory mechanisms. However, studies have not shown a direct link between HMB supplementation and tumor growth (Tian et al., 2019; Prado et al., 2022).

HMB has also been proposed to operate through its capacity to stabilize the sarcolemma via cholesterol synthesis. It has been shown that the majority of HMB is converted into 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, which is the limiting step of cholesterol synthesis. As such, increased intramuscular levels of HMB may serve as the available substrate for cholesterol synthesis, and therefore for formation and stabilization of sarcolemma (Rossi et al., 2017). HMB is recognized as an effective anti-catabolic agent, reducing protein degradation while enhancing protein synthesis.

The currently recommended daily dose is 3 g/day (Muscaritoli et al., 2021).

1.4 Glutamine supplementation

Glutamine is the most abundant amino acid in the plasma and in the skeletal muscle (Cruzat et al., 2018). In healthy individuals this amino acid plays an essential role in the regulation of cellular functions, among which immune cell activity, energy metabolism, maintenance of intestinal mucosal integrity, and protein synthesis (Newsholme et al., 2003). Glutamine metabolism is, however, disturbed in cancer patients, resulting in dysregulated protein synthesis, which can contribute to muscle wasting (Cruzat et al., 2018). Thus, the therapeutic potential of glutamine supplementation in cancer patients has been widely discussed, under the light of contributing to the preservation of muscle mass and warranting quality of life in patients with cancer-related wasting (Pradhan et al., 2024).

A cohort of 44 surgery patients with head and neck cancer received enteral glutamine supplementation for 4 weeks, having achieved significantly higher nutritional status and improved clinical outcomes than the control group. Notably, the intervention group maintained lean body mass, which correlated with a higher quality of life score during the postoperative period (Azman et al., 2015). Furthermore, other trials have investigated the effects of glutamine supplementation in combination with HMB and arginine, attenuating cancer-related wasting in association with increased free fat mass in patients with advanced disease, as observed by May et al. (2002).

In animal models, several studies demonstrated improvement in energy balance and inhibition of tumor growth following glutamine supplementation, as reviewed by Van de Worp et al. (2020). Walker-256 tumor-bearing rats supplemented with 2% l-glutamine exhibited reduced body weight loss and a lower percentage (%) loss of body mass index (BMI) (cachexia index), calculated according to tumor mass (Fracaro et al., 2016). These studies suggest that glutamine supplementation may improve muscle preservation and energy metabolism in CC patients.

Besides the promising findings, glutamine supplementation raises concerns due to its role in tumor growth. Since there is metabolic competition for glutamine between host cells and tumor cells, it is crucial to find a balance that supports normal cell function without promoting tumorigenesis during supplementation (Wang et al., 2024; Muranaka et al., 2024). Therefore, glutamine supplementation needs to be carefully monitored, and further research evaluating the potential benefits related to muscle loss in cancer-associated cachexia is necessary.

1.5 Arginine supplementation

Arginine is a conditionally essential amino acid that depends on metabolic status. Arginine stimulates cell growth and protein synthesis by inducing the activation of the mTOR pathway in the muscle (Panwar et al., 2023). In addition, arginine serves as a substrate for nitric oxide synthesis, an important signaling molecule that affects immune function, vasodilation, and cicatrization (Wu et al., 2021). Considering its importance in several biological functions, this amino acid has been suggested to improve patient outcome in CC by regulating tumor growth and promoting anabolic effects in the muscle (Pradhan et al., 2024). In addition, in immune system cells, arginine supplementation has been shown to promote beneficial immunomodulatory gain. Finally, it has been shown to improve nutritional status (Soares et al., 2020).

Under catabolic conditions in cancer, arginine levels in plasma are decreased, as observed in patients with breast cancer, colon cancer, and pancreatic cancer, independent of weight loss or tumor stage (Vissers et al., 2005). This result indicates disturbed arginine metabolism in the disease.

A study with arginine supplementation during the perioperative period showed no beneficial effect upon outcome in patients with head and neck cancer; nonetheless, the arginine-supplemented group demonstrated a trend toward improved survival span (Van Bokhorst-de van der Schueren et al., 2001), consistent with findings reported by the same group in a previous study (Buijs et al., 2010). Antoun and Raynard (2018) discussed that arginine supplementation still lacks robust clinical evidence regarding its benefits on muscle wasting. Randomized trials with larger sample sizes, focusing on arginine supplementation in patients with cancer-associated cachexia, may in the future elucidate arginine's direct and indirect effects on wasting conditions.

1.6 Creatine supplementation

Creatine is synthesized endogenously from three amino acids. The initial stages occur in the kidney, where arginine and glycine are involved, and the subsequent steps in the liver, with the participation of methionine. It can also be acquired through an animal protein-rich diet (Tanaka et al., 2022). Most of creatine is absorbed, stored, and used by the skeletal muscle (Jung et al., 2013; Gualano et al., 2012), suggesting creatine supplementation to be able to increase muscle strength and lean body mass in myopathies (Harris et al., 1992).

The effects of creatine supplementation in cachectic patients must still be well established. A study with patients with colorectal cancer under chemotherapy and with CC showed no changes in neither muscle mass, nor body composition (Jatoi et al., 2017). Another randomized and double-blind clinical trial was carried out with 30 individuals with stage III-IV colorectal cancer who received creatine or placebo over an 8-week period. The creatine supplementation protocol included an initial loading phase of 4 × 5 g per day during the first week, followed by a maintenance phase of 2 × 2.5 g per day. Creatine was not effective in improving muscle mass gain or its function. In addition, there were no changes in the quality of life of these patients. However, it enhanced the bioimpedance phase angle, which is related to improved prognosis (Norman et al., 2006).

Studies in rats with cachexia showed that creatine supplementation attenuated weight loss and decreased tumor growth (Cella et al., 2020; Campos-Ferraz et al., 2016; Deminice et al., 2016; Wei et al., 2022). In addition, supplementation promoted lower plasma concentration of TNF-α and IL-6 (pro-inflammatory cytokines), while increasing the concentration of IL-10 (anti-inflammatory cytokine), and preventing atrogin-1 and MuRF-1,

key regulators of muscle atrophy in the skeletal muscle (Cella et al., 2020).

Although the recommended dose for humans is 3–5 g/day (Tanaka et al., 2022), this dose did not show the same results in patients with cachexia (Jatoi et al., 2017; Norman et al., 2006), suggesting that changes in the metabolic pathways associated with the disease may limit the effects of creatine (Tanaka et al., 2022).

1.7 L-carnitine supplementation

L-carnitine (LC) is among the most studied nutritional supplements in advanced cancer patients with malnutrition (Johal et al., 2022) due to its antioxidant activity and possible anti-wasting effect (Alhasaniah, 2023). It is synthesized in the liver and kidney by converting the amino acids lysine and methionine. Its primary function is to facilitate the transport of long-chain fatty acids to the mitochondrial matrix for β-oxidation and subsequent energy production (Alhasaniah, 2023; Longo et al., 2016). Failure in this process can lead to increased oxidative stress, metabolic dysfunctions, and increased pro-inflammatory cytokines. In this context, LC supplementation can reduce oxidative stress and the inflammatory response, leading to potential clinical benefits (Longo et al., 2016).

Gramignano et al. (2006) evaluated the effect of LC supplement intake in 12 patients with advanced cancer, having observed a significant increase in lean mass and an improvement in appetite after 4 weeks, suggesting that LC can mitigate muscle loss and improve nutritional status. In a phase III study by Mantovani et al. (2010), although the use of a LC supplement (4 g/day) for 4 months did not significantly improve primary outcomes, such as lean mass and fatigue, a positive impact on secondary indicators such as the Glasgow Prognostic Score and ECOG (Eastern Cooperative Oncology Group) performance status was reported. Similarly, the study by Kraft et al. (2012) in patients with advanced pancreatic cancer showed that LC supplementation (4 g/day) for 12 weeks increased BMI and improvement in quality of life, further supporting the therapeutic potential of LC in patients with cancer.

In experimental models, LC treatment improved food intake, reduced muscle mass loss, and led to the downregulation of atrogin-1 and MuRF1, biomarkers of skeletal muscle atrophy. In addition, LC intake decreased proteasome activity (degradation pathway) in the gastrocnemius muscle of cachectic animals (Busquets et al., 2012; Busquets et al., 2020) and increased physical activity levels by improving energy production (Busquets et al., 2012). Another intervention with the LC supplement led to an increase in both food intake and muscle mass. The study also examined the activity of carnitine palmitoyltransferase-1 (CPT-1), a marker of the effects of carnitine. The results demonstrated an upregulation of CPT-1, associated with reduced plasma levels of IL-6 and TNF-α (Liu et al., 2011). Other experimental studies support the finding that CPT-1 activity is higher in the muscles of cachectic animals supplemented with LC (Busquets et al., 2020; Silvério et al., 2012) in relation to controls. In cachectic animals, CPT-1 expression is typically reduced, contributing to an accumulation of triacylglycerols in the liver. A 28-day supplementation of LC administered intragastrically at a dose of 1 g/kg body weight/day, effectively restored CPT-1 activity, thereby preserving hepatic lipid metabolism. Additionally, LC modulated CPT I enzymatic activity and MTP gene expression, further supporting healthy hepatic lipid regulation. The supplementation also enabled normal weight gain in tumor-bearing animals and significantly inhibited tumor growth, demonstrating LC's potential as a therapeutic strategy for managing cachexia (Silvério et al., 2012).

The available evidence suggests that LC has the potential to mitigate muscle mass loss, modulate lipid metabolism, and reduce inflammation associated with CC. Nonetheless, further studies are required to investigate the underlying molecular mechanisms to understand its therapeutic effects better.

1.8 Omega-3 fatty acids supplementation

Marine long-chain omega-3 (n-3) fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are well-known for their anti-inflammatory properties. N-3 has been shown to decrease tumor angiogenesis and invasiveness (McCarty, 1996). N-3 supplementation can alter inflammatory markers in patients with colorectal cancer, decreasing IL-6 and increasing albuminemia (Camargo et al., 2016). Additionally, n-3 polyunsaturated fatty acids can induce ferroptosis through lipid peroxidation in acidic tumor environments, selectively promoting cancer cell death (Dierge et al., 2021), while also inhibiting tumor proliferation and modulating pro-resolving lipid mediators, which help suppress chronic inflammation (Liput et al., 2021).

We have previously discussed in a meta-analysis that n-3 fatty supplementation was not able to lower circulating inflammatory markers in cachexia (de Castro et al., 2022). Nevertheless, due to the low number of studies that matched our inclusion and exclusion criteria, only six articles were included in the systematic review, disallowing a strong conclusion (de Castro et al., 2022). Oral nutritional supplements with high protein and n-3 content have been shown to preserve lean body mass during chemo (radio) therapy (de van der Schueren et al., 2018). However, another meta-analysis could not support this result, indicating that oral n-3 supplements could not maintain muscle and body weight mass nor improve the quality of life in patients with cancer (Lam et al., 2021).

Despite these contradictions, the European Society for Clinical Nutrition and Metabolism (ESPEN) advocates, although with a weak recommendation, the supplementation of n-3 for some patients with cancer (Muscaritoli et al., 2021). Notably, patients with advanced cancer undergoing chemotherapy and endangered by weight loss or malnutrition could benefit from n-3 supplementation due to its ability to improve appetite, food consumption, lean body mass, and body weight (Muscaritoli et al., 2021). The safety and tolerability of n-3 supplements were also highlighted (Muscaritoli et al., 2021).

1.9 Antioxidants

An increase in oxidative stress occurs when the normal redox equilibrium is altered, with increased presence of oxidative species and lower antioxidant defense status (Li et al., 2022). Patients with lung cancer and cachexia have higher skeletal muscle protein

carbonyls and superoxide anions content compared with healthy controls (Puig-Vilanova et al., 2015). It has been proposed that dietary antioxidants may modulate the oxidative stress in CC (Li et al., 2022).

A comprehensive meta-analysis including approximately 68 clinical trials did not identify protective effects of antioxidants against cancer. On the contrary, an increase in mortality was observed in individuals who consumed β-carotene, vitamin A or vitamin E, as evidenced by Bjelakovic et al. (2007). These findings can be partially explained by the critical role of free radicals as signaling molecules in anabolic processes. Excess antioxidants can negatively interfere with these signaling pathways, compromising essential cellular mechanisms (Higgins et al., 2020).

Reactive oxygen species (ROS) play a complex role in cancer biology. Elevated ROS levels are essential for tumor cell proliferation, survival, and progression by activating signaling pathways such as NF-κB, STAT3, and MAPK, which promote cell growth, angiogenesis, and metastasis. Paradoxically, ROS are critical for the efficacy of chemotherapy, as many anticancer treatments rely on elevating ROS to toxic levels to induce oxidative stress and trigger tumor cell death. Excessive antioxidant supplementation can disrupt this delicate balance by lowering ROS levels below the threshold necessary for effective chemotherapeutic action (Moloney and Cotter, 2018). This highlights the complex role of ROS and the potential risks of antioxidant overuse in compromising tumor control and treatment outcomes.

1.9.1 Resveratrol (trans-3,4′,5-trihydroxystilbene) supplementation

Resveratrol is a polyphenol in the skin of red grapes and other fruits. It has been shown to inhibit NF-κB (p65) activity and gene expression of MURF1 in the skeletal muscle of mice bearing C-26 adenocarcinoma (Shadfar et al., 2011). In mice with lung cancer and cachexia, resveratrol (20 mg/kg body weight/day, for 15 days) was able to reduce tumor mass and preserve body weight, soleus and gastrocnemius weight, myofiber cross-sectional area, and decrease Atrogin and MURF1- protein (Penedo-Vázquez et al., 2021). However, in a previous study, resveratrol administered to rats bearing the Yoshida AH-130 ascites hepatoma (1 mg/kg body weight/day for 7 days) and mice bearing the Lewis lung carcinoma (at doses of 5 and 25 mg/kg body weight/day for 15 days) was not able to preserve skeletal muscle and body weight mass (Busquets et al., 2007). Of concern, rats bearing the Yoshida AH-130 ascites hepatoma that received resveratrol showed lower food intake, lower gastrocnemius mass, heart mass, and white adipose tissue and liver weight, compared with rats bearing the tumor that did not receive resveratrol (Busquets et al., 2007).

1.9.2 Curcumin supplementation

Curcumin is a polyphenolic compound found in turmeric. It is recognized as a potent antioxidant and has been studied in the context of CC (Li et al., 2022). Patients with pancreatic cancer who received 8 g of curcumin per day for up to 18 months showed lower expression of NF-κB proteins, cyclooxygenase-2, and phosphorylated signal transducer and activator of transcription 3 in peripheral blood mononuclear cells (Dhillon et al., 2008). In a lung cancer-induced cachexia model, treatment with curcumin (1 mg/kg body weight/day for 15 days) effectively mitigated muscle wasting. Curcumin effectively preserved body weight and increased the weight of both the gastrocnemius and soleus muscles. Additionally, it enhanced the cross-sectional area of type I and type II muscle fibers, indicating reduced muscle atrophy. Furthermore, it attenuated proteolysis by reducing the levels of Atrogin-1 and MuRF-1, while also improving overall muscle structure and function (Penedo-Vázquez et al., 2021). Patients with advanced colorectal cancer received doses between 0.45 and 3.6 g per day for up to 4 months (Sharma et al., 2004). A dose of 3.6 g per day decreased the production of a lipid mediator derived from arachidonic acid, prostaglandin E2 (PGE2), in blood samples taken 1 h after the dose on days 1 and 29, compared to the PGE2 concentration found before treatment (Sharma et al., 2004).

1.9.3 Epigallocatechin-3-gallate supplementation

Epigallocatechin-3-gallate (EGCG), a polyphenol derived from green tea, shows promising properties in the context of cancer and cachexia. Studies have shown that EGCG negatively regulates the expression of genes associated with Atrogin-1, a key protein in the ubiquitin-mediated protein degradation process, while also acting on other proteins of the F-BOX family, contributing to the prevention or mitigation of the progression of cachexia both *in vitro* and *in vivo* (Wang et al., 2011). Additionally, EGCG exhibits anti-inflammatory properties, which may alleviate the systemic inflammation characteristic of cancer-associated cachexia, thus reducing the overall metabolic burden of the organism (Loyala et al., 2024).

Furthermore, EGCG inhibits tumor growth by inducing apoptosis through the mitochondrial pathway, arresting the cell cycle, and modulating key signaling pathways such as EGFR/RAS/RAF/MEK/ERK. These combined effects highlight its potential as a therapeutic agent against both cancer progression and muscle wasting (Sharifi-Rad et al., 2020).

A randomized, placebo-controlled trial assessed the safety of green tea catechins (Polyphenon E®) in 97 men with high-grade prostatic intraepithelial neoplasia (HGPIN) or atypical small acinar proliferation (ASAP) over 1 year. Participants received 200 mg of EGCG twice daily with food. The supplement was well-tolerated, with no significant treatment-related adverse events, including liver toxicity, compared to placebo. Plasma EGCG levels were significantly higher in the treatment group, confirming compliance (Kumar et al., 2016). The same group also showed that the supplemented group had lower rates of prostate cancer and Gleason scores after 1 year. Notably, in men with HGPIN but not ASAP at baseline, no progression to ASAP occurred in the treatment group compared to 20% in the placebo group (Kumar et al., 2016) These findings highlight the potential of green tea catechins to slow the progression of precursor lesions without increasing the risk of high-grade disease.

Despite these potential benefits, the mechanisms of action of EGCG are not yet fully elucidated. Green tea catechins exhibit very low stability after digestion, with EGCG being particularly sensitive. Its poor bioavailability restricts its therapeutic potential; however, it can be amplified when combined with other compounds, such as vitamin C, which has been shown to boost its biological activity in experimental models (Furniturewalla and Barve, 2022).

1.10 Vitamin D supplementation

Patients with metastatic prostate cancer who received 2000 units of vitamin D per day for 12 weeks showed an improvement in muscle strength. Among the 16 patients enrolled, half of them showed an improvement in the timed chair rises, and the other half improved timed 10-meter walk (Van Veldhuizen et al., 2000). A meta-analysis evaluated the vitamin D levels and the outcomes in patients with lung cancer (Feng et al., 2017). This study showed that circulating 25-hydroxyvitamin D concentration was inversely associated with lung cancer risk and mortality but not with overall survival (Feng et al., 2017).

Higher (4000 IU) compared to standard dose (400 IU) of vitamin D supplementation together with chemotherapy to treat head and neck cancer was not able to improve patients' body weight, BMI, muscle area, muscle attenuation, visceral adipose tissue area, or subcutaneous adipose tissue area after the first eight cycles of chemotherapy (Brown et al., 2020).

1.11 Combined nutrient supplementation

Cancer patients received arginine associated with omega-3 fatty acids and dietary nucleotides 5 days before and after radical cystectomy. They were compared with the control group that received BOOST Plus®, a commercially available nutritional drink (Hamilton-Reeves et al., 2018). The supplementation increased arginine levels from baseline to day 30 postoperative and immune support by maintaining the Th1-Th2 balance during surgery, leading to lower plasma IL-6 levels. Despite the advantageous modulation of the inflammatory response, there were no significant differences in appendicular muscle loss between the two groups (Hamilton-Reeves et al., 2018).

Chitapanarux et al. (2020) investigated the effects of arginine, glutamine, and fish oil supplementation in 88 cancer patients with head and neck cancer (45%), esophageal cancer (32%), and cervical cancer (23%) undergoing concurrent chemoradiotherapy (CCRT). The intervention group received 250 mL of supplementation twice daily, providing 500 kcal/day with additional protein (106.25 g/day total) for 40 days on average, corresponding to the full course of CCRT. The intervention group showed significantly lower severe hematologic toxicities (5% vs 23%, $p = 0.03$) and improved treatment completion rates compared to the placebo group. The study highlights the potential of immune-enhancing supplements to reduce CCRT-related toxicities and improve treatment adherence. Nonetheless, it may not be the exclusive beneficial factor, as it was not an isocaloric study, and the supplemented group received additional protein and calories (500 kcal/d) from the arginine, glutamine, and fish oil supplementation.

May et al. (2002) conducted a randomized, double-blind, controlled study to assess the effects of HMB, arginine, and glutamine supplementation in patients with advanced cancer cachexia. Thirty-two patients with solid tumors received either the HMB/Arg/Gln mixture (3 g HMB, 14 g arginine, 14 g glutamine daily) or an isonitrogenous control for 24 weeks. The supplemented group experienced an increase in body weight, primarily due to gains in lean body mass, while the control group showed continued muscle loss. These improvements were maintained throughout the study, and body composition changes were validated through multiple assessment methods. The supplementation was well-tolerated, with no adverse effects reported. Although no significant changes in fat mass or quality of life were observed, the results suggest that HMB, arginine, and glutamine may help counteract muscle wasting in CC, possibly by reducing protein breakdown and enhancing protein synthesis (May et al., 2002).

Changes in muscle mass and function were evaluated in 55 pre-cachectic and cachectic patients with lung cancer, who received an oral nutritional supplement containing ≈200 kcal, 10 g whey protein, 2.0 g eicosapentaenoic acid/docosahexaenoic acid in fish oil, and 10 μg 25-hydroxy-vitamin D3 during a 12-week randomized, double-blind, controlled pilot trial. The group of patients that received the supplement did not show differences in waist and calf circumference, appendicular lean body mass, grip strength, or daily walking distance compared to the control group (Laviano et al., 2020).

Another study evaluated the effects of an oral nutritional supplement containing several compounds, including vitamin C, vitamin B5, vitamin B9, and vitamin D alongside nutritional counseling, offered to 30 patients with lung cancer under chemotherapy (Torricelli et al., 2020). Compared to the control group, the patients who received the nutritional supplement maintained or increased their body weight after 90 days and reported improvements in chemotherapy-related symptoms, including anorexia, weakness, dyspnea, pain, and hemoptysis, leading to enhanced quality of life (Torricelli et al., 2020).

2 Conclusion

Nutritional intervention strategies have been explored owing to the potential impact on mitigation of muscle loss in patients with CC (Figure 1), a multifactorial condition marked by accelerated muscle protein degradation. Most cancer patients face treatment and disease side effects, compromising food intake of calories, protein, and bioactive compounds, worsening the nutritional status and aggravating the inflammatory and catabolic state.

To maximize the anabolic potential, it is crucial to detect risks of significant weight loss at the time of diagnosis and, through continuous nutritional screening, provide early nutritional intervention to prevent compromising of clinical outcome.

High-protein diets were reported to be associated with maintenance of muscle mass, while WP supplementation, a good strategy to increase protein intake, stands out for allowing rapid absorption and high plasma concentration of essential amino acids. WP improved body composition, muscle strength, and overall treatment tolerance in cancer patients undergoing chemotherapy. Additionally, HMB emerges as a promising option due to its dual action of promoting protein synthesis and inhibiting muscle degradation.

Creatine supplementation has yielded inconsistent results in human studies, with no significant improvements in muscle mass or quality of life observed in colorectal cancer patients. Despite promising results in improving lean mass and reducing fatigue in advanced cancer patients, LC requires further investigation to confirm efficacy.

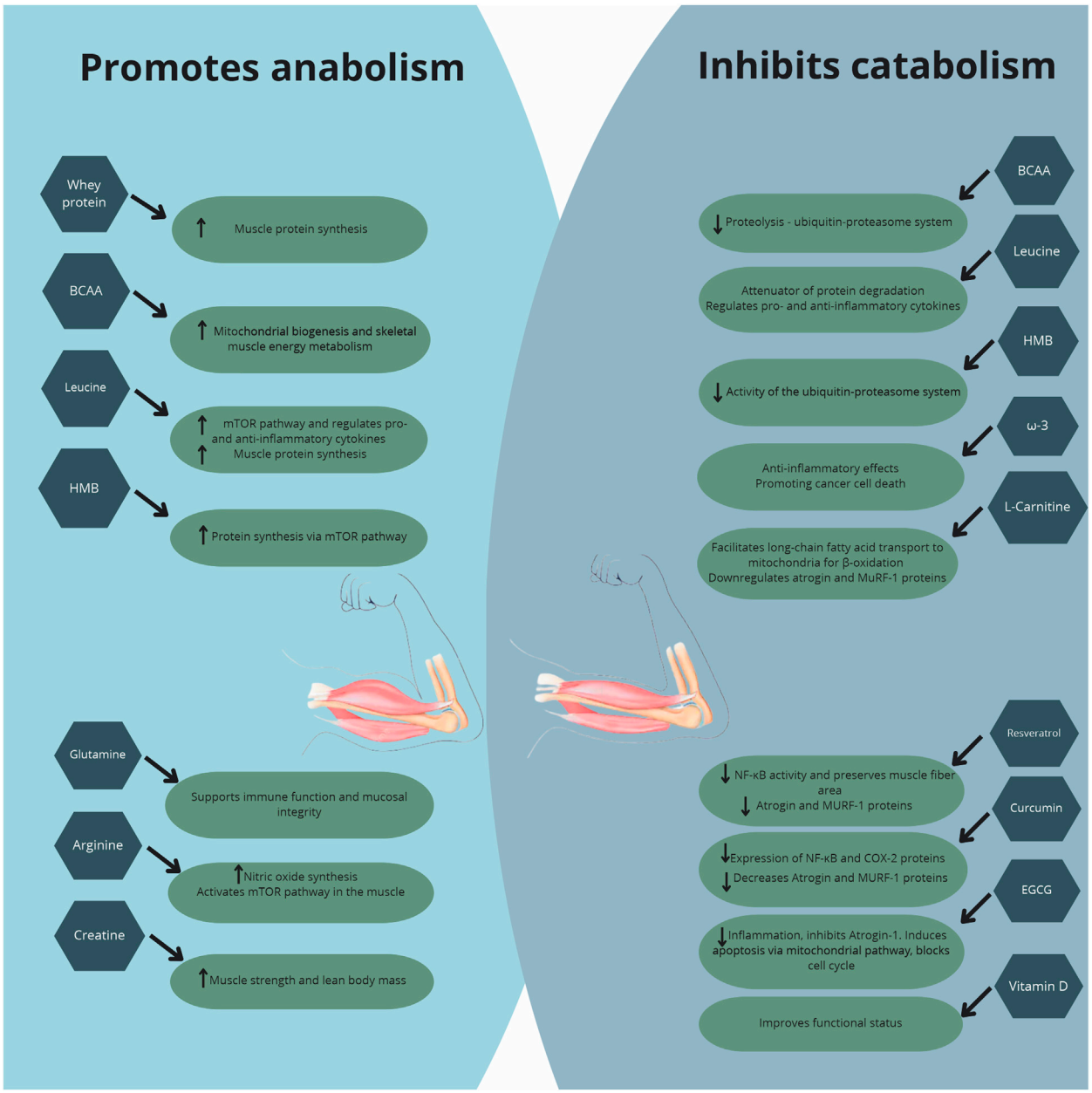

FIGURE 1
Major effects of nutritional supplementation in cancer cachexia.

The use of omega-3 fatty acids has shown contradictory results. When effectiveness was evaluated by meta-analysis, supplementation had no significant impact on inflammatory markers or muscle mass maintenance. Similarly, while antioxidants like resveratrol, curcumin, and EGCG have been explored for potential benefits, the clinical evidence in humans remained limited and inconclusive.

Combined nutrient supplementation, including arginine, omega-3 fatty acids, glutamine, and HMB, has demonstrated varying degrees of benefit in cancer patients by modulating the inflammatory response, reducing treatment-related toxicities, and preserving lean body mass. While improvements in immune function, symptom control, and quality of life were observed, the impact on muscle preservation and physical function remains inconsistent across studies.

In conclusion, the management of cancer-associated cachexia requires an individualized approach associated with nutritional counseling to manage treatment and disease side effects and to ensure feasible ways to achieve dietary requirements. An early intervention is mandatory or, at least, as soon as possible. The total protein intake matters and a dose of 1.4–2 g/kg of body weight is more effective in preventing muscle loss. When combined with other nutrients, it may improve the inflammatory profile. Further research, particularly well-designed clinical trials, is needed to determine optimal dosages and evaluate long-term outcomes, especially across different stages of cachexia and in conjunction

with other therapeutic approaches to establish their full potential and clinical utility.

Author contributions

JF: Conceptualization, Writing–original draft, Writing–review and editing. MFA: Conceptualization, Writing–original draft, Writing–review and editing. GC: Conceptualization, Writing–original draft, Writing–review and editing. JR: Writing–original draft, Writing–review and editing. MC: Writing–original draft, Writing–review and editing. GA: Writing–original draft, Writing–review and editing. MS: Conceptualization, Supervision, Writing–original draft, Writing–review and editing.

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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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Sodium nitrate regulates senescence accompanied by aortic atherosclerosis in ApoE$^{-/-}$ mice through the miR-34a/FGF-21 axis

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Introduction: Increasing evidence indicates that cellular senescence is a significant risk factor for atherosclerosis (AS).

Methods: In the present study, we used an apolipoprotein E knockout (ApoE$^{-/-}$) mouse model to address the effect of sodium nitrate on senescence accompanied by atherosclerosis. After sodium nitrate intervention, the degree of AS pathological and cellular senescence changes was evaluated in mouse aortic. At the same time, an H_2O_2-induced human arterial endothelial cell (HAoEC) senescence model was established to verify the role of miR-34a in AS-associated senescence.

Results: We observed that sodium nitrate decreased the Oil Red O-positive area, reduced the serum cholesterol (CHO) and triglyceride (TG) concentrations, and relieved inflammatory reactions in ApoE$^{-/-}$ mice. Moreover, the SA-β-Gal-positive area, the expression of cell cycle regulation-related genes and miR-34a in the aorta decreased after sodium nitrate treatment. Furthermore, sodium nitrate upregulated the expression of FGF21 by inhibiting the expression of miR-34a, thereby rescuing the senescent phenotype of HAoECs. These results suggested that sodium nitrate could rescue the endothelial cell senescence phenotype and alleviate aortic atherosclerosis in ApoE$^{-/-}$ mice by regulating the miR-34a/FGF21 axis.

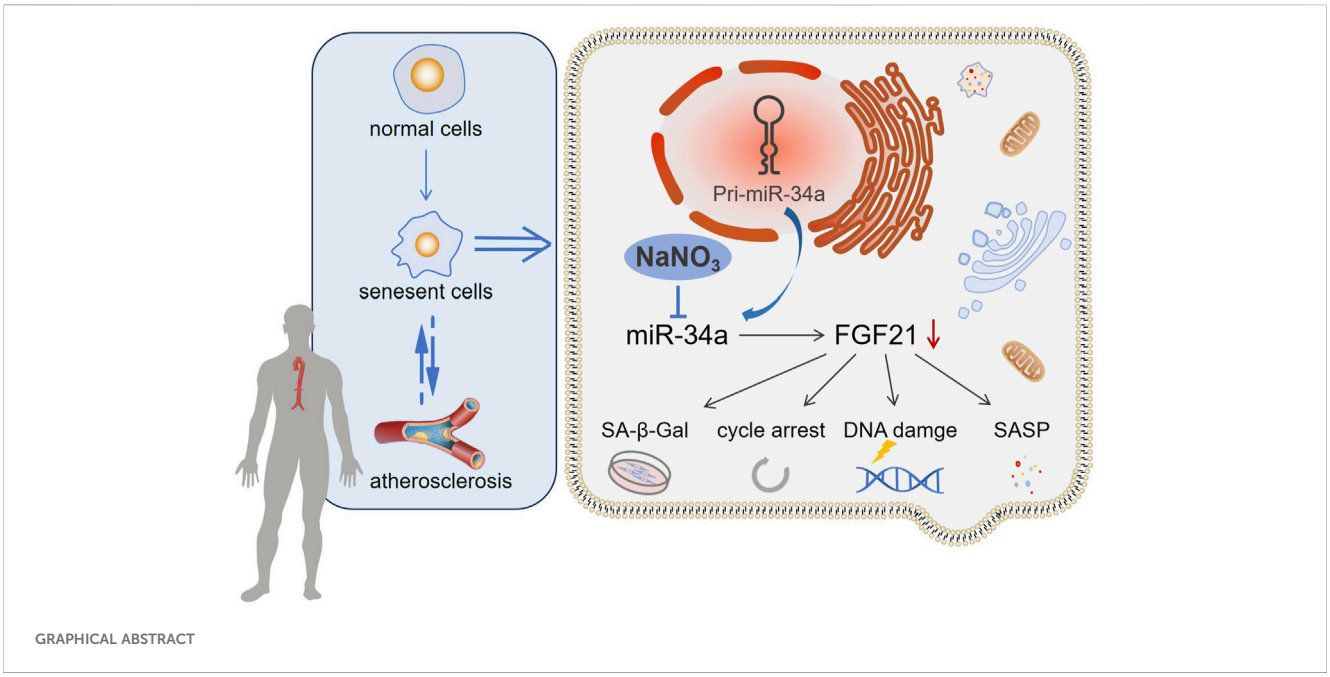
Discussion: These findings might lead to the introduction of a new therapy for senescence-related diseases in the future.

KEYWORDS

atherosclerosis, senescence, sodium nitrate, miR-34a, FGF21

1 Introduction

Atherosclerosis (AS) is a chronic progressive pathological process that refers to the continuous deposition and aggregation of lipids in the intima of arteries and some other components in the blood; this process leads to damage of the intima and proliferation and movement of smooth muscle cells and collagen fibers to the intima and is accompanied by



GRAPHICAL ABSTRACT

various degrees of vascular pathological processes, such as necrosis and calcification (Libby et al., 2019). Inflammation theory has been the most recognized theory in recent years. AS is not a simple lipid deposition disease. It is considered a long-term, chronic, low-level inflammatory response process (Tedgui and Mallat, 2006). Inflammation plays a vital role in the occurrence and development of AS and is a marker and important driver factor of pathological vascular remodeling in AS.

Senescence, a kind of stress-induced and persistent cell cycle arrest, is induced by many stimulating factors, including carcinogenic signal transduction, telomere dysfunction, DNA damage, mitochondrial dysfunction, and oxidative stress (He and Sharpless, 2017; Lopez-Otin et al., 2023). During the senescence process, cells secrete various characteristic factors, collectively known as the senescence-related secretory phenotype (SASP), such as chemokines, interleukins, and proteases (Borghesan et al., 2020; De Cecco et al., 2019). These factors promote the senescence of local cells and may even cause aging of the system via a paracrine pathway (Tasdemir and Lowe, 2013). Increasing evidence indicates that cellular senescence and aging are closely related to the occurrence and development of various age-related diseases (Wiley and Campisi, 2021).

Many studies have verified that AS is closely related to premature aging in organisms, and AS plaques also exhibit phenotypes related to cell senescence, such as decreased cell proliferation ability, growth arrest and apoptosis, DNA damage, and mitochondrial dysfunction (Xu et al., 2020). Simultaneously, various inflammatory factors are secreted to promote the development of AS and inhibit plaque repair. In addition, a variety of metalloproteinases are secreted by senescent endothelial cells, monocytes, macrophages, and foam cells and can promote the thinning of fiber caps and cause instability of atherosclerotic plaque (Wang et al., 2015). This means that senescent cells may be an important source of local, chronic, and low-level inflammatory and plaque instability factors while providing various additional plaque instability factors (Stojanovic et al., 2020; Childs et al., 2018). Senescence may play an important role in the entire process, from the occurrence of AS to plaque rupture. Different types of senescent cells play important roles in the process of atherosclerosis (Childs et al., 2016). The senescence of endothelial cells, vascular smooth muscle cells, macrophages, and other cells can be observed at the early stage of AS. These senescent cells can further expand the necrotic core, accelerate the degeneration of the extracellular matrix, decrease the thickness of the fiber cap, erode it, calcify it, and assist angiogenesis in plaques (Bentzon et al., 2014).

The beneficial effects of inorganic nitrates on various biological processes have been revealed in recent decades. Dietary nitrate can act as a reservoir of NO in the body through the nitrate-nitrite-NO pathway. Nitrate has a variety of biological activities similar to NO, such as improving exercise ability, protecting the digestive system, lowering blood pressure, and assisting in tumor treatment (Qin and Wang, 2022).

Bakker et al. reported that dietary nitrate can play an anti-inflammatory role by regulating the expression level of inflammatory factors (Bakker et al., 2016). Clinical data have shown that long-term nitrate supplementation can improve the degree of arteriosclerosis and reduce systolic blood pressure and proinflammatory factor levels in elderly volunteers (Rammos et al., 2014). Daily supplementation with nitrate can alleviate cellular senescence induced by D-galactose and natural senescence in mice and inhibit the occurrence of age-related lesions in the liver

Abbreviations: AS, atherosclerosis; ApoE$^{-/-}$, apolipoprotein E knockout; QPCR, Quantitative Real-time PCR; NOCs, N-nitroso compounds; SA-β-Gal, Senescence-associated β-D-Galactosidase; HAoECs, human arterial endothelial cells; FGF21, fibroblast growth factor 21; CHO, cholesterol; TG, triglyceride; SASP, senescence-related secretory phenotype; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; rhFGF21, recombinant human protein of FGF21.

TABLE 1 The sequences of the miR-34a mimic and miR-34a inhibitor.

Name	Sequence
miR-34a mimic NC	Sense: UUGUACUACACAAAAGUACUG Antisense: GUACUUUUGUGUAGUACAAUU
miR-34a mimic	Sense: UGGCAGUGUCUUAGCUGGUUGU Antisense: AACCAGCUAAGACACUGCCAUU
miR-34a inhibitor NC	CAGUACUUUUGUGUAGUACAA
miR-34a inhibitor	ACAACCAGCUAAGACACUGCCA

(Wang et al., 2018). However, the effect of dietary nitrate on senescence accompanied by AS and the underlying mechanism is still unknown.

In this study, ApoE$^{-/-}$ mice fed a high-fat diet were used to establish a mouse model of aortic AS, and the therapeutic effect of sodium nitrate on AS and accompanying cell senescence was evaluated. In addition, we established an H_2O_2-induced senescence model in HAoECs to clarify the mechanism through which sodium nitrate alleviates cell senescence.

2 Materials and methods

2.1 Cell culture

Human aortic endothelial cells (HAoECs) were purchased from ATCC (United States). HAoECs were cultured in Dulbecco's modified Eagle's medium (DMEM, 11965092, Gibco, United States) supplemented with 10% fetal bovine serum (FBS, 12003C, Sigma, United States).

2.2 Cell senescence model and treatment

HAoECs were treated with H_2O_2 (600 μM, 7722-84-1, Sinopharm Chemical Reagent, China) for 24 h to establish a cell senescence model and then cocultured with 50 μM sodium nitrate (221341, Sigma, United States). To verify the specific effects of miR-34a, HAoECs were transfected with 10 nM miR-34a mimic, inhibitor or corresponding NC (Sangon Biotech, China) according to the manufacturer's instructions for the RNATransMate reagent (E607402, Sangon Biotech, China). All four miRNA were dissolved in DEPC water provided with the kit. FGF21 recombinant protein (50 ng/mL) (rhFGF21, HY-P7345, MedChemExpress, United States) were also used to treat HAoECs concomitantly with miR-34a mimic. The sequences of the miR-34a mimic, inhibitor and the corresponding control sequences are listed in Table 1.

2.3 Animal studies

ApoE$^{-/-}$ mice (8 weeks old, male, with a C57BL/6 background) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (China). All animals were maintained on a 12 h/12 h light/dark cycle, with a temperature of 22°C ± 2°C and controlled humidity ranging from 50% to 60%. 36 ApoE$^{-/-}$ mice were randomly divided into 3 groups (12 mice per group: three aorta of mice were used for oil red o-staining, three for SA-β-Gal staining, three for immunohistochemical detection, and the rest for RNA extraction): normal group (NFD), fed normal chow (1022, Beijing HFK Bioscience CO., LTD, China) and double distilled water for 12 weeks; high-fat group (HFD), fed high-fat chow (H10540, Beijing HFK Bioscience CO., LTD, China) containing 40 kcal % fat (1.254% cholesterol by weight), 20% kcal protein and 40 kcal % carbohydrates for 12 weeks; and sodium nitrate treated group (HFD + $NaNO_3$), fed high-fat chow and drank 1 g/mL sodium nitrate for 12 weeks. All animal experiments were performed under protocols approved by the Institutional Animal Care and Use Committee of the Laboratory Animal Center (IACUC DWZX-2021-714).

2.4 Serum lipid profile

Whole blood was collected from the canthal vein of mice at weeks 0, 3, 6, 9, and 12, and left at room temperature for 30 min, then at 4°C for 30 min, and finally centrifuged at 3,000 rpm for 10 min at 4°C to isolate serum. Total cholesterol (CHO) (S03042, Leidu, China) and triglyceride (TG) were measured using a total cholesterol determination kit and triglyceride determination kit (S03027, Leidu, China), respectively.

2.5 ELISA

Blood was collected from the angular vein of ApoE$^{-/-}$ mice at weeks 3, 6, 9, and 12, and serum was sampled. The expression of IL-6 and TNF-α was detected by using an ELISA kit (MEK1016, Boster, United States) according to the manufacturer's protocol. Briefly, the precoated 96-well plate was equilibrated to room temperature and incubated with 50 μL of diluted standard and mouse serum. The cells were washed three times with 1× wash buffer for 1 min each. Then, 50 μL of biotin-labeled antibody was added to the 96-well plate and incubated for 60 min. After washing with wash buffer, 50 μL of streptavidin-labeled horseradish peroxidase was added, and the mixture was incubated for 15 min. Then, the cells were washed again, and 50 μL of mixed substrate A and substrate B were added to each well (Substrate A: Substrate B = 1:1). All of the above operations were performed at room temperature. Finally, the analysis was performed using a Q-View Imager LS (QuansysBio, United States).

2.6 Oil red O staining

To quantify the burden of atherosclerotic plaques, the adventitial fat was removed from the harvested aortas before fixation. After fixation in a neutral formalin solution overnight, the aortas were washed with PBS and then incubated in Oil Red O solution (G1015, Servicebio, China) at RT for 1 h. After the PBS washes, the samples were imaged via optical microscopy (Nikon, Japan), and the percentage of positive staining was analyzed via ImageJ software (NIH, United States).

2.7 SA-β-Gal staining

At the endpoint of the experiment, the mice were sacrificed under excessive anesthesia with pentobarbital sodium. After the aortas were isolated, SA-β-Gal staining was performed following the manufacturer's instructions (C0602, Beyotime, China), and the results were visualized via optical microscopy (Nikon, Japan). The percentage of positive staining was calculated using ImageJ software (NIH, United States).

2.8 Immunohistochemistry

At the endpoint of the experiment, the aortas of the $ApoE^{-/-}$ mice were removed after cardiac perfusion with sterile saline, and the surface adipose tissue was removed in PBS. The incised aortas were fixed in 4% paraformaldehyde solution and embedded in paraffin before sectioning. After antigen retrieval, the sections were placed in a 3% hydrogen peroxide solution that blocks endogenous peroxidases. The paraffin sections were blocked with a 3% BSA solution (GC305010, Servicebio, China) for 30 min at RT and incubated with anti-P16 (dilution ratio: 1:600; ab241543, abcam, UK) overnight at 4°C. The next day, the sections were incubated with goat anti-rabbit IgG-HRP (dilution ratio: 1:200; GB23303, Servicebio, China) for 50 min at RT. Then, a DAB chromogenic solution (G1212, Servicebio, China) was added dropwise for chromogenic development, and a hematoxylin staining solution (G1004, Servicebio, China) was used for counterstaining. After the tissue was covered with mounting gel (G1404, Servicebio, China), it was photographed under a light microscope (Nikon, Japan) for observation. The P16-positive area ratio (positive area ratio = positive area/tissue area) was calculated with Aipathwell software (Servicebio, China), and the P16 expression level was evaluated.

2.9 Intracellular SA-β-Gal detection

HAoECs were cultured in 12-well plates at a density of 100,000 cells/well and treated with the corresponding reagents described in Section 2.2 for 24 h. Then, the culture medium was discarded, the cells were fixed with 0.5 mL of staining fixation solution for 15 min, and the cells were subsequently incubated with 0.5 mL of SA-β-Gal staining working fluid overnight. Five visual fields were observed through an optical microscope, and the percentage of positively stained areas was calculated.

2.10 Cell proliferation assay

HAoECs were seeded in 96-well plates at a density of 8,000 cells/well. After cell adhesion, the corresponding reagents described in Section 2.2 was added, and the cells were cultured for 96 h. After the cells were incubated with CCK-8 working solution (C0602, Beyotime, China) at 37°C for 1.5 h, the optical density (OD) at a wavelength of 450 nm was measured with a microplate reader (Thermo, United States).

2.11 Real-time PCR analysis

An RNA Rapid Extraction Kit (RN001, ES Science, China) was used to extract RNA from mouse aortas or HAoECs according to the manufacturer's protocol. A Rapid Reverse Transcription Kit (RT001, ES Science, China) was used to synthesize complementary DNA, and PCR was performed on an ABI 7500 Fast (Applied Biosystems, United States) using qPCR SYBR Green Master Mix (11184ES03, Yeasen, China). All the results were calculated using the $2^{-\Delta\Delta CT}$ method by normalization to the expression of GAPDH (human), Actin (mouse) or U6. The sequences of primers used are listed in Table 2 (the primers for the internal reference gene U6 was obtained from the reagent kit).

2.12 Western blot

HAoECs were collected and lysed in RIPA buffer (P0013C, Beyotime, China) supplemented with protease inhibitors, after which the protein concentration was detected using a BCA protein quantitative kit (23225, Thermo Scientific, United States). The proteins were separated by 12% SDS–PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. Then, the PVDF membrane was blocked with 5% skim milk powder and incubated overnight with the corresponding primary antibodies at 4 °C: anti-RB (dilution ratio: 1:500, #9309; Cell Signaling Technology, United States), anti-P53 (dilution ratio: 1:1000, #48818; Cell Signaling Technology, United States), anti-P21 (dilution ratio: 1:1000, #80772; Cell Signaling Technology, United States), and anti-GAPDH (dilution ratio: 1:5000, #10494-1-AP; Proteintech, China). The next day, the membranes were washed with TBST four times for 5 min each time and incubated with the following secondary antibodies for 1 h at RT: goat anti-rabbit IgG-HRP (dilution ratio: 1:3000, #GB23303; Servicebio, China) and goat anti-mouse IgG-HRP (dilution ratio: 1:000, #GB23301; Servicebio, China). After washing with TBST four times, the signal was detected by using an enhanced chemiluminescence (ECL) kit (PE0010, Solarbio, China).

2.13 Immunofluorescence

The DNA damage foci of HAoECs were detected using a DNA Damage Assay Kit (C2035S, Beyotime, China) following the manufacturer's instructions. Briefly, the treated cells were fixed with 4% paraformaldehyde at RT and coincubated with the rabbit monoclonal antibody γ-H2AX overnight at 4°C. Then, an anti-rabbit secondary antibody was added for 1 hour at RT in the dark. After 10 min of coincubation with DAPI to stain the nuclei, images were acquired using fluorescence confocal microscopy (CrestOptics, Italy). ImageJ software (National Institutes of Health, United States) was used to count the number of DNA damage foci (National Institutes of Health, United States).

TABLE 2 The sequences of the real-time PCR primers.

Primer name[a]	Forward primer sequence	Reverse primer sequence
Actin-Mus	TCTTTGCAGCTCCTTCGT	GACCCATTCCCACCATC
P53-Mus	AACGCTTCGAGATGTTCC	GTTTGGGCTTTCCTCCTT
P21-Mus	GATCCCCTTTGCCACTC	TCACCAGATTAACCCTCCA
P16-Mus	CGTGCGATATTTGCGTTC	ACGTTCCCAGCGGTACA
RB-Mus	CAAAAGAAGTGCTGAAGGC	CCGCTGGGAGATGTTTAC
IL-1β-Mus	TGTCCTGATGAGAGCATCC	AAGGTCCACGGGAAAGAC
IL-6-Mus	AGCCCACCAAGAACGATAG	GGTTGTCACCAGCATCAGT
IL-8-Mus	CACTCCACTATGGGCTGTT	TGGGGCACTGAAGACAA
TNF-α-Mus	CGCTGAGGTCAATCTGC	GGCTGGGTAGAGAATGGA
FGF21-Mus	GGTCAAGTCCGGCAGAG	CGCCTACCACTGTTCCAT
GAPDH-Hu	CCTTCCGTGTCCCCACT	GCCTGCTTCACCACCTTC
P53-Hu	GAGGTTGGCTCTGACTGTACC	TCCGTCCCAGTAGATTACCAC
P21-Hu	ATGA-GTTGGGAGGAGGCA	CTGAGCGAGGCACAAGG
P16-Hu	GATCCAGGTGGGTAGAAGGTC	CCCCTGCAAACTTCGTCCT
RB-Hu	TAAGAATGGCCCTAGAGTGG	TGCTACAAAAGAAGGCAAAGT
IL-1β-Hu	TGTCCTGATGAGAGCATCC	AAGGTCCACGGGAAAGAC
IL-6-Hu	TGAGGAGACTTGCCTGGT	GGGTCAGGGGTGGTTATT
IL-8-Hu	CAGTGAAGATGCCAGTGAAA	CAACCCTACAACAGACCCA
TNF-α-Hu	GAGGCCAAGCCCTGGTATG	CGGGCCGATTGATCTCAGC
FGF21-Hu	TTTCTGTGCTGGCTGGTC	GCTGGGCATCATCTGTGT
miR-34a-5p	TGGCAGTGTCTTAGCTGGTTG	Provided by the kit

[a]Full name of gene: Actin (actin, beta), P53 (transformation related protein 53), P21 (cyclin dependent kinase inhibitor 1A), P16 (cyclin dependent kinase inhibitor 2A), RB (RB transcriptional corepressor 1), IL-1β (interleukin 1 beta), IL-6 (interleukin 6), IL-8 (C-X-C motif chemokine ligand 15), TNF-α (tumor necrosis factor), FGF21 (fibroblast growth factor 21), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), miR-34a-5p (microRNA 34a).

2.14 Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 (San Diego, United States). One-way analysis of variance (ANOVA) was used to compare multiple groups, and a t-test was used to compare two groups. The data are expressed as the mean ± SD, and a P value less than 0.05 was used to indicate statistical significance.

3 Results

3.1 Sodium nitrate treatment alleviated aortic atherosclerosis in ApoE$^{-/-}$ mice

An AS mouse model was established through the consumption of a high-fat diet for 12 weeks to determine the effect of sodium nitrate on senescence accompanied by AS (Figure 1A). Mouse serum was collected at weeks 0, 3, 6, 9, and 12, and the mouse aorta was removed at the experimental endpoint. Our results showed that after the consumption of a high-fat diet, the concentrations of serum CHO (Figure 1B) and TG (Figure 1C) increased significantly, and sodium nitrate treatment decreased them. However, compared to the HFD group, the $NaNO_3$ group had no statistically significant difference in TG concentration at 6 weeks and CHO concentration at 12 weeks. The Oil Red O staining area is a key indicator for evaluating the size of AS plaques, and our results showed that, compared with that in the HFD group, the area stained with Oil Red O decreased by approximately 30% in the sodium nitrate treatment group (Figures 1D, E). To evaluate the effect of sodium nitrate on atherosclerosis, we detected inflammatory cytokine expression in mouse aortas by using QPCR and found that the expression levels of IL-1β, IL-6, IL-8, and TNF-α, especially IL-8, were significantly decreased in the sodium nitrate treatment group (Figure 1F). We also detected the expression levels of the serum inflammatory factors IL-6 and TNF-α by using ELISA. The results showed that sodium nitrate treatment reduced the concentrations of serum IL-6 (Figure 1G) and TNF-α (Figure 1H). These results indicated that sodium nitrate could effectively alleviate atherosclerosis and reduce systemic and local inflammatory reactions.

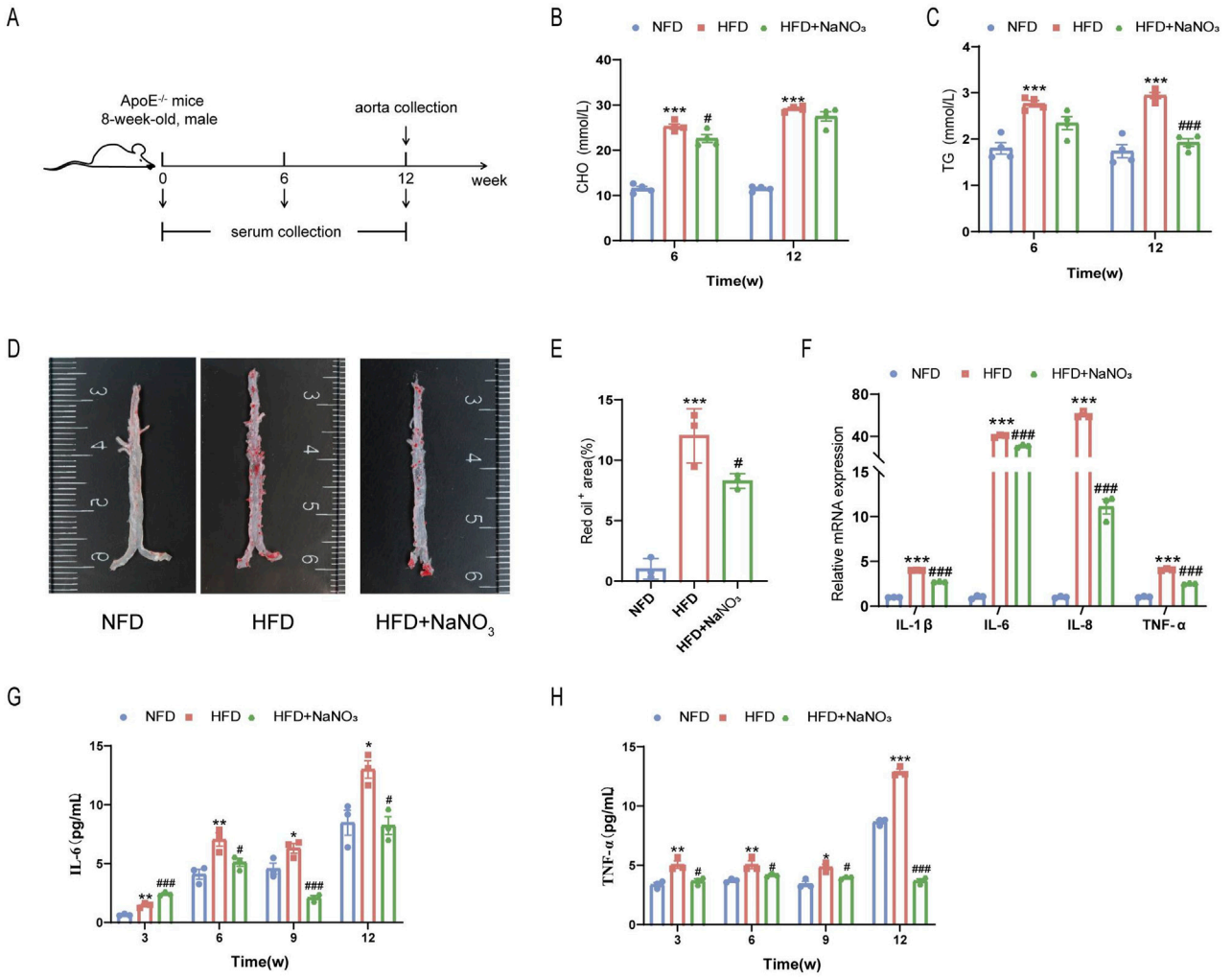


FIGURE 1
The effect of sodium nitrate on the AS. **(A)** Mice were treated as shown in the scheme. The concentrations of CHO **(B)** and TG **(C)** in the serum of the mice were detected. n = 4. **(D)** Representative image of Oil Red O staining of the aorta. **(E)** Statistical analysis of the data in **(D)**. n = 3. **(F)** QPCR was used to detect the expression levels of inflammatory factors in the aortas of the mice. n = 3. **(F)** The expression levels of the serum inflammatory factors IL-6 **(G)** and TNF-α **(H)** were detected. n = 3. *p < 0.05, **p < 0.01, ***p < 0.001, vs. the NFD group; #p < 0.05, ###p < 0.001, vs. HFD group.

3.2 Sodium nitrate treatment alleviated cellular senescence in ApoE$^{-/-}$ mice

To investigate the effect of sodium nitrate on the senescence of ApoE$^{-/-}$ mice, we collected aortas from the mice for SA-β-Gal staining (Figures 2A, B). The results showed that a high-fat diet significantly increased the percentage of SA-β-Gal-positive cells, while sodium nitrate treatment significantly decreased the percentage of SA-β-Gal-positive cells. The immunohistochemistry results showed that the expression level of P16, a classic indicator of cellular senescence, was significantly lower in the sodium nitrate treatment group than in the HFD group (Figures 2C, D). Compared to those in the NFD group, the expression levels of p53, p21, p16, and Rb in the aorta were upregulated in the HFD group. Sodium nitrate treatment downregulated the expression of these senescence-related genes (Figure 2E). These results showed that sodium nitrate could alleviate cell senescence accompanied by aortic atherosclerosis in ApoE$^{-/-}$ mice.

3.3 Sodium nitrate treatment alleviated the senescence of HAoECs

To further verify the effect of sodium nitrate on cell senescence, 600 μM H_2O_2 was used to induce senescence in the HAoECs. We examined the cell proliferation ability of HAoECs induced by H_2O_2 using a CCK-8 kit. H_2O_2 significantly inhibited the proliferation of HAoECs, and sodium nitrate treatment increased this ability by approximately 40% at 96 h (Figures 3A, B). The SA-β-Gal staining results also showed that sodium nitrate could reduce the increase in the percentage of H_2O_2-treated HAoECs (Figures 3C, D). The number of DNA damage foci induced by H_2O_2 was significantly reduced after sodium nitrate treatment (Figures 3E, F). Sodium nitrate treatment also downregulated the expression of the inflammatory cytokines IL-1β, IL-6, IL-8, and TNF-a (Figure 3G). Moreover, compared to those in the H2O2 group, the expression levels of cell cycle-related genes at the mRNA (p53, p21, p16, and Rb) levels were lower in the sodium nitrate treatment

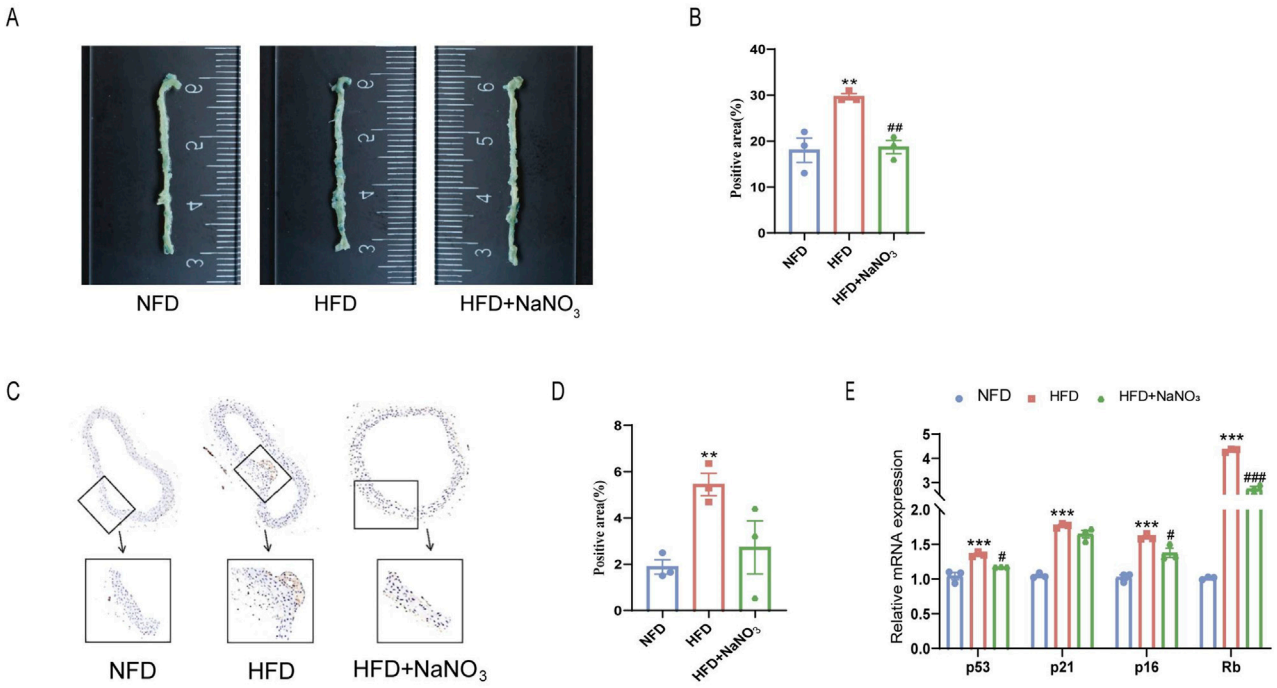


FIGURE 2
Effect of sodium nitrate on the senescence of aortic cells in ApoE$^{-/-}$ mice. **(A)** Representative image of SA-β-Gal staining of the aorta. **(B)** Statistical analysis of the staining-positive area in **(A)**. n = 3. **(C)** Representative images of P16 expression in the aorta detected by immunohistochemistry. **(D)** Statistical analysis of the stained area in **(C)**. n = 3. **(E)** The expression levels of p53, p21, p16 and Rb. n = 3. **p < 0.01, ***p < 0.001, vs. the NFD group; $^{\#}$p < 0.05, $^{\#\#}$p < 0.01, $^{\#\#\#}$p < 0.001, vs. HFD group.

group (Figure 3H). But no statistical differences in the expression of the cell cycle-related protein between groups (Figures 3I, J).

3.4 Sodium nitrate could regulate the expression of miR-34a and its downstream gene FGF21

To further explore the mechanism of action of Sodium nitrate in alleviating AS, we searched the GeneCards database for atherosclerosis or aging. The results showed that 276 of the 450 miRNA involved in AS regulation are closely related to aging (Figure 4A). Previous research and retrieval data suggest that AS and aging are inextricably linked (Wang and Bennett, 2012; Björkegren and Lusis, 2022). Therefore, we hypothesize whether sodium nitrate alleviates AS by inhibiting aging. Consequently, we selected miR-34a, which with the highest aging relevance score among the 276 common miRNAs, for following studies, (Figure 4B). To investigate whether miR-34a is involved in the anti-senescent effect of sodium nitrate, we detected the expression levels of miR-34a in the aortic tissue of ApoE$^{-/-}$ mice and HAoECs. The results showed that a 12-week high-fat diet and H_2O_2 treatment upregulated the expression of miR-34a, while sodium nitrate treatment downregulated its expression (Figure 4C). We also examined the expression of FGF21, the downstream gene of miR-34a. The results showed that sodium nitrate treatment upregulated the expression of FGF21 both in aortic tissue and in HAoECs (Figure 4D). Based on these results, we speculated that sodium nitrate could regulate the expression of miR-34a and FGF21 and that miR-34a is upstream of FGF21. To verify this hypothesis, a miR-34a-5p mimic and inhibitor were used to treat HAoECs. The results showed that the expression level of FGF21 decreased after miR-34a-5p mimic treatment and increased after miR-34a-5p inhibitor treatment (Figure 4E). The above results suggested that sodium nitrate may alleviate senescence by inhibiting the expression of miR-34a, thereby upregulating the expression of its downstream gene FGF21.

3.5 miR-34a could regulate the senescence of HAoECs

To further clarify the role of miR-34a in cellular senescence, miR-34a mimic and inhibitor were used to discover senescence-related phenotypes in HAoECs. The CCK-8 assay results showed that the miR-34a mimic could significantly inhibit the proliferation of HAoECs, and the HAoECs showed almost no proliferative ability after 48 h of treatment (Figure 4G). The miR-34a inhibitor had no effect on the proliferation of HAoECs (Figure 4H). The results also showed that the miR-34a mimic could significantly increase the percentage of SA-β-Gal- and γ-H2AX-positive cells compared to that in the mimic-NC group (Figures 4I–L). Moreover, compared with the inhibitor-NC, the miR-34a inhibitor significantly reduced the percentage of SA-β-Gal- and γ-H2AX-positive cells. The miR-34a mimic upregulated the expression of the inflammatory factors IL-1β, IL-6, IL-8 and TNF-a (Figure 4M) and further exacerbated the

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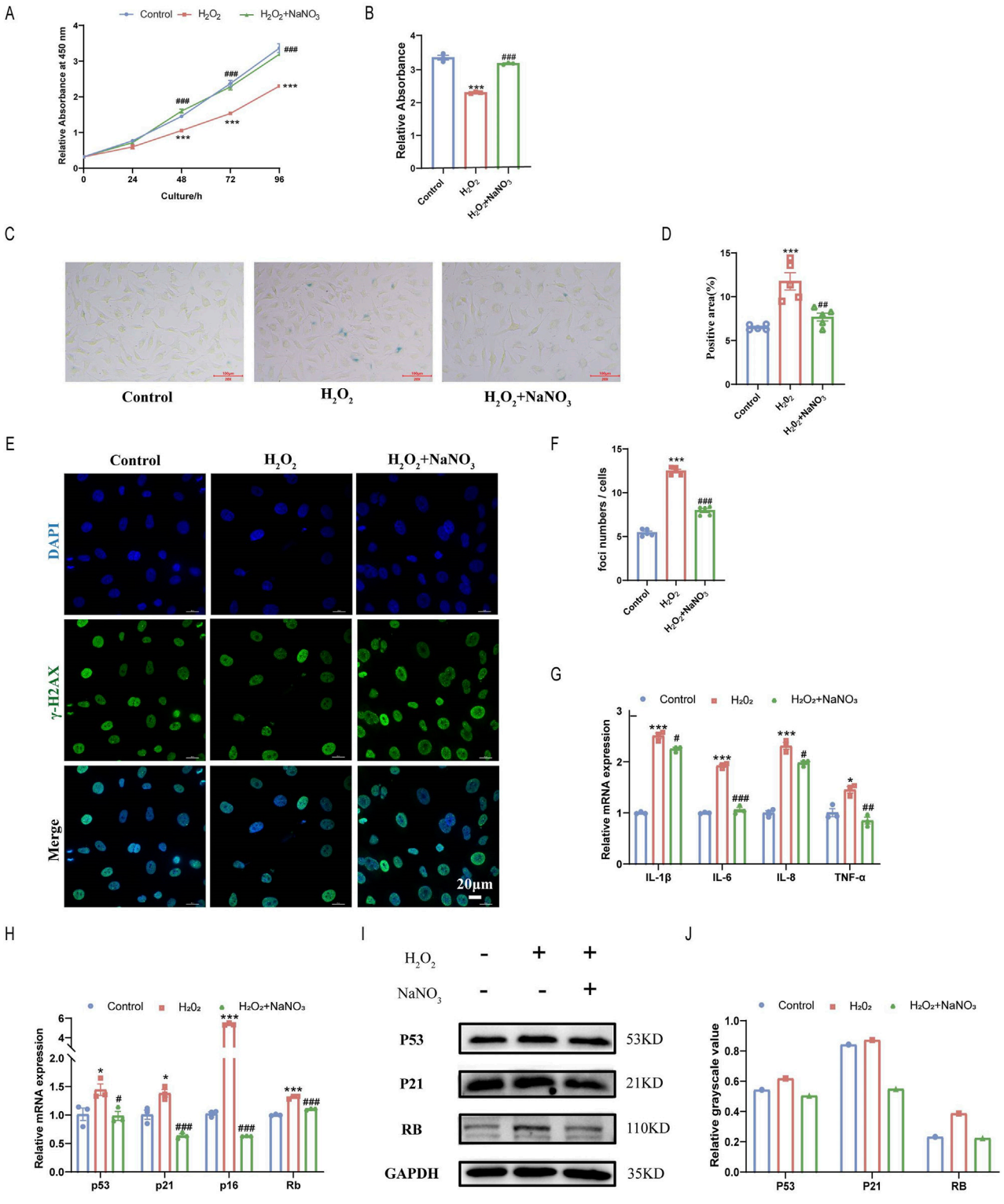

FIGURE 3
Effect of sodium nitrate on H_2O_2-induced senescence in HAoECs. **(A)** The CCK-8 method was used to evaluate the effect of sodium nitrate on the proliferation of HAoECs. **(B)** Statistical analysis of cell proliferation at 96 h **(A)**. n = 3. **(C)** Representative images of SA-β-Gal-stained HAoECs. Bar = 100 μm. **(D)** Statistical analysis of the stained area in **(C)**. n = 5. **(E)** Representative images of γ-H2AX staining of HAoECs. Bar = 10 μm. **(F)** Statistical analysis of the DNA damage foci in **(E)**. n = 5. **(G)** QPCR analysis of the mRNA expression of the inflammatory factors IL-1β, IL-6, IL-8, and TNF-α in HAoECs. n = 3. **(H)** QPCR was used to detect the relative expression levels of the cell cycle-related genes p53, p21, p16 and Rb in HAoECs. n = 3. **(I)** Western blotting was used to detect the expression of P53, P21, and RB in HAoECs. n = 3 **(J)** Quantitative analysis of the results in **(I)**. *p < 0.05, ***p < 0.001, vs. the control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, vs. H_2O_2 group.

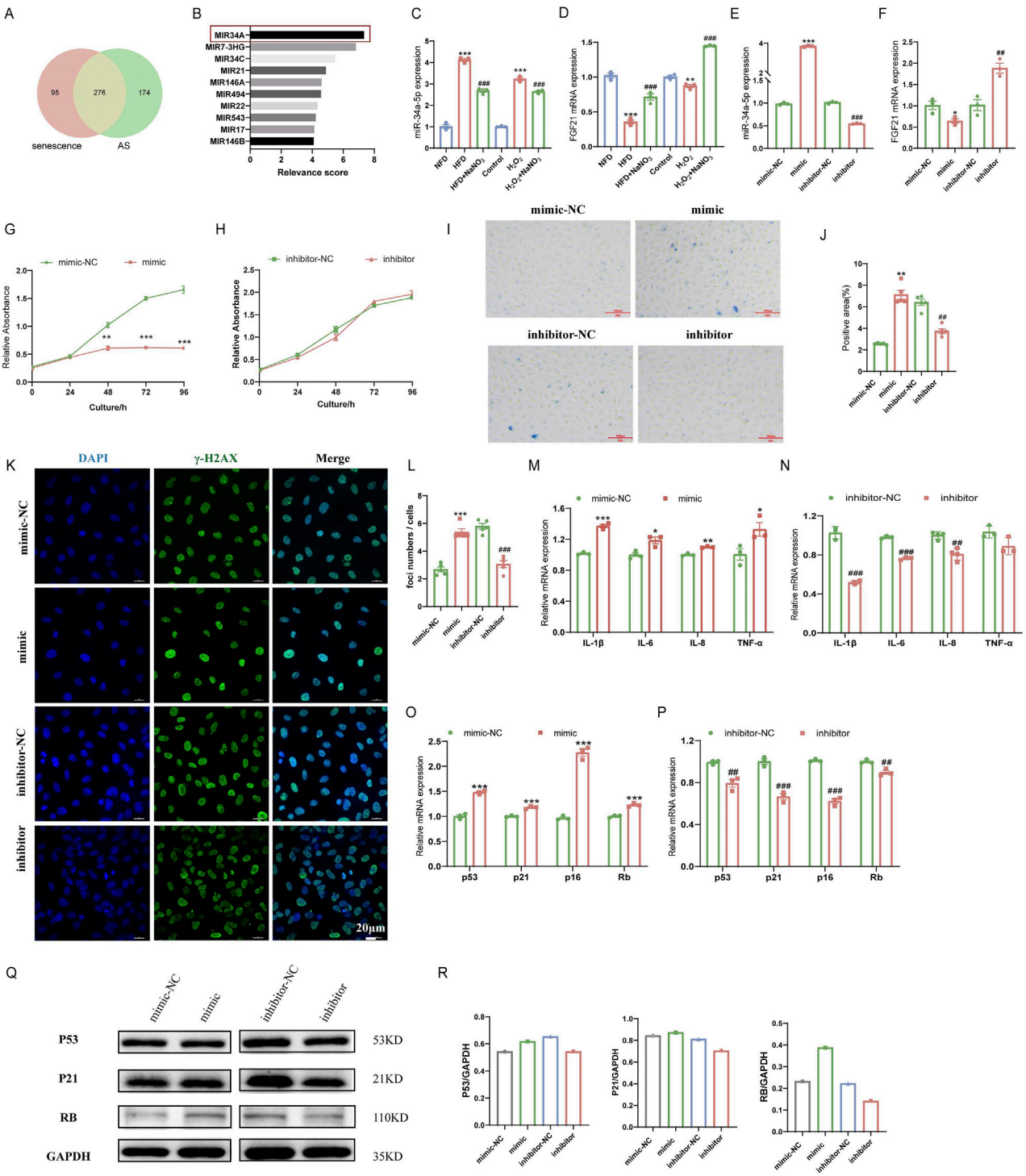

FIGURE 4
Effects of miR-34a and FGF21 in ApoE$^{-/-}$ mice and HAoECs. **(A)** Venn plots for GeneCards database search for senescence and AS-related miRNA. **(B)** Top 10 miRNAs with the highest correlation scores associated with senescence in the common 276 miRNAs. **(C)** QPCR was used to detect the expression level of miR-34a in ApoE$^{-/-}$ mice at 12 weeks and HAoECs at 24 h. n = 3. **(D)** QPCR analysis of FGF21 mRNA expression in ApoE$^{-/-}$ mice at 12 weeks and HAoECs at 24 h. n = 3. **(E)** QPCR was used to detect the expression levels of miR-34a in HAoECs after treatment with the miR-34a mimic or inhibitor at 24 h. n = 3. **(F)** QPCR analysis of FGF21 mRNA expression in HAoECs after treatment with the miR-34a mimic or inhibitor at 24 h. n = 3. **(G)** The CCK-8 method was used to evaluate the effect of the miR-34a mimic on the proliferation of HAoECs at 96 h. n = 3. **(H)** CCK-8 was used to detect the effect of the miR-34a inhibitor on the proliferation of HAoECs at 96 h. n = 3. **(I)** A representative image of SA-β-Gal-stained HAoECs after treatment with the miR-34a mimic or inhibitor at 24 h. n = 5. Bar = 100 μm. **(J)** Statistical analysis of the stained area in **(I)**. n = 5. **(K)** A representative image of γ-H2AX staining of HAoECs after treatment with the miR-34a mimic or inhibitor at 24 h. Bar = 20 μm. **(L)** Statistical analysis of the number of DNA damage foci in **(K)**. n = 5. **(M)** QPCR analysis of the mRNA expression of the inflammatory factors IL-1β, IL-6, IL-8 and TNF-α in HAoECs after miR-34a mimic treatment at 24 h. n = 3. **(N)** QPCR analysis of the mRNA expression of the inflammatory factors IL-1β, IL-6, IL-8 and TNF-α in HAoECs after miR-34a inhibitor treatment at 24 h. n = 3. **(O)** QPCR was used to detect the relative expression levels of the cell cycle-related genes p53, p21, p16 and Rb in HAoECs after miR-34a mimic

(*Continued*)

FIGURE 4 (Continued)
treatment at 24 h. n = 3. **(P)** QPCR was used to detect the relative expression levels of the cell cycle-related genes p53, p21, p16 and Rb in HAoECs in different treatment groups after miR-34a-5p inhibitor treatment at 24 h. n = 3. **(Q)** Western blotting was used to detect the protein expression of p53, p21, and Rb in HAoECs after miR-34a mimic or inhibitor treatment at 24 h. n = 3. **(R)** Quantitative analysis of the results in **(Q)**. *p < 0.05, **p < 0.01, ***p < 0.001, vs. the NFD group or the control group or mimic-NC; ##p < 0.01, ###p < 0.001, vs. HFD group or H_2O_2 group or inhibitor-NC.

inflammatory response. However, the miR-34a inhibitor downregulated the expression of IL-1β, IL-6, IL-8 and TNF-a (Figure 4N). Moreover, the expression levels of the senescence-related genes p53, p21, p16 and Rb were significantly upregulated after treatment with the miR-34a mimic (Figure 4O) and downregulated after treatment with the miR-34a inhibitor (Figure 4P). Changes in the expression of P53, P21 and RB at the protein level were consistent with the above results (Figures 4Q, R). These results indicated that high expression of miR-34a could accelerate cellular senescence in HAoECs, while inhibiting the expression of miR-34a could alleviate cell senescence.

3.6 FGF21 is involved in cell senescence

To further validate the antiaging effect of FGF21, the recombinant human protein of FGF21 (rhFGF21) was added to treat HAoECs. rhFGF21 reversed the decrease in HAoEC proliferation caused by the miR-34a mimic (Figures 5A, B). In addition, compared with the miR-34a mimic, rhFGF21 treatment reduced the percentage of SA-β-Gal-positive cells (Figures 5C, D) and γ-H2AX-positive cells (Figures 5E, F), decreased the expression of the proinflammatory factors IL-1β, IL-6, IL-8 and TNF-α (Figure 5G), downregulated the mRNA (p53, p21, p16 and Rb) and protein (P53, P21 and RB) expression of the cell cycle-related genes (Figures 5H–J). These results suggested that FGF21 was important in the anti-senescence effect of sodium nitrate.

4 Discussion

Cardiovascular disease has become the leading cause of death in humans. Among them, arteriosclerotic cardiovascular disease accounts for the most significant proportion of cases, and the incidence and mortality rate increase annually (Benjamin et al., 2019). The traditional treatment methods for AS mainly include drug treatment, surgical treatment, and lifestyle changes, which have shortcomings, such as limited treatment effects and high risk. In recent years, scholars have found that AS is a class of aging-related diseases that are not only more common in elderly individuals but also accompanied by the senescence of various cells (Tyrrell and Goldstein, 2020). Many treatments for young and middle-aged people have little effect on older people. Therefore, there is an urgent need to develop a new therapy to control this disease, especially when senescence occurs. Elucidating the mechanisms by which senescence promotes atherosclerotic cardiovascular disease will be fundamental to developing new treatments to reduce the burden of atherosclerosis caused by senescence.

Several animals, such as mice, rats, rabbits, and pigs, are often used to establish AS models at present. Among these models, the $ApoE^{-/-}$ mouse has become a standard animal model for AS research because of its simple modeling process and the advantages of vascular lesions being very similar to those of the human body (Emini et al., 2017). In this study, AS model was established by feeding 8-week-old male $ApoE^{-/-}$ mice a high-fat diet for 12 weeks, and each mouse ate about 4 g of diet and drank 5 mL of water per day. During the experiment, mouse body weights were measured at 0, 6 and 12 W. The experimental results showed that the weight of the $ApoE^{-/-}$ mice in the HFD group increased slightly at 6 W and decreased at 12 W. There was no significant change in the body weight of the mice after NaNO3 treatment when compared with HFD group. Lipid-related biochemical indices showed that a high-fat diet could increase the serum levels of CHO and TG. Moreover, it increased the area of aortic Oil Red O-stained plaques and upregulated the expression of TNF-α and other proinflammatory factors in the serum and aorta. This evidence indicated that we successfully established a mouse model of AS, and at the same time, we evaluated the relevant indicators of cell senescence in $ApoE^{-/-}$ mice. The results showed that the percentage of SA-β-Gal-positive cells was increased, and the expression levels of cell cycle-related genes (p53, p21, p16 and Rb) were also increased in the aortas of high-fat diet-fed mice. The above results suggested that $ApoE^{-/-}$ mice exhibit a certain degree of cellular senescence when AS occurs. Nitrates are widely distributed in natural environments, such as food, water, and air. Nitrates used as drugs for cardiovascular diseases can be traced back to ancient China in the 8th century AD (Lundberg et al., 2008). Nitrate effectively alleviates cell senescence and improves cardiovascular function. Therefore, sodium nitrate was selected to intervene in AS and its accompanying cellular senescence in $ApoE^{-/-}$ mice. Lu et al. found that high dietary sodium intake increased systolic blood pressure, but low dietary sodium intake exacerbated atherosclerosis in mice with hypercholesterolemia (Lu et al., 2013). In this study, we selected an intermediate sodium content between high and low concentrations for experimentation. However, we did not study the effects of different nitrate concentrations on aortic atherosclerosis in mice, and we will pay attention to this issue in future experiments. We found that sodium nitrate decreased the concentrations of CHO and TG in the serum of mice, reduced the percentage of positive aortic Oil Red O staining, and decreased the expression levels of inflammatory factors in the serum and aorta in our study. The above results showed that sodium nitrate could alleviate systemic and local inflammation in the aorta and has an anti-AS effect. This observation is not in agreement with the findings of Khambata RS et al. in $ApoE^{-/-}$ mice, where a 12-week nitrate treatment did not alter plaque size in HFD-fed mice (Khambata et al., 2017). This inconsistency may be due to the different components of the high-fat feed used. In Khambata RS' study, the high-fat feed contained 42 kcal % fat and ~0.2% cholesterol by weight, while in our study it contained 40 kcal % fat and ~1.254% cholesterol by weight. There are also studies that are consistent with our experimental results, such as Peng et al.'s

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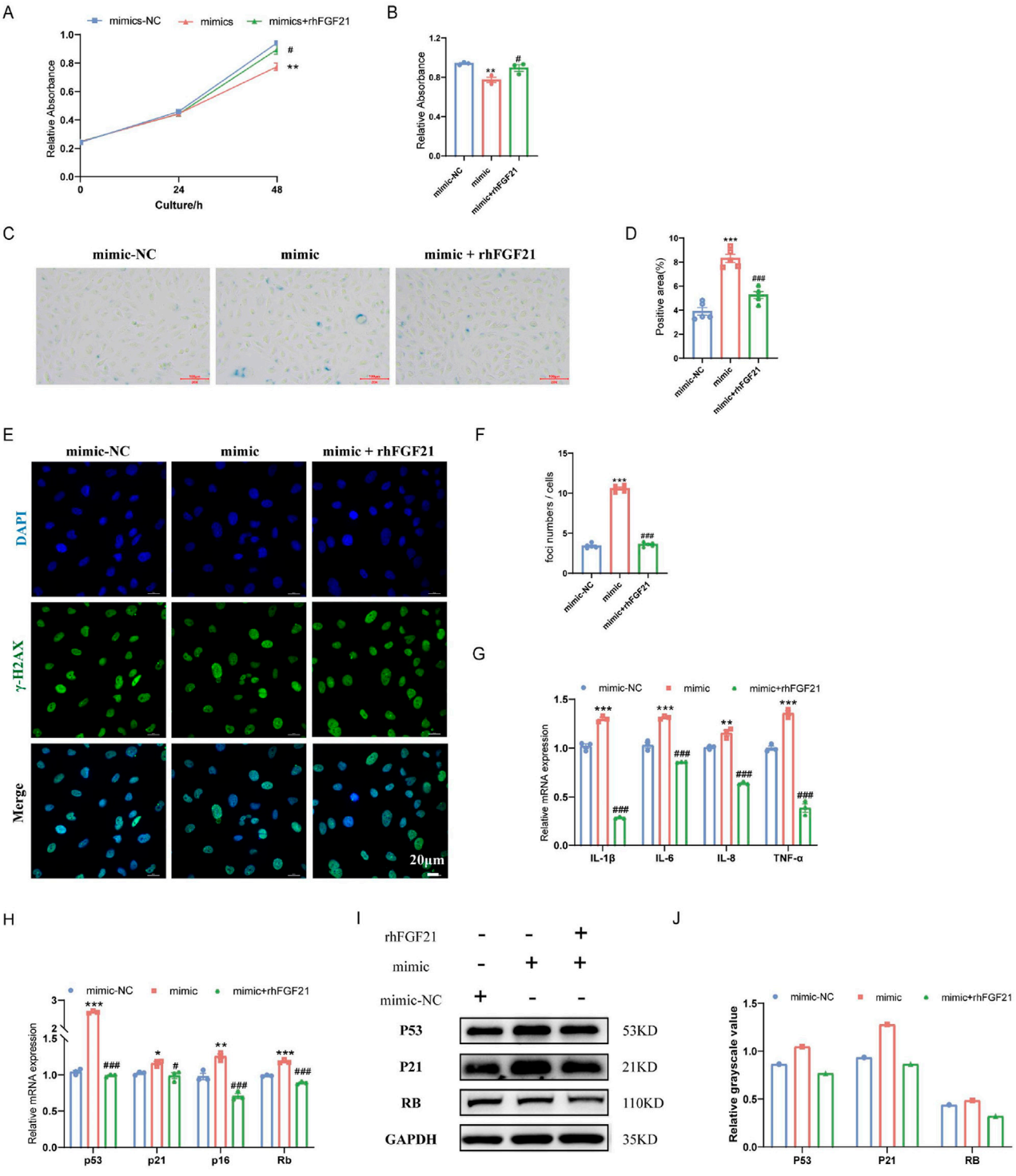

FIGURE 5
Effect of FGF21 on the senescence of HAoECs. **(A)** A CCK-8 kit was used to detect the effect of rhFGF21 on the proliferation of HAoECs at 48 h. **(B)** Statistical analysis of the HAoEC proliferation ability at 48 h **(A)**. n = 3. **(C)** Representative images of SA-β-Gal-stained HAoECs after treatment with the miRNA-34a mimic or rhFGF21 at 24 h. Bar = 100 μm. **(D)** Statistical analysis of the stained area in **(C)**. n = 5. **(E)** Representative images of γ-H2AX staining of HAoECs after miRNA-34a mimic and rhFGF21 treatment at 24 h. Bar = 20 μm. **(F)** Statistical analysis of the number of DNA damage foci in **(E)**. n = 5. **(G)** QPCR analysis of the mRNA expression of the inflammatory factors IL-1β, IL-6, IL-8 and TNF-α in HAoECs from different treatment groups at 24 h. n = 3. **(H)** QPCR was used to detect the relative mRNA expression of p53, p21, p16 and Rb in HAoECs after treatment with the miRNA-34a mimic or rhFGF21 at 24 h n = 3. **(I)** Western blotting was used to detect the protein expression of P53, P21, and RB in HAoECs after treatment with the miRNA-34a mimic or rhFGF21 at 24 h n = 3. **(J)** Quantitative analysis of the results in **(I)**. *p < 0.05, **p < 0.01, ***p < 0.001, vs. the mimic-NC group; $^{\#}$p < 0.05, $^{\#\#\#}$p < 0.001, vs. the mimic group.

research showed that dietary nitrate could reduce the size of atherosclerotic plaques in ApoE$^{-/-}$ mice fed an HFD (Peng et al., 2020). More research is needed to clarify the reasons for the differences between these studies.

Cellular senescence occurs throughout the whole process of AS occurrence and development. DNA damage, cell cycle arrest, massive secretion of inflammatory factors, enhanced SA-β-Gal activity, and mitochondrial dysfunction are the main features of cellular senescence (Lopez-Otin et al., 2023). Based on these findings, researchers have established a number of methods for identifying cellular senescence. However, due to the complexity of cellular senescence regulation, there is currently no way to assess cellular senescence alone. The combination of SA-β-Gal staining, cell cycle arrest, and inflammatory factor expression level is currently a well-recognized method for identifying cellular senescence (Schellenberg et al., 2012). Our experiments showed that sodium nitrate could reduce the percentage of positive aortic SA-β-Gal staining area and downregulate the expression of cell cycle-related genes (p53, p21, p16, and Rb). Therefore, sodium nitrate could alleviate the cellular senescence associated with AS.

H_2O_2 is a commonly used inducer that can cause senescence in various cells in a short period of time, simulating the process of oxidative stress experienced by cells during AS occurrence (Wang et al., 2013). Vascular endothelial cells cover the innermost part of the vascular lumen, and structural and functional impairment of these cells could initiate AS (Björkegren and Lusis, 2022). Therefore, HAoECs, a kind of aortic endothelial cell line, were used to establish a cell model induced by H_2O_2. Our results showed that H_2O_2 could successfully induce cellular senescence in HAoECs. However, after sodium nitrate treatment, cell proliferation was restored, the percentage of SA-β-Gal-positive cells and the number of DNA damage foci decreased, and the expression levels of cell cycle-related genes and inflammatory factors decreased. These results preliminarily validated the results of *in vivo* experiments and suggested that sodium nitrate could alleviate cellular senescence.

miRNAs are noncoding RNA segments with a length of approximately 22 nucleotides that can bind to corresponding mRNAs to regulate gene expression (Ghafouri-Fard et al., 2021). Studies have shown that inhibition of miR-34a expression has therapeutic effects in a variety of aging-related disease models. For example, inhibition of the miR-34a-mediated SIRT1/mTOR signaling pathway attenuates D-gal-induced senescence in rat brain tissue, and alveolar epithelial cell dysfunction is alleviated in aged miR-34a$^{-/-}$ pulmonary fibrosis mice (Kou et al., 2016; Cui et al., 2017). Clinical research data have also shown that the upregulation of miR-34a/b/c expression in peripheral blood mononuclear cells promotes vascular aging and the occurrence of atherosclerotic vascular disease (Gatsiou et al., 2021). Therefore, our experiments explored whether miR-34a can play an important role in the treatment of aortic atherosclerosis through anti-cellular senescence. We found that the expression level of miR-34a was significantly upregulated in ApoE$^{-/-}$ mice fed a high-fat diet, while miR-34a was downregulated considerably after dietary sodium nitrate treatment in our study. To further validate the role of miR-34a in therapeutic effect of $NaNO_3$, we first treated HAoECs with a miR-34a mimic and inhibitor. After miR-34a mimic treatment, the percentage of SA-β-Gal-positive cells was increased, cell proliferation was suppressed, the number of DNA damage foci was increased, and the expression levels of cell cycle-related genes and inflammatory factors were upregulated in HAoECs. The effects of the miR-34a inhibitor on HAoECs verified the ability of miR-34a to promote cell senescence. Our results demonstrated the critical role of miR-34a in AS-associated cell senescence.

Studies have shown that miR-34a is involved in regulating the expression of FGF21 (Fu et al., 2014). FGF21 is a hormone-like member of the FGF family, and as a stress hormone, it can be induced by endoplasmic reticulum stress, mitochondrial dysfunction, and autophagy disorders (Salminen et al., 2017). Research has shown that FGF21 can alleviate many age-related metabolic diseases (Youm et al., 2016). To explore whether $NaNO_3$ exerts anti-senescence effects by regulating the expression of miR-34a and its downstream gene FGF21, we tested the expression levels of FGF21 in ApoE$^{-/-}$ mice. The results showed that the high-fat diet significantly reduced the expression level of FGF21, and $NaNO_3$ treatment upregulated the expression of FGF21, which was consistent with the results at the cellular level.

To further clarify the role of FGF21 in the anti-senescence effect of $NaNO_3$, recombinant FGF21 protein was used to treat HAoECs. rhFGF21 rescued the senescence phenotype caused by the miR-34a mimic, as indicated by decreased cell proliferation, increased SA-β-Gal activity, upregulated cell cycle-related genes, and increased inflammatory factor secretion. Thus far, our results suggest that $NaNO_3$ might exert anti-senescence effects by regulating the expression of miR-34a and its downstream gene FGF21.

5 Conclusion

In conclusion, the results showed that $NaNO_3$ could reduce the size of aortic atherosclerotic plaque, inhibit local cell senescence, alleviate local and systemic inflammatory reactions, and downregulate the expression of miR-34a in ApoE$^{-/-}$ mice. Moreover, $NaNO_3$ inhibited the expression of miR-34a, upregulated the expression of the downstream gene FGF21, and alleviated H_2O_2-induced cellular senescence in HAoECs. This is the first report that $NaNO_3$ can reduce cell senescence accompanied by AS through the miR-34a/FGF-21 axis. These findings might lead to the introduction of a new therapy for senescence-related diseases in the future.

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 authors.

Ethics statement

The animal study was approved by Animal Care and Use Committee of the Laboratory Animal Center of AMMS. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

NT: Formal Analysis, Investigation, Methodology, Writing–original draft. ZH: Formal Analysis, Investigation, Methodology, Writing–original draft. HD: Formal Analysis, Investigation, Writing–original draft. LW: Formal Analysis, Investigation, Writing–original draft. JY: Formal Analysis, Investigation, Writing–original draft. JS: Formal Analysis, Investigation, Writing–original draft. LL: Formal Analysis, Investigation, Writing–original draft. JD: Formal Analysis, Investigation, Writing–original draft. HC: Formal Analysis, Investigation, Writing–original draft. XD: Conceptualization, Funding acquisition, Writing–review and editing. HW: Conceptualization, Resources, Visualization, Writing–review and editing.

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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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Carnosine alleviates oxidative stress to prevent cellular senescence by regulating Nrf2/HO-1 pathway: a promising anti-aging strategy for oral mucosa

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Introduction: Aging is associated with significant metabolic alterations that contribute to cellular senescence and age-related functional decline. As individuals age, an increased prevalence of oral diseases and a gradual decline in oral functions are observed. However, the metabolic shifts underlying oral mucosal aging remain unexplored.

Methods: We initially conducted histological analyses on the tongues from young (4-week-old), adult (4-month-old) and old (20-month-old) C57BL/6 mice to identify age-related alterations in the tongue mucosa. Subsequently, metabolomics analysis was performed to characterize metabolic profiles of mouse tongues across these age groups and identify metabolic biomarkers of oral mucosal aging. Then we validate the anti-senescence effect of carnosine and investigate its underlying mechanisms using a tert-butyl hydroperoxide (tBHP)-induced cellular senescence model in vitro. Finally, metabolomics analyses of human saliva and blood were conducted to explore associations between carnosine levels and systemic aging.

Results: Compared to young and adult mice, we observed epithelial atrophy and an accumulation of senescent cells in the tongue mucosa of old mice. After that, we found significant differences in the metabolic profiles among the young, adult, and old mouse tongues. Carnosine was identified as a potential biomarker of oral mucosal aging, as its levels declined significantly with age. Consistently, carnosine synthase 1 (CARNS1) activity decreased, and carnosinase 2 (CNDP2) activity increased with age in the tongue mucosa. Furthermore, carnosine protected oral epithelial cells from tBHP-induced cellular senescence by reducing oxidative stress, mitigating DNA damage, and downregulating Nrf2/HO-1 pathway. In humans, salivary and blood carnosine levels also declined with age and were significantly associated with age-related diseases.

Discussion: Our findings reveal dynamic metabolic reprogramming during natural oral mucosal aging and highlight the dual role of carnosine as both an aging biomarker and a therapeutic target for combating age-related mucosal degeneration. These insights support the development of novel carnosine-based

interventions to preserve oral mucosal function, prevent age-related oral diseases, and improve oral health in the aging population, thereby advancing healthy aging.

KEYWORDS

carnosine, senescence, aging, metabolomics, oral mucosa

1 Introduction

The globe meets the challenges of an aging population. According to the World Population Prospects 2024, the global average life expectancy will reach 73.3 years in 2024. It is expected that by the end of the 2070s, the global population aged 65 and above will reach 2.2 billion, surpassing the number of people under the age of 18 (United Nations Department of Economic and Social Affairs, 2024). Aging is described as an inevitable time-dependent functional decline that affects all living organisms (López-Otín et al., 2023). Aging-related changes lead to declined functions and increased risks of diseases and mortality. Cellular senescence, a hallmark of aging, is described as prolonged and generally irreversible cell cycle arrest (Gorgoulis et al., 2019). Although cellular senescence is beneficial to embryonic development, host immunity, tumor suppression and wound healing, the aberrant accumulation of senescent cells has been identified as the primary cause of age-related diseases, including chronic obstructive pulmonary disease (COPD), neurodegenerative diseases, atherosclerosis and cancers (Di Micco et al., 2021). Recently, clearing senescent cells emerged as a promising therapy to extend lifespan, alleviate age-related functional decline and improve other health indicators (Chaib et al., 2022).

Aging significantly affects the oral cavity (Ib et al., 2016). A previous study showed significant accumulation of senescent cells in monkeys' gingival epithelium with aging (Hu et al., 2024). A recent study showed senescent cells accumulated in periodontal ligament and alveolar bone with aging and exacerbated the chronic inflammation in periodontal tissue through secreting senescence-associated secretory phenotype (SASP) and interacting with bacteria (Chen et al., 2022). In pulp tissue, $CD51^{+}$/PDGFR-α^{+} human dental pulp stromal cells (hDPSCs), which were promising seeding cells for regenerative medicine, decreased in chronological senescence. While the chronologically senescent hDPSCs showed impaired self-renewal and higher ossificatory differentiation (Yao et al., 2023). With aging, oral mucosa exhibits an increased incidence of multiple diseases, including infectious diseases, oral potentially malignant disorders (OPMDs) and oral squamous cell carcinoma (OSCC) (Ib et al., 2016). The incidence of OSCC rises dramatically after the age of 40–49 years and reaches a plateau around the age of 70–79 years (Hussein et al., 2017). Additionally, older age at diagnosis of OSCC is associated with lower overall survival (OS) (Zanoni et al., 2019). Furthermore, the aging oral mucosa is characterized by altered mucosal sensation, increased permeability, and delayed wound healing (Ib et al., 2016). However, the mechanism of oral mucosal aging and the treatment against age-related changes remain unclear.

Emerging evidence indicated that metabolic dysregulations contributed to aging (Hamrick and Stranahan, 2020). Senescent cells undergo extensive metabolic reprogramming to survive through avoiding apoptosis (Wiley and Campisi, 2021). Recent studies indicated that metabolic profiles of plasma in older individuals are significantly different from those in younger people (Tian et al., 2022). Furthermore, the metabolic profiles of healthy agers differ from those of rapid agers (Hamsanathan et al., 2024). Additionally, it seems plausible that metabolites can serve as biomarkers of aging and metabolomics shows great promise in assessing biological age (Rist et al., 2017; Robinson et al., 2020). However, most metabolomic analyses focus on the alterations of global plasma and other biofluids (Pietzner et al., 2021), yet biofluid metabolomics lacks the capacity to offer the detailed insights into the aberrant metabolism occurring in local disease pathogenesis that tissue metabolomic analysis can provide (Saoi and Britz-McKibbin, 2021). To date, no metabolomic analysis has been conducted on oral mucosal aging, and the mechanisms linking metabolic changes to oral mucosal aging remain unknown.

Carnosine is a dipeptide composed of β-alanine and L-histidine, with high levels in skeletal muscle, myocardium and brain, especially in the olfactory bulb (Boldyrev et al., 2013). It is synthesized by CARNS1 and degraded by carnosinase (CN) (Boldyrev et al., 2013). Carnosine has been proven to have many functions, including regulating excitation-contraction coupling, exhibiting antioxidant activity, chelating metal ions, inhibiting protein carbonylation and acting as a pH buffer (Boldyrev et al., 2013). In aging research, carnosine shows promising potential for treating age-related disorders (José et al., 2015), including pulmonary fibrosis, myocardial ischemia reperfusion injury and age-related cataract (Dubois and Bastawrous, 2017; Zhao et al., 2020; Park et al., 2022). Additionally, it protects against neurodegenerative diseases such as Alzheimer's disease, dementia, and Parkinson's disease by inhibiting amyloid-β (Aβ) aggregation and reducing neuroinflammation (Banerjee et al., 2021; Spaas et al., 2021). However, the specific role of carnosine in oral mucosal aging remains unexplored.

This study was designed to investigate metabolic alterations during aging in mouse tongues and to identify potential metabolic biomarkers and therapeutic agents to mitigate oral mucosal aging. For the first time, we observed epithelial atrophy and the accumulation of senescent cells in the tongue mucosa of aging mice. Through untargeted metabolomic analysis, we identified carnosine as a key biomarker of oral mucosal aging, showing a significant age-related decline in its level. Furthermore, we found that carnosine alleviated tBHP-induced cellular senescence by reducing oxidative stress, mitigating DNA damage, and downregulating the Nrf2/HO-1 pathway. Our work provided a comprehensive understanding of oral mucosal aging at the metabolic level and established carnosine as a promising therapeutic candidate for developing new strategies to prevent and treat aging-related oral mucosal diseases.

2 Materials and methods

2.1 Animals

All mice involved in this study were C57BL/6 acquired from the Laboratory Animal Center, Sun Yat-sen University. Mice were

housed under specific pathogen-free circumstances with a 12-h light/dark cycle and free access to tap water and food. Experimental mice were divided into three age groups: young mice (YM, 4-week-old), adult mice (AM, 4-month-old), and old mice (OM, 20-month-old). Mice were sacrificed via cervical vertebra decoupling under isoflurane inhalation anesthesia, and tongue tissues were collected immediately and then stored at −80°C. All animal experiments were executed with the approval of the Institutional Animal Care and Use Committee of Sun Yat-sen University (SYSU-IACUC-2024-001928).

2.2 Metabolomics analysis

Sample processing and analyses were performed by Luming Biological Technology, Inc. (Shanghai, China). The brief analysis processes are as follows, and detailed steps and parameters are available upon request. We collected tongue tissue from six mice in each group. For liquid chromatography-mass spectrometry (LC-MS) analysis, a Dionex Ultimate 3000 RS UHPLC with a Q-Exactive quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, United States) was used, operating in both ESI positive and negative ion modes. An ACQUITY UPLC HSS T3 column (1.8 μm, 2.1 × 100 mm) was employed. For gas chromatography-mass spectrometry (GC-MS) analysis, derivatized samples were analyzed on an Agilent 7890B gas chromatography system coupled to an Agilent 5977B MSD system (Agilent Technologies Inc., CA, United States). An HP-5MS fused-silica capillary column (30 m × 0.25 mm × 0.25 μm, Agilent J & W Scientific, Folsom, CA, United States) was used for separation. QC samples were injected at regular intervals throughout the analytical run to determine repeatability.

The original GC-MS and LC-MS data were processed using Analysis Base File Converter, MS-DIAL, and Progenesis QI for format conversion, baseline filtering, peak identification, integration, retention time correction, peak alignment, and normalization. After normalization, redundancy removal and peak merging were performed to obtain the data matrix. The data matrix was imported into R for analyzing and visualizing. Differential metabolites between groups were identified using one-way ANOVA ($p < 0.05$) and selected based on VIP values >1.0. Metabolic pathway enrichment analysis was performed using MetaboAnalyst 4.0 and the KEGG database, applying a threshold of impact values >0.1 and −log(P) values >2.0. Correlation analysis between differential metabolites was conducted using Spearman's rank correlation coefficient in MetaboAnalyst 4.0 ($p < 0.05$; $\rho < -0.5$ or $\rho > 0.5$) and visualized as chord diagrams with the R package "circlize." And the classification was based on Human Metabolome Database (HMDB) classification. Receiver operating characteristic (ROC) curve analysis was also performed using MetaboAnalyst 4.0.

Moreover, we extracted the data of carnosine-related metabolites in human serum and saliva from MetaboLights (MTBLS265 and MTBLS2108) and performed ROC curve analysis using MetaboAnalyst 4.0. Data from the association matrix, including standardized regression coefficients (β-estimates) and nominal *p*-values, were extracted from the open-access web server (https://omicscience.org/apps/mwasdisease/). Regression coefficients and nominal *p*-values were plotted in a heatmap using R version 4.1.0.

TABLE 1 Primary antibodies used in the study.

Name	Company and Cat no.	Application
Anti-p21^{Waf1}	Santa Cruz, sc-6246	1:200 (IHC), 1:500 (WB)
Anti-Ki-67	Abcam, ab16667	1:1,000 (IHC)
Anti-CARNS1	Abbexa, abx129855	1:50 (IHC)
Anti-CNDP2	Proteintech, 14925-1-AP	1:1,000 (IHC)
Anti-γH2A.X	Servicebio, GB11365-100	1:100 (IF), 1:500 (WB)
Anti-Nrf2	Santa Cruz, sc-365949	1:500 (WB)
Anti-HO-1	Abcam, ab189491	1:2000 (WB)
Anti-β-actin	Proteintech, 66009-1-Ig	1:5,000 (WB)
Anti-8-OHdG	Santa Cruz, sc-66036	1:300 (IF)

2.3 Cell culture

The cell lines used in this study were human dermal keratinocyte (HaCaT) and human oral epithelial cell (HOEC), which were kindly provided by Professor Xianyue Ren and Professor Guiqing Liao (Guanghua School of Stomatology, Sun Yat-sen University), respectively. Both cell lines were grown in DMEM (Gibco, #11965092) containing 10% FBS (TransGenBiotech, FS401) and 100 IU/mL penicillin/streptomycin at 37°C in a 5% CO_2 humidified incubator. HaCaT and HOEC were treated with tBHP (Sigma Aldrich, #458139) to induce cellular senescence. Cells were seeded and cultivated in complete culture medium until they reached 50% confluence, with or without carnosine (Sigma-Aldrich, C9625) and N-acetyl-L-cysteine (NAC, Sigma-Aldrich, A2835) supplementation. tBHP was administered at 250 μM for 2 hours and 200 μM for 6 hours to HaCaT and HOEC, respectively (Li et al., 2020). Then cells were washed three times with PBS (Gibco, #10010023) and cultured for another 2 days with or without carnosine and NAC supplementation.

2.4 Histological analysis

Tongue tissues were collected from mice, rinsed three times with PBS, fixed in 4% paraformaldehyde (PFA) for 24 h at 4°C, and dehydrated in a 30% sucrose in PBS solution for 24–48 h. The fixed tongues were embedded in paraffin, cut into 4-μm-thick sections using a rotary microtome (Leica, AUTOCUTE), and stained with hematoxylin and eosin (H&E). Histologic images were captured with a slide scanner (Leica, Aperio AT2) and analyzed using ImageScope 11.0 software. The thickness of the tongue epithelium, defined as the total thickness of the stratum granulosum, stratum spinosum, and stratum basale (Wakamori et al., 2022), was measured at 15 random points per whole tongue.

2.5 Immunohistochemistry (IHC)

Paraffin-embedded tissues were dewaxed with Histoclear (Solarbio, YA0031) and rehydrated with a gradient of ethanol. Antigen was retrieved by incubating the sections for 5–10 min in

0.01 M citrate buffer (pH = 6.0). The slides were immersed in 0.1% Triton X-100 for 15 min, then blocked with normal goat serum (BOSTER, AR0009) for 60 min at room temperature. Sections were then incubated with primary antibodies at 4°C overnight and then were washed with PBS three times. The primary antibodies used in this experiment are shown in Table 1. Afterward, sections were incubated in 3% H_2O_2 for 30 min to block endogenous peroxidase activity. Secondary antibodies were incubated on slides at room temperature for 60 min. Tissue sections were stained with diaminobenzidine (DAB, Servicebio, G1212) and counterstained with hematoxylin. Images were captured using a slide scanner (Leica, Aperio AT2) and analyzed by ImageScope 11.0. The Ki-67 or $p21^{Waf1}$ positive cells were counted manually and averaged in six high-power fields (HPFs, 400×). The expression levels of CARNS1 and CNDP2 were measured by H-score plugins in ImageScope 11.0.

2.6 Immunofluorescence (IF)

Paraffin-embedded tissue sections were dewaxed, rehydrated, and antigen-retrieved as previously described in our IHC protocol. The slides were immersed in 0.3% Triton X-100 for 10–15 min, then blocked with 5% BSA for 60 min at room temperature. Slides were gently washed with PBS, followed by incubating with the primary antibody at 4°C overnight. Following primary antibody removal, the slides were incubated with the appropriate secondary antibodies for 1 h at room temperature in the dark. Nuclei were stained with DAPI (Beyotime, C1002) for 5 min. After washing, the sections were mounted with anti-fade mounting medium (Servicebio, G1401). Images were captured using a ZEISS Axio microscope and an Olympus FV3000 confocal microscope. Fluorescent signal intensity was analyzed using ImageJ. The primary antibodies used in this experiment were shown in Table 1. The fluorescent intensity was measured by ImageJ.

2.7 CCK-8 viability assay

HaCaT and HOEC cells were seeded in 96-well plates at a density of 1×10^4 cells/cm^2 and cultured in DMEM medium with gradient concentrations of carnosine (0, 2, 5, 10, 20 mM). After 2 days, the medium was replaced with 200 μL of fresh medium containing 20 μL of CCK-8 reagent (DOJINDO). Following a 2-h incubation, the absorbance at 450 nm was measured using a spectrophotometric microplate reader (BioTek Synergy H1).

2.8 Western blot

Total protein was extracted by RIPA lysis buffer (CWBIO, CW2333S), whose concentrations were quantified by BCA protein assay kit (CWBIO, CW0014S). Proteins were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% milk for 1 h at room temperature. Then the membranes were incubated with primary antibodies overnight at 4°C. Following washing with TBST, the membranes were incubated with appropriate secondary antibodies.

TABLE 2 Primer sequences for qRT-PCR.

Homo gene	Primer sequences (5′ > 3′)	
IL-1β	Forward	TGTACCTGTCCTGCGTGTTG
	Reverse	ACGGGCATGTTTTCTGCTTG
IL-6	Forward	CAATGAGGAGACTTGCCTGGT
	Reverse	GCAGGAACTGGATCAGGACT
IL-8	Forward	ACACTGCGCCAACACAGAAATTA
	Reverse	TTTGCTTGAAGTTTCACTGGCATC
IL-18	Forward	TCTTCATTGACCAAGGAAATCGG
	Reverse	TCCGGGGTGCATTATCTCTAC
TNF-α	Forward	TATCCTGGGGGACCCAATGT
	Reverse	AAAAGAAGGCACAGAGGCCA
Nrf2	Forward	GTGTGGCATCACCAGAACAC
	Reverse	GACACTTCCAGGGGCACTAT
HO-1	Forward	AGTCTTCGCCCCTGTCTACT
	Reverse	CTTCACATAGCGCTGCATGG
NQO1	Forward	AACACTGCCCTCTTGTGGTG
	Reverse	GCTCGGTCCAATCCCTTCAT

Detection of labeled proteins was carried out using Immobilon Western Chemiluminescent HRP Substrate (Millipore, WBKLS0050). The antibodies utilized in this experiment were detailed in Table 1.

2.9 RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated and purified from cell samples using the Easy Pure Fast Cell RNA Kit (Transgene, ER111), and the concentrations of RNA were measured by NanoDrop (Thermo). DNase treatment and reverse transcription were performed using the HiScript III All-in-one RT SuperMix (Vazyme, R333). The obtained templates were used for qRT-PCR with SYBR MasterMix (Vazyme, Q511) on an ABI Q5 (Thermo). All samples were normalized to β-actin. Primer sequences for qRT-PCR are shown in Table 2.

2.10 Senescence-associated β-Galactosidase (SA-β-Gal) staining

As previously described, SA-β-Gal staining was carried out on human cells at pH 6.0 and on mouse tissue at pH 5.5 (Amor et al., 2020). To stain the tongue with SA-β-Gal, the tissue was embedded in OCT, sectioned at a thickness of 12 μm, and stored at −20°C. After rehydration in PBS, staining was performed using the SA-β-Gal Staining Kit (CST, #9860) with a 15-min fixation followed by incubating in the staining solution at 37°C without CO_2 for 96 h. Tissue sections were counterstained with 0.1% Nuclear Fast Red

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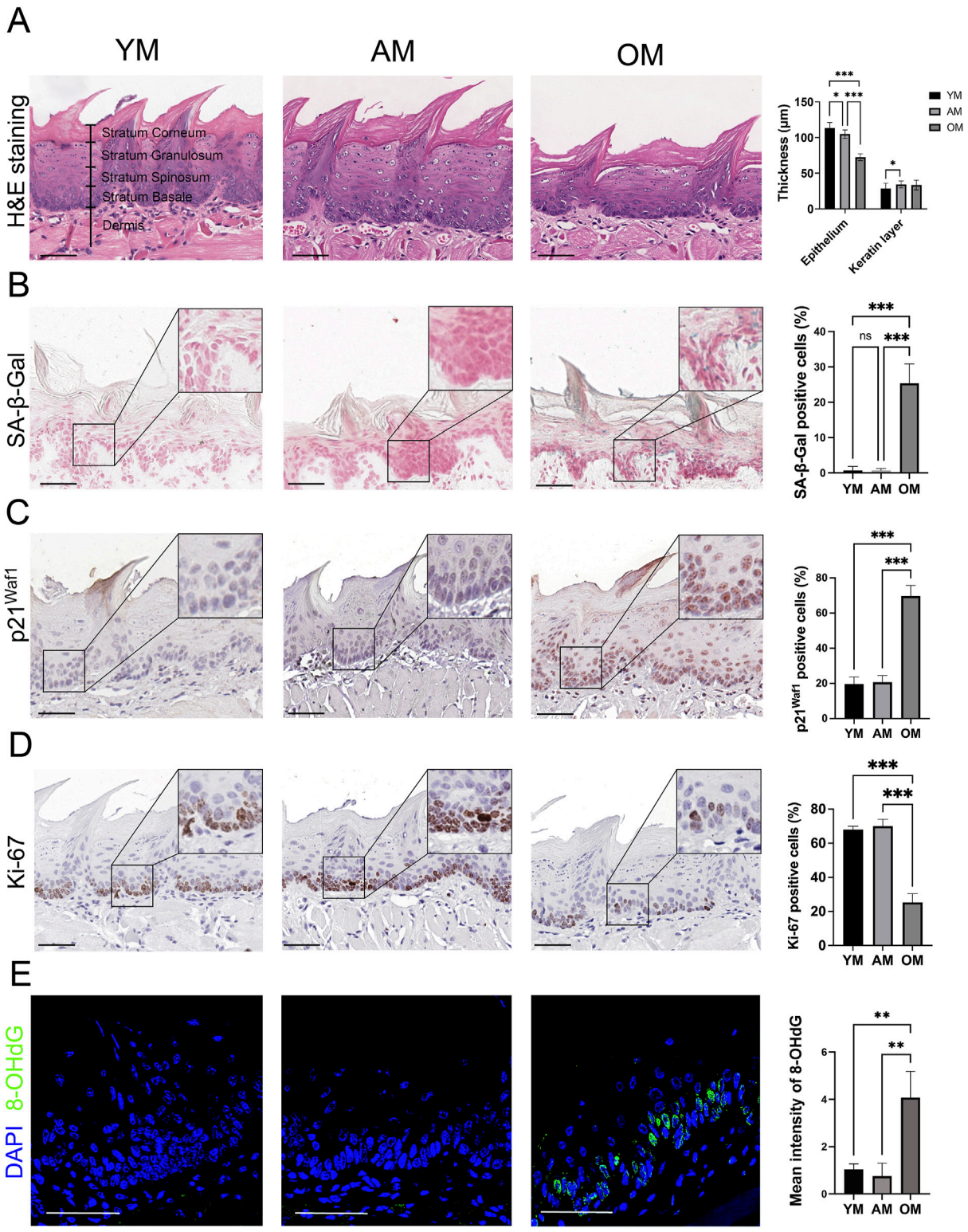

FIGURE 1
Epithelial atrophy and accumulation of senescent cells in the tongue mucosa of old mice. **(A)** Representative H&E images and quantification of epithelial thickness and keratin layer thickness in the tongue from young, adult and old mice. **(B)** Representative images of the dorsal surface of the tongue epithelium from YM, AM and OM assayed for SA-β-Gal activity are shown. In addition, quantification of the proportion of SA-β-Gal positive cells in YM, AM and OM is provided. **(C,D)** Representative images of immunohistochemical staining of **(C)** $p21^{Waf1}$ and **(D)** Ki-67 in tongue epithelium of YM, AM and OM are shown. Quantification of the proportion of positive cells in YM, AM and OM is provided. **(E)** Representative images and quantification of immunofluorescence of 8-OHdG in the tongue epithelium from YM, AM and OM are shown. For all relevant figures, scale bars: 50 μm. Data are represented as mean ± SEM (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

Solution (Beytime, G1320). Adherent cells were fixed for 10 min before being immersed in the SA-β-Gal staining solution at 37°C without CO_2 for 48 h. Cells with cytoplasmic staining were scored as positive. Five high-power fields per well were counted and averaged to quantify the percentage of SA-β-Gal positive cells.

2.11 Determination of ROS

The intracellular ROS level was measured using the Reactive Oxygen Species Assay Kit (Beyotime, S0064S). Cells were collected 48 h after treatment with tBHP, carnosine and NAC. Afterward, cells were incubated with 10 μM Dihydroethidium (DHE) in the dark at 37°C for 20 min. Following washes with PBS, the mean fluorescence intensity (MFI) was then detected using flow cytometry.

2.12 Statistical analysis

Statistical analysis was performed using SPSS version 22.0. Statistical significance was determined using one-way ANOVA with Bonferroni correction. *P* value <0.05 was considered to indicate statistical significance. Results are illustrated using GraphPad Prism 10. The error bars indicate the standard error of the mean (SEM).

3 Results

3.1 Epithelial atrophy and the accumulation of senescent cells in the tongue mucosa of elderly mice

H&E staining revealed significant epithelial atrophy in the tongue mucosa of OM, with a notable reduction in epithelial thickness compared to YM and AM. However, there is no significant reduction in the thickness of the keratin layer with aging (Figure 1A). Then we further detected cellular senescence with SA-β-Gal staining and immunohistochemical staining of $p21^{Waf1}$. Few SA-β-Gal positive cells were observed in the tongue mucosa of YM and AM, whereas the SA-β-Gal positive epithelial cells significantly increased in the tongue mucosa of OM (Figure 1B). A significant increase of $p21^{Waf1}$ positive cells was observed in the epithelial cells of tongue mucosa of OM compared with YM and AM, with no significant difference between YM and AM (Figure 1C). Additionally, the proportion of Ki-67 positive cells in tongue epithelium declined significantly in OM compared with YM and AM (Figure 1D). DNA damage in the tongue mucosa was assessed by measuring 8-OHdG levels, which were significantly elevated in the tongue epithelium of OM compared to YM and AM (Figure 1E). These results suggest that epithelial atrophy, accumulation of senescent cells, and increased DNA damage occur in the tongue mucosa of OM, but not in YM and AM.

3.2 Metabolic alterations in tongue mucosal aging in mice

To investigate the metabolic perturbations in oral mucosa during aging, we performed untargeted metabolomics analysis on tongues from young, adult and old mice for the first time. We finally identified 4,399 and 251 metabolite features in LC-MS and GC-MS, respectively. Following the removal of the duplicate data, the GC-MS and LC-MS data were merged, and finally, 335 differential metabolites were identified. Then we performed an unsupervised principal component analysis (PCA) to identify potential effects of age on the metabolomic profiles alteration. The PCA model showed clear separation among YM, AM and OM (Figure 2A). There were 47 metabolites with significant differences overlapping the three groups (Figure 2B). The levels of these 47 metabolites were illustrated in a heatmap (Figure 2C). Moreover, orthogonal partial least squares discriminant analysis (OPLS-DA) showed significant differences between each pair of groups, which were YM vs. AM, YM vs. OM, and AM vs. OM (Figures 2D–F). The volcano plots showed the differential metabolites with decreased expression were notably dominant, especially in the comparison of AM and OM (Figures 2G–I). Notably, carnosine consistently ranked first in all three groups, exhibiting the highest significance. Furthermore, we ranked the top 15 differential metabolites contributing to group separation according to the VIP values in each pair of groups (Figures 2J–L). We found that amino acids, peptides, and carbohydrates accounted for most of the top 15 metabolites, and carnosine, L-2-amino-3-methylenehexanoic acid, 2E,7-octadienoic acid, methyl-tetrahydrophenanthrenone, and gluconic acid were found to overlap among the three groups.

Hierarchical clustering and heatmap profiling of the top 50 differential metabolites among the three paired groups (Figure 3A) demonstrated three main patterns of metabolic perturbation: a steady decrease with age, a continuous increase with age, and an increase from young to adult followed by a decrease from adult to elderly. Most of the differential metabolites decreased continuously with age. Pathway enrichment analysis highlighted the critical role of amino acid metabolism, particularly beta-alanine metabolism and histidine metabolism, both of which are linked to carnosine metabolism (Figure 3B). According to the chord diagrams, lipids and lipid-like molecules showed strong correlations across all three comparisons, indicating that these molecules may play a significant regulatory role in the aging process (Figures 4A–C). The correlation heatmap showed a high correlation between phosphocholine and lysophosphatidylethanolamine during aging (Figures 4D–F). Collectively, we found significant metabolic alterations associated with aging in the tongue mucosa of mice. During aging, the levels of most differential metabolites gradually decreased, with amino acid metabolism and carbohydrate metabolism playing important roles in determining the overall metabolic pattern.

3.3 Downregulation of carnosine metabolism in mouse tongue mucosa with age

According to the metabolomics data, the levels of carnosine, L-histidine and β-alanine in mouse tongues decreased with age (Figure 5A). Notably, the fold change of carnosine level between OM and YM is 0.23. Additionally, ROC curve analysis was performed to evaluate the potential of carnosine as a biomarker for oral mucosal

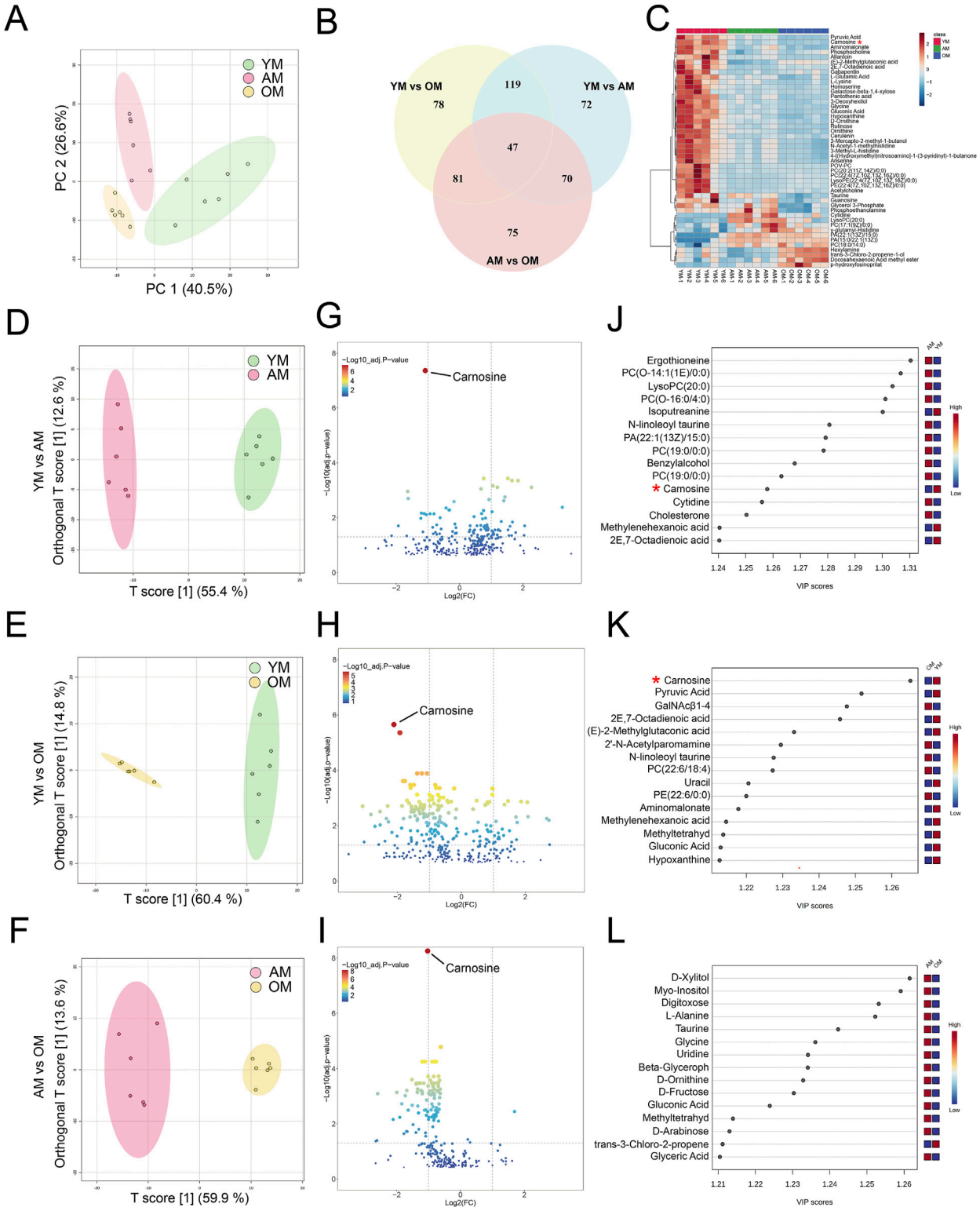

FIGURE 2
Metabolomic features associated with aging in mouse tongues. **(A)** PCA reveals significant variance in metabolites among YM, AM and OM. PC1: 40.5%, PC2: 26.6%. **(B)** A Venn plot comparing the numbers of differential metabolites showed variations between each pair of groups. **(C)** Heatmap of 47 differential metabolites overlapping in YM vs. AM, YM vs. OM and AM vs. OM. **(D–F)** Differences in metabolomic profiles between **(D)** YM and AM, **(E)** YM and OM, and **(F)** AM and OM were illustrated by the OPLS-DA score plots. **(G–I)** Volcano plots showed the alteration of metabolite concentrations between **(G)** YM and AM, **(H)** YM and OM, and **(I)** AM and OM. The points that represented carnosine were annotated. The horizontal reference dashed line indicates the adjusted *p*-value equal to 0.05. The vertical reference dashed line indicates $\log_2$FC values equal to −1 and 1. The size of the points is proportional to the absolute value of $\log_2$FC. **(J–L)** Top 15 metabolites were identified by VIP score plots, ranked by VIP values from OPLS-DA, between **(J)** YM and AM, **(K)** YM and OM, and **(L)** AM and OM.

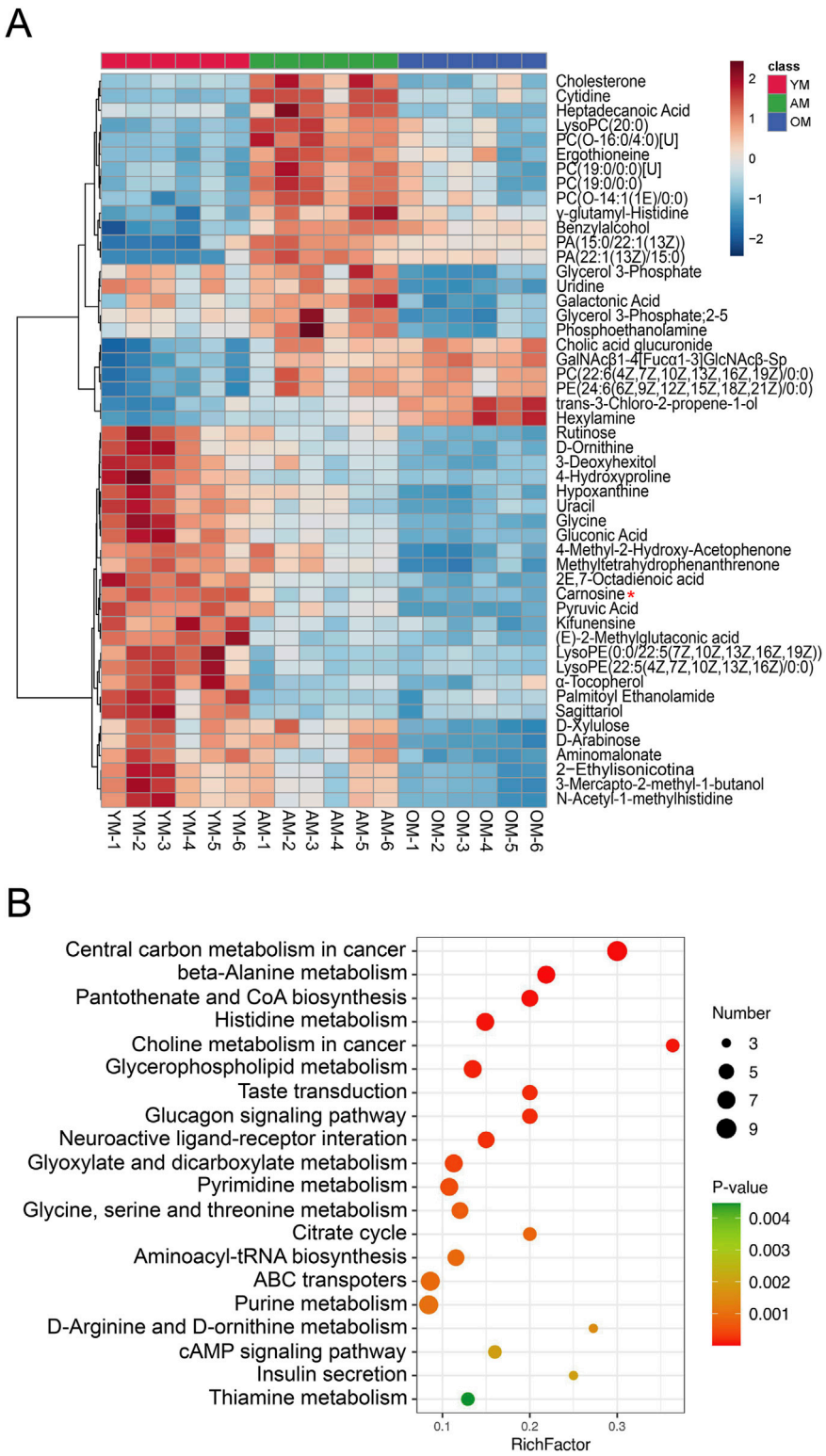

FIGURE 3
Cluster analysis and pathway enrichment analysis. **(A)** Heatmap of the top 50 significant metabolites across three different age groups, ranked by adjusted *p*-value. **(B)** Pathway enrichment analysis visualized by a bubble plot, highlighting the top 20 significant pathways. The size of the points is proportional to the number of metabolites enriched in specific pathways, while the color indicates the *p*-value. The X-axis represents the rich factor.

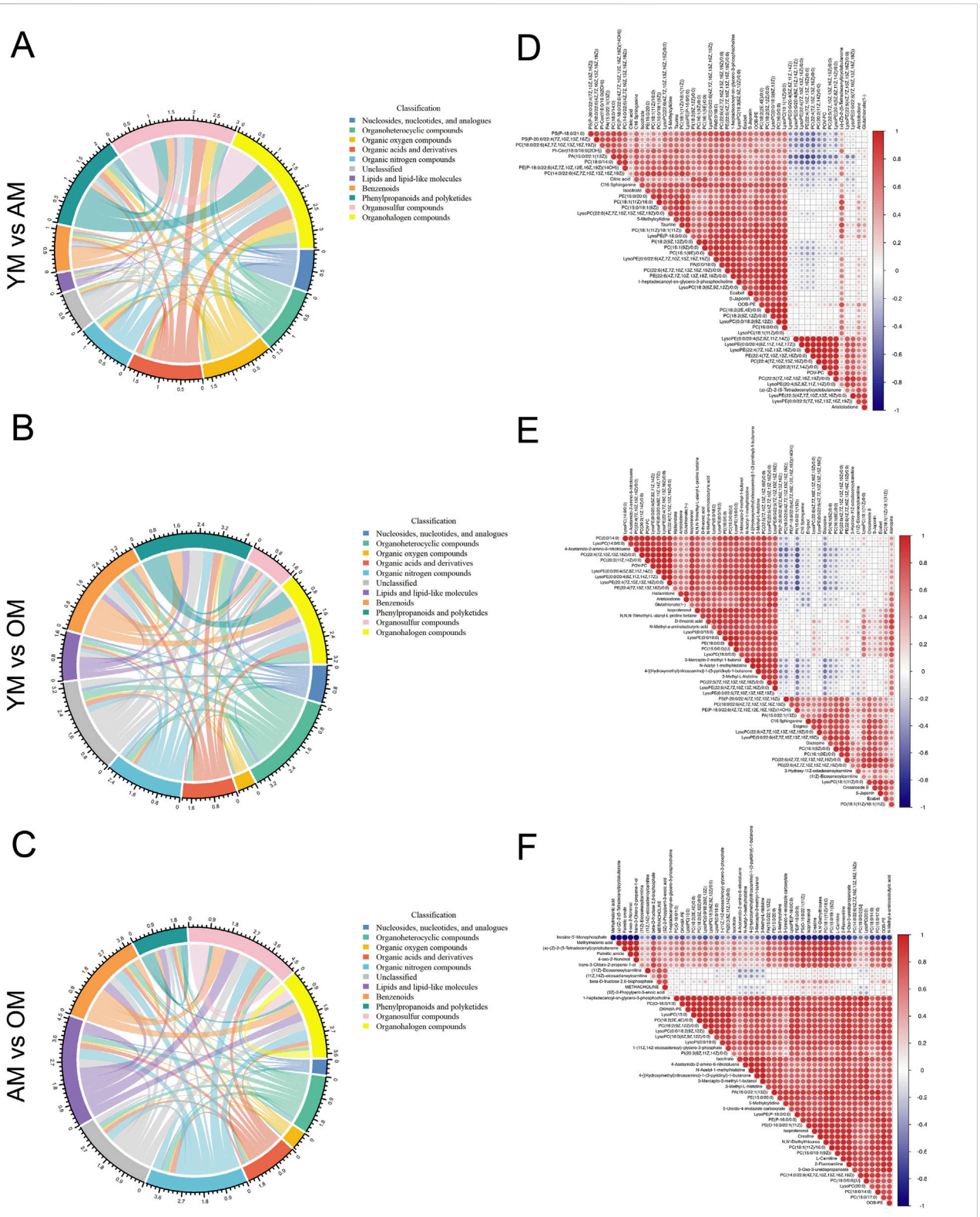

FIGURE 4
Correlation analysis of differential metabolites associated with aging. **(A–C)** The metabolic correlations between **(A)** YM and AM, **(B)** YM and OM, and **(C)** AM and OM were shown by chord diagrams. The outer ring illustrates the sum of Spearman's correlation efficiency for each metabolic classification according to HMDB classification. The width of the bands indicates the mean correlations efficiency, and the color indicates the classification of metabolites. *P*-value cut-off was set to $p < 0.05$ and *p*-value cut-off was set to $\rho < -0.5$ or $\rho > 0.5$. **(D–F)** Correlation heatmaps show the Spearman's rank correlation coefficients of the top 50 metabolites between **(D)** YM and AM, **(E)** YM and OM and **(F)** AM and OM. Red indicates positive correlations and blue indicates negative correlations.

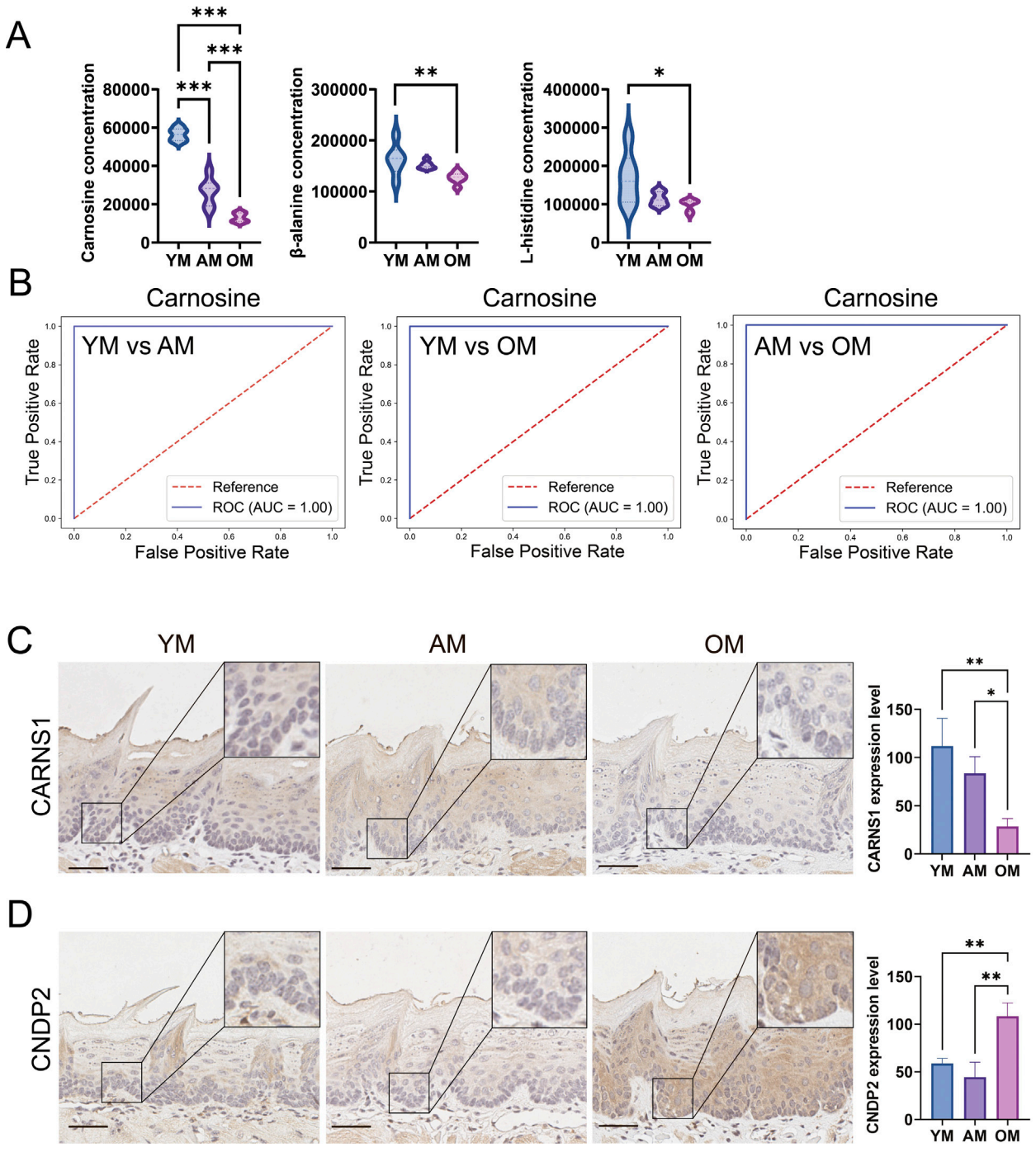

FIGURE 5
Downregulation of carnosine metabolism in mouse tongue mucosa with age. **(A)** Relative levels of carnosine, β-alanine and L-histidine in YM, AM and OM. **(B)** ROC curve analysis evaluated the accuracy of carnosine as a biomarker in distinguishing YM, AM and OM. In the YM vs. AM, YM vs. OM, and AM vs. OM groups, the AUC value was equal to 1, indicating that the predictive model based on the concentration of carnosine exhibited extremely high accuracy in distinguishing among the three groups. **(C,D)** Representative images of immunohistochemical staining and semi-quantification of **(C)** CARNS1 and **(D)** CNDP2 in tongue epithelium from young, adult and old mice. Scale bars: 50 μm (Mean ± SEM, one-way ANOVA, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$, multiple correction test by Bonferroni correction).

aging. In the YM vs. AM, YM vs. OM, and AM vs. OM groups, the AUC value was equal to 1, indicating that the predictive model based on the concentration of carnosine exhibited extremely high accuracy in distinguishing among the three groups (Figure 5B). To further investigate the metabolic activity of carnosine, we measured the expression of CARNS1 and CNDP2 in the tongue mucosa of mice. CARNS1 expression significantly declined in OM, with no significant differences between YM and AM (Figure 5C). Conversely, CNDP2 expression increased significantly in OM, with no notable changes between YM and AM (Figure 5D).

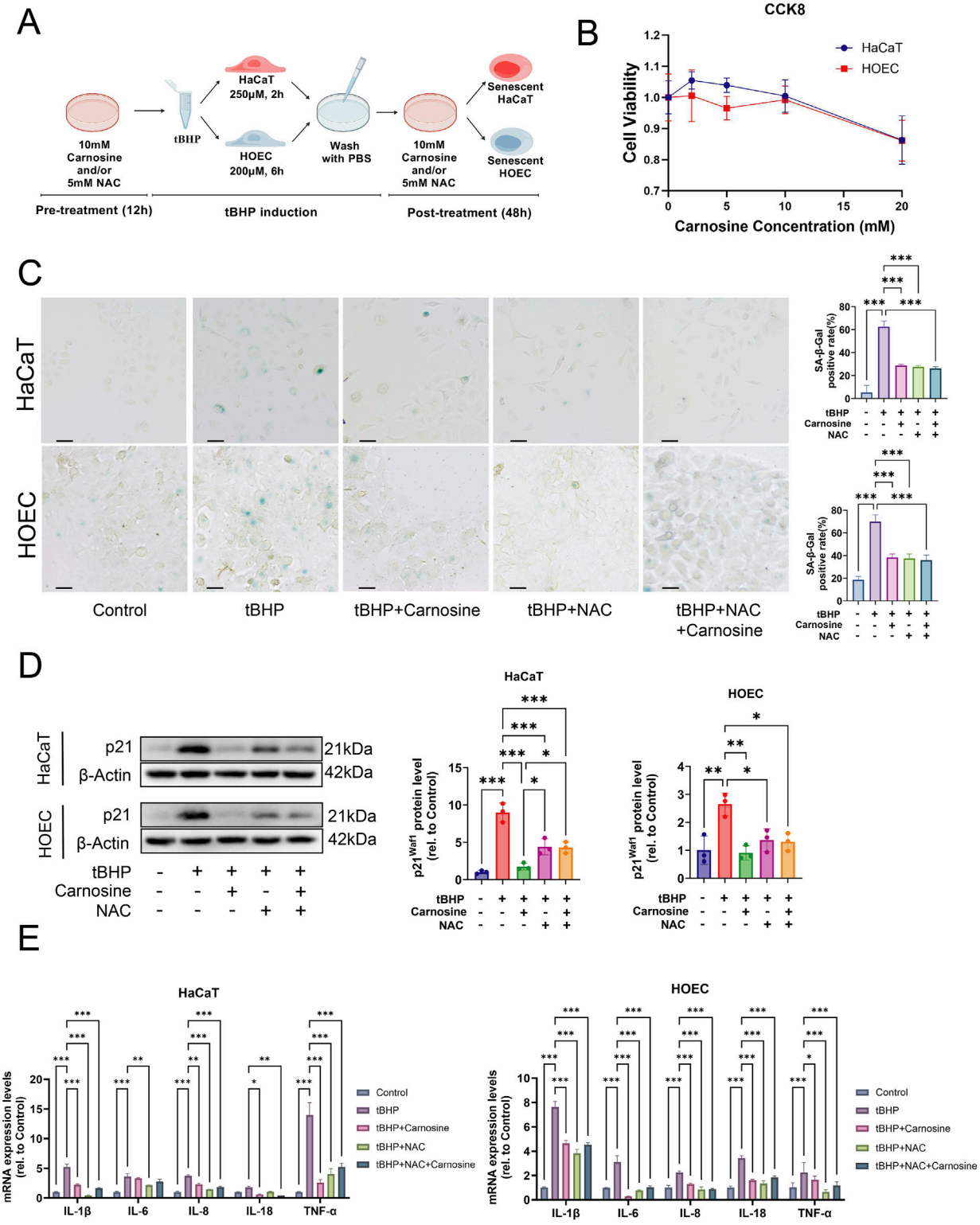

FIGURE 6
Carnosine inhibits cellular senescence *in vitro*. **(A)** Schematic diagram outlining the experimental protocol for inducing cellular senescence *in vitro*. Created with https://BioGDP.com. **(B)** Cell viability of epithelial cells after carnosine supplementation, as determined by the CCK-8 assay (n = 3). **(C)** Representative images of SA-β-Gal staining were observed under a light microscope and a quantitative analysis of the proportion of SA-β-Gal positive cells in HaCaT and HOEC was performed, respectively (n = 3). Scale bar: 100 μm. **(D)** The expression levels of $p21^{Waf1}$ were measured by Western blot and semi-quantification in HaCaT and HOEC, respectively (n = 3). **(E)** Relative mRNA expression levels of IL-1β, IL-6, IL-8, IL-18 and TNF-α were determined by qRT-PCR in HaCaT and HOEC, respectively (n = 3) [Data represent the Mean ± SEM from n (described above) independent experiments; one-way ANOVA, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$].

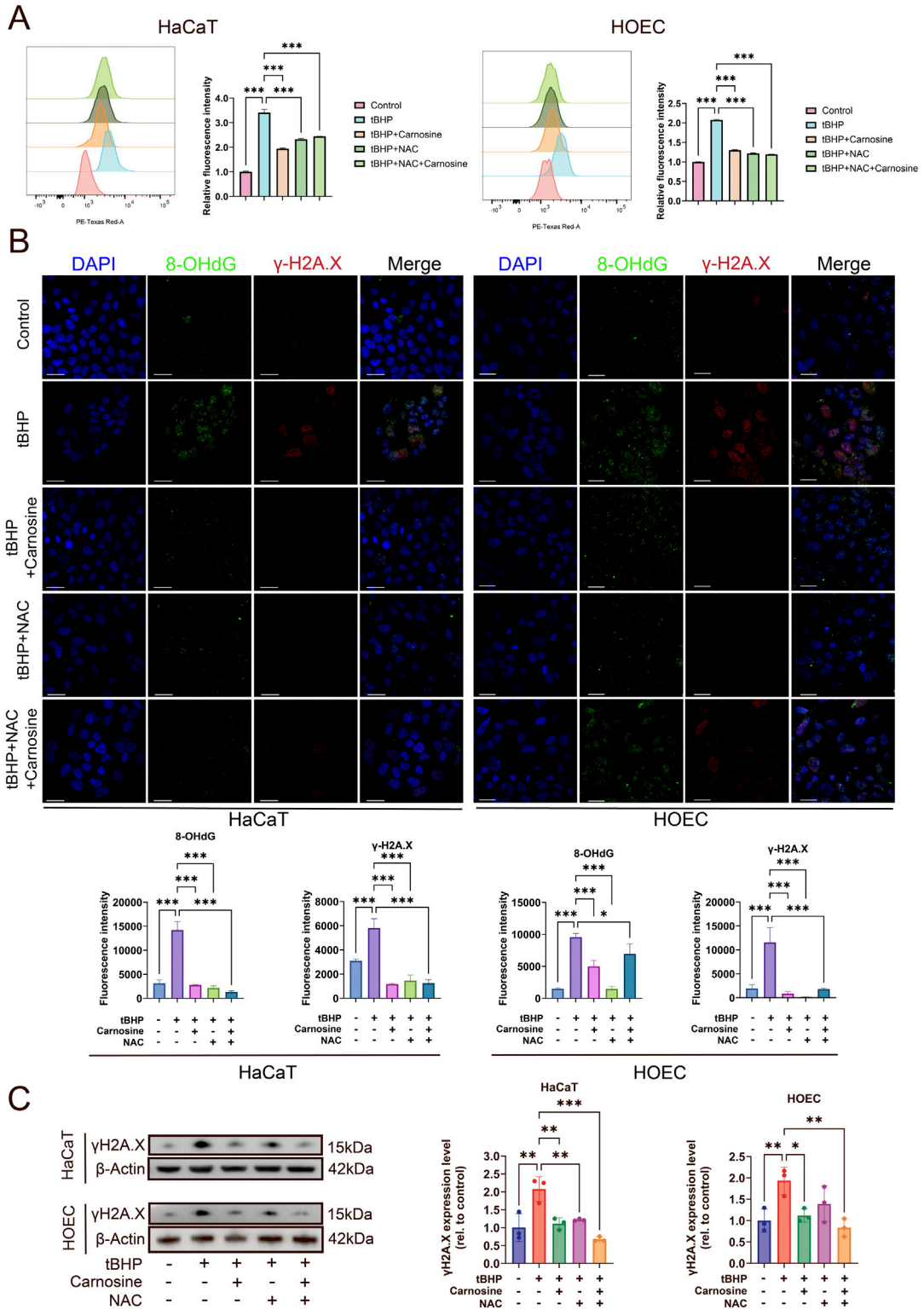

FIGURE 7
Carnosine inhibits ROS accumulation and DNA damage in tBHP-induced cellular senescence. **(A)** ROS levels were determined by flow cytometry and illustrated by histograms in HaCaT and HOEC (n = 3). ROS levels were increased significantly by tBHP induction and were relieved after carnosine and NAC intervention individually or in combination. **(B)** Representative IF images of 8-OHdG and γH2A.X in HaCaT and HOEC in each group (n = 3). Nuclei were stained with DAPI. Scale bar: 30 μm. Quantification of the mean fluorescent intensity of each group was shown below the images. **(C)** γH2A.X expression levels were determined by Western blot (n = 3) [Data represent the Mean ± SEM from n (described above) independent experiments; one-way ANOVA, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$].

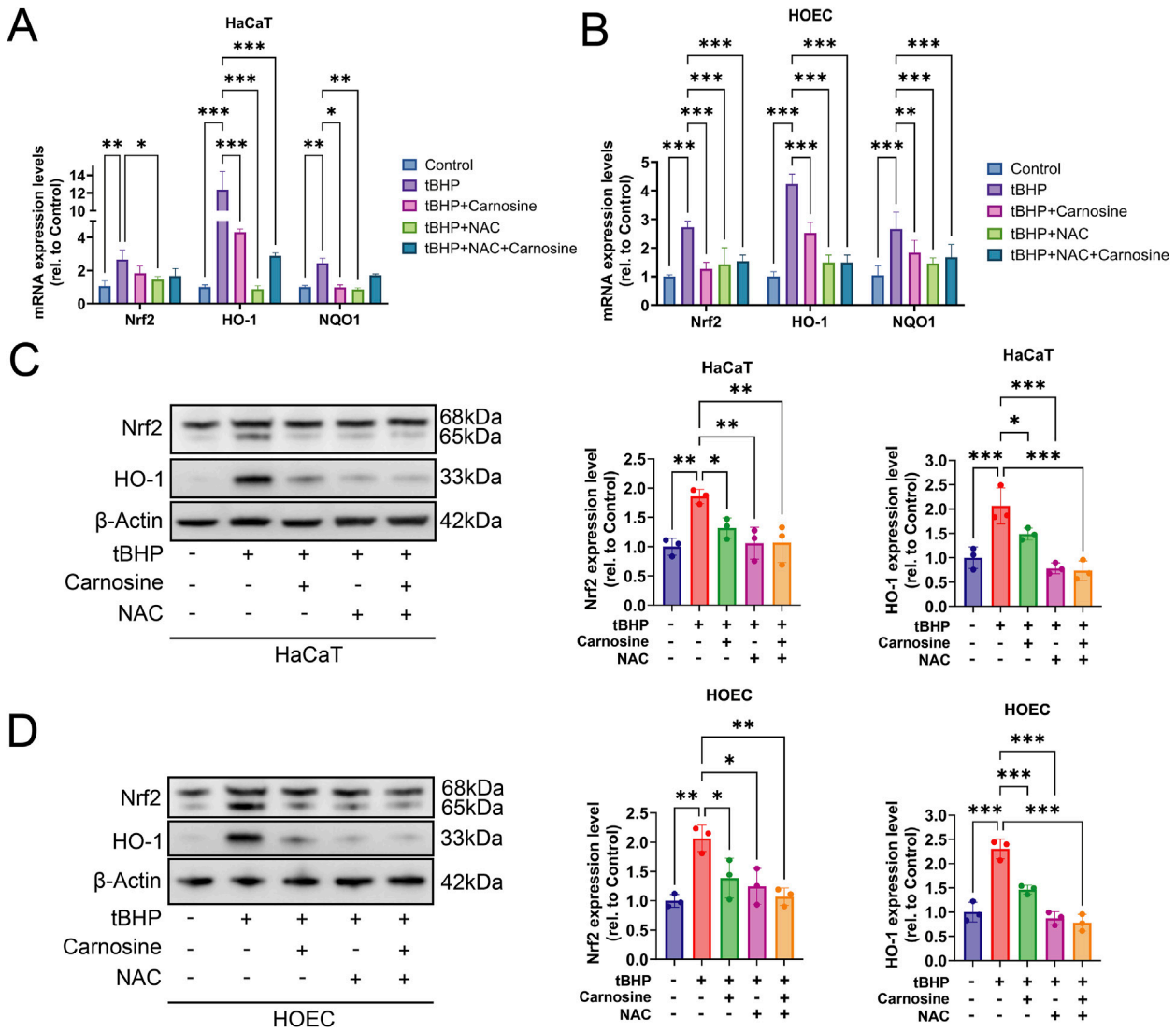

FIGURE 8
Carnosine inhibits tBHP-induced cellular senescence through downregulation of the Nrf2/HO-1 pathway. **(A,B)** mRNA expression levels of Nrf2, HO-1 and NQO1 were significantly increased in tBHP-induced cellular senescence and significantly decreased after carnosine and/or NAC intervention in HaCaT and HOEC, respectively (n = 3). **(C,D)** Protein expression levels of Nrf2 and HO-1 were decreased significantly following carnosine and/or NAC treatment, as determined by Western blot, in HaCaT and HOEC (n = 3) [Data represent the Mean ± SEM from n (described above) independent experiments; one-way ANOVA, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$].

These results suggested that the age-related decline in carnosine level in the tongue mucosa might be attributed to increased hydrolysis and reduced synthesis. Overall, these findings highlight the potential of carnosine as a biomarker of tongue mucosal aging.

3.4 Carnosine alleviates cellular senescence induced by tBHP

To investigate whether carnosine supplementation can inhibit cellular senescence, we used tBHP to establish an oxidative stress-induced cellular senescence model in HaCaT and HOEC *in vitro*, as depicted in the schematic diagram (Figure 6A). Based on the CCK-8 assay, the maximum non-toxic concentration of carnosine was determined to be 10 mM for both HaCaT and HOEC (Figure 6B). Our previous study showed the optimal NAC concentration to inhibit oxidative stress in oral epithelial cells is 5 mM (Lu et al., 2023). As hypothesized, the proportion of SA-β-Gal positive cells increased significantly after tBHP induction and decreased significantly following carnosine and/or NAC supplementation (Figure 6C). Additionally, the expression of the senescence-associated protein $p21^{Waf1}$ increased in the tBHP group and decreased following carnosine and/or NAC intervention (Figure 6D). Notably, carnosine decreased the expression of $p21^{Waf1}$ more effectively than NAC. Furthermore, we investigated the transcriptional activity of SASP factors, including IL-1β, IL-6, IL-8, IL-18 and TNF-α. Except for IL-6 in HaCaT, the mRNA levels of all SASP factors were significantly upregulated in tBHP-induced cellular senescence and downregulated after carnosine and/or NAC treatment (Figure 6E). These findings indicated carnosine

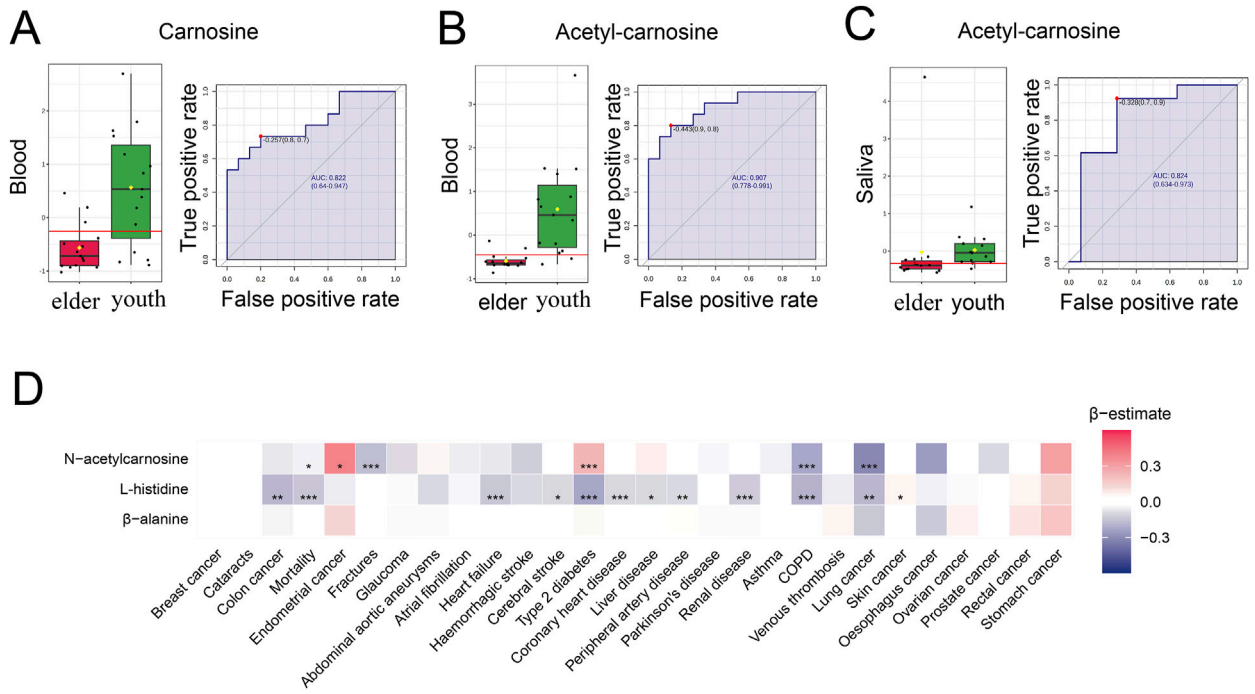

FIGURE 9
Carnosine and its derivatives decline with age and are reduced in age-associated pathologies in humans. **(A,B)** ROC curves for carnosine and acetyl-carnosine and their distribution profiles in youth and older individuals' blood (n = 30). Both carnosine and acetyl-carnosine are decreased in elder individuals. **(C)** ROC curves for acetyl-carnosine and its distribution profile in youth and elder individuals' saliva (n = 27). Acetyl-carnosine was decreased in elder individuals' saliva and the AUC value was 0.824 (95% confidence interval: 0.634–0.973). **(D)** Heatmap showing the results from Cox-proportional hazard models for assessing the associations between 27 NCDs and carnosine-related metabolites (N-acetylcarnosine, histidine, and β-alanine) in blood from 11 966 subjects in the EPIC-Norfolk study. Effect size and direction of these associations are given by the β-estimates resulting from these regression models. A negative β-estimate (blue color) indicates an inverse association and a positive β-estimate (red color) indicates a positive association (Mean ± SEM, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$).

significantly alleviated tBHP-induced cellular senescence in HaCaT and HOEC.

3.5 Carnosine inhibits ROS accumulation and DNA damage in tBHP-induced cellular senescence

Firstly, the ROS levels, determined by DHE, were increased significantly after tBHP induction and were reduced significantly following carnosine and/or NAC intervention (Figure 7A). However, the combination of NAC and carnosine did not provide additional effects beyond either treatment alone in reducing ROS levels. Secondly, to further assess the DNA damage induced by ROS, we performed immunofluorescence staining of 8-OHdG and γH2A.X, which were the markers of double-strand break and DNA damage response (Figure 7B). The expression of 8-OHdG and γH2A.X was significantly increased following tBHP induction and significantly decreased after treatment with carnosine and/or NAC. Thirdly, the results of the Western blot further confirmed that the expression of γH2A.X was significantly decreased following carnosine and/or NAC intervention (Figure 7C). These findings suggest that ROS levels and oxidative stress-induced DNA damage are increased in the tBHP-induced cellular senescence and alleviated by carnosine intervention.

3.6 Carnosine inhibits tBHP-induced cellular senescence through downregulation of the Nrf2/HO-1 pathway

To further investigate whether the Nrf2 signaling pathway participates in the carnosine activity on cellular senescence, we evaluated the mRNA expression levels of Nrf2, HO-1 and NQO1 through qRT-PCR. As anticipated, the mRNA expression levels of Nrf2, HO-1 and NQO1 increased after tBHP induction and decreased following carnosine and/or NAC intervention (Figures 8A,B). We further confirmed the expression of Nrf2 and HO-1 at the protein level using Western blot analysis, which showed a significant increase in the tBHP-induced group and a significant decrease in the groups treated with carnosine and/or NAC (Figures 8C,D). Collectively, these results suggest that carnosine inhibits tBHP-induced cellular senescence in HaCaT and HOEC by downregulation of the Nrf2/HO-1 pathway.

3.7 Carnosine and its derivatives decline with age and are reduced in age-associated pathologies in human

Next, we sought to further investigate the relationship between carnosine and aging in the human body. Firstly, we performed ROC

analysis of the metabolome in blood samples from 15 young and 15 elderly individuals (Chaleckis et al., 2016). We found the levels of carnosine and acetyl-carnosine were significantly decreased in the blood of elder individuals compared to young individuals. The AUC values for carnosine and acetyl-carnosine were 0.822 and 0.929, respectively (Figures 9A,B). Secondly, we investigated the alterations of acetyl-carnosine level in the metabolome of saliva samples from 13 young and 14 elderly individuals (Teruya et al., 2021). Acetyl-carnosine levels decreased significantly in the saliva of elderly people, with an AUC value of 0.824 (Figure 9C). Next, to explore the relationship between carnosine-related metabolites (N-acetylcarnosine, histidine, and β-alanine) and health variables in humans, we performed an association analysis between the levels of these metabolites and the risk of 27 incident non-communicable diseases (NCDs) as well as all-cause mortality in 11,966 subjects of the EPIC-Norfolk study (Figure 9D) (Pietzner et al., 2021). We found that higher levels of N-acetylcarnosine were associated with a lower prevalence of fractures, COPD and lung cancer, as well as reduced mortality. Furthermore, histidine blood level showed a negative correlation with mortality and multiple age-related diseases, such as heart failure, cerebral stroke, type 2 diabetes, coronary heart disease and COPD. Together, these findings suggest that carnosine can serve as a potential biomarker of aging, which is significantly associated with age-related pathologies in humans.

4 Discussion

During aging, the human oral mucosa undergoes a series of physiological and pathological alterations (Ib et al., 2016). In terms of histology, the oral mucosa in humans demonstrates decreased elastic fibers and disorganization of collagen bundles in the connective tissue (Ib et al., 2016). A recent study on geriatric cynomolgus monkeys indicated that the thickness of gingival lamina propria significantly decreased with aging, and collagen bundles significantly decreased in aged males but not in aged females (Hu et al., 2024). Previous studies have proved decreased epithelial thickness accompanied with increased thickness of the keratin layer in the mouse tongue mucosa during aging (Carrard et al., 2008). Nevertheless, in our study, we found epithelial thickness decreased significantly while the thickness of the keratin layer did not increase significantly in OM compared with YM and AM. This contrary evidence suggested that the adaptive response, which was characterized by an increased keratin layer in response to reduced epithelial thickness, was not as significant as previously thought and that further studies with larger samples were needed.

In addition, the oral mucosal epithelium contains a substantial reservoir of epithelial stem cells essential for maintaining epithelial homeostasis (Papagerakis et al., 2014). The basal cells of oral mucosal epithelium become larger and flatter with aging, accompanied by a decreased cell proliferation rate (Carrard et al., 2008). In our study, we observed an accumulation of senescent cells in tongue epithelium, as indicated by a higher positive rate of SA-β-Gal and $p21^{Waf1}$ in elderly mice compared with young and adult mice. DNA damage, a well-established hallmark of aging (López-Otín et al., 2023), was also found to be increased in the tongue epithelium of elderly mice, evidenced by increased levels of 8-OHdG in comparison to the younger mice. Moreover, the reduced proliferation rate was supported by a decreased Ki-67 positive rate in epithelial cells during aging. Functional decline is another characteristic of oral mucosal aging. For instance, gingival wound healing is severely delayed with aging (Smith et al., 2015). Our findings suggest that the accumulation of senescent cells in epithelium may contribute to the impaired wound healing in oral mucosa during aging.

To find out the age-associated metabolic alterations *in situ*, we comprehensively analyzed the metabolic characteristics of aging in mouse tongues for the first time and identified a total of 4,650 metabolite features using a combination of LC-MS and GC-MS methods. Our findings provide specific insights into age-related metabolic changes in mouse tongues, suggesting that aging has a profound effect on the metabolic characteristics of this tissue. Most of the differential metabolites decreased in OM compared with YM and AM. According to the results of pathway enrichment analysis and correlation analysis, alterations in amino acid metabolism and carbohydrate metabolism play a pivotal role in aging in mouse tongues. We also identified carnosine as a potential biomarker of oral mucosal aging. Carnosine is a naturally occurring dipeptide that has been used as an antioxidant and anti-glycating agent to protect against age-related disorders and facilitate healthy aging (Turner et al., 2021). A recent study indicated that carnosine stimulated macrophage-mediated clearance of senescent skin cells, though it did not directly eliminate senescent cells after tBHP induction (Li et al., 2020). However, our results demonstrated that carnosine supplementation significantly protected against tBHP-induced cellular senescence in HaCaT and HOEC. These findings suggest that carnosine protected cells from cellular senescence through blocking senescence induction rather than clearing senescent cells.

Carnosine not only directly scavenges free radicals but also indirectly enhances endogenous antioxidant systems, chelates metal ions, and inhibits protein carbonylation and glycoxidation (Caruso et al., 2023). The primary antioxidant capacity of carnosine is attributed to the imidazole ring of its L-histidine residue, which reacts directly with ROS to diminish the oxidative reactivity of free radicals. Notably, carnosine has been reported to react with singlet oxygen two-to four-fold faster than free L-histidine (Boldyrev et al., 2013). In this study, carnosine significantly reduced ROS levels in senescent cells. This finding was further supported by Western blot and qRT-PCR results, which showed that carnosine markedly decreased the activation of Nrf2 and its downstream molecules in senescent cells. These results suggest that carnosine inhibits cellular senescence by directly scavenging ROS rather than by indirectly activating endogenous antioxidant systems. Moreover, carnosine is an effective quencher of hydroxyl radicals (·OH), which is a primary cause of DNA damage. DNA damage triggers a DNA damage response and subsequently activates downstream signaling cascades involving p53/p16 and p21/Rb that lead to cell cycle arrest (Shmulevich and Krizhanovsky, 2021). Our results of immunofluorescence and Western blot analysis showed that both carnosine and NAC significantly reduced the expression of DNA damage markers, including 8-OHdG and γH2A.X, in senescent cells. Although the effects of carnosine on ROS scavenging and DNA protection were comparable to those of NAC, carnosine more effectively downregulated the expression of senescence markers such as $p21^{Waf1}$. These results suggest that carnosine is more effective than NAC at inhibiting cellular senescence, likely due to its additional ability to inhibit protein carbonylation and glycoxidation, thereby preserving protein structure and function (Hipkiss et al., 2016).

Nrf2 is a critical transcription factor that regulates more than 600 genes involved in responses to oxidative stress (Wang et al., 2022). It plays a protective role in inhibiting age-related pathologies caused by oxidative damage and inflammation, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, stroke, and multiple sclerosis (George et al., 2022). Nrf2 has been shown to protect against oral mucositis via antioxidation and keratin layer thickening (Wakamori et al., 2022). While Nrf2 activation is generally linked to enhanced defense against oxidative stress, our study showed that tBHP induction increased Nrf2 and HO-1 expression. After carnosine and/or NAC intervention, the expression levels of both Nrf2 and HO-1 were significantly reduced, indicating that carnosine and/or NAC alleviated oxidative stress. Our findings were in line with earlier research on oxidative stress-induced skin damage (Wang et al., 2020). These findings indicated that carnosine downregulated the Nrf2 signaling pathway in keratinocytes to inhibit oxidative stress-induced cellular senescence. It is also important to note that prolonged activation of Nrf2 has negative effects. Long-term activation of Nrf2 in keratinocytes can result in epidermal thickening, hyperkeratosis and inflammation in mice (Schäfer et al., 2012). Activation of Nrf2 in fibroblasts induces cellular senescence and the expression of genes characteristic for cancer-associated fibroblasts (Hiebert et al., 2018). These findings suggested that preventing aberrant Nrf2 pathway activity in aging may be beneficial. To sum up, the mechanisms of Nrf2 under aging and cellular senescence are not fully understood and require further study.

In our study, we further investigated the levels of carnosine-related metabolites in the blood and saliva during human aging. We found that carnosine and acetyl-carnosine significantly decreased with aging. The following ROC analysis proved the high predictive accuracy of these two metabolites as a biomarker of aging. Recent studies have shown that carnosine supplementation is beneficial to several age-related disorders, such as Alzheimer's disease, dementia, Parkinson's disease and type 2 diabetes (Attanasio et al., 2013; de Courten et al., 2016; Caruso et al., 2021; Hariharan et al., 2024). We found that higher N-acetylcarnosine and histidine levels in blood were associated with lower prevalence of some age-related diseases, such as COPD, lung cancer, fractures and heart failure. This evidence suggests that carnosine has the potential to serve as a biomarker of aging, both in the oral epithelium and systemically.

Carnosine was well tolerated and exhibited no toxicity. In rat models, intravenous administration of carnosine at doses ranging from 100 to 2,000 mg/kg did not result in any adverse effects. Parameters such as body weight, food consumption, activity, and mortality did not differ significantly from those in the control group, and no organ lesions were observed (Bae et al., 2013). Moreover, no severe or systemic adverse events were attributed to the subcutaneous injection of a hyaluronic acid containing L-carnosine (2.00 mg/mL) for the treatment of neck wrinkles in humans, and no allergic reactions were reported (Yue and Ju, 2022). Furthermore, Polaprezinc (PZN), a mucosal protective zinc L-carnosine complex used to treat *Helicobacter pylori* infections without the risk of resistance in humans, has also demonstrated an excellent safety profile. The typical PZN dose is 150 mg, which contains 34 mg of zinc and 116 mg of L-carnosine, and no significant adverse events were observed following its oral administration (Mahmoud et al., 2022). PZN lozenge (75 mg/day for 35 days) is effective for prophylaxis against oral mucositis associated with chemotherapy without any side effects (Cesak et al., 2023). Based on the above evidence, carnosine demonstrates high safety and tolerability and can be utilized for the treatment of oral mucosal diseases. In the future, carnosine appears to be a promising and safe therapeutic agent for addressing oral mucosal aging and aging-related disorders.

A limitation of this study is the lack of supplementation experiments to directly verify carnosine's effect on oral mucosal aging in mouse tongues. Future studies should address this through animal models. In conclusion, our study reveals dynamic metabolic reprogramming during natural oral mucosal aging, thereby establishing carnosine's dual role as both an aging biomarker and a therapeutic target for combating age-related mucosal degeneration. Our work not only elucidates the specific mechanism underlying the anti-senescence effect of carnosine, which involves alleviating oxidative stress, reducing DNA damage and downregulating Nrf2/HO-1 pathway, but also provides critical insight into how carnosine may function as an anti-aging pharmaceutical agent. Ultimately, our work lays the foundation for the development of innovative carnosine-based strategies aimed at preserving oral mucosal function, preventing age-related oral diseases, and enhancing oral health in the aging population. In doing so, it contributes to the broader goal of healthy aging.

Data availability statement

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

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. The animal study was approved by Institutional Animal Care and Use Committee of Sun Yat-Sen University (SYSU-IACUC-2024-001928). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

HH: Data curation, Investigation, Writing – original draft. CL: Formal Analysis, Writing – original draft. YX: Software, Validation, Writing – original draft. WL: Methodology, Writing – original draft. ZL: Visualization, Writing – review and editing. BC: Supervision, Writing – review and editing. XT: Conceptualization, Funding acquisition, Writing – review and editing.

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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.

Generative AI statement

The authors declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

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Harnessing nutrients and natural products for sustainable drug development against aging

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Developing treatments for age-related diseases requires cost-effective and efficient approaches. Nutrients and natural metabolites offer safer alternatives to synthetic drugs. Aging increases the need for solutions that protect health and repair cells. Recent studies show that nutrients and natural products reduce oxidative stress, regulate metabolism, and influence longevity-related genes. This review focuses on vitamins, minerals, antioxidants, and natural products that improve healthspan and combat aging. It also discusses challenges such as standardization, clinical validation, and regulatory approval. Finally, emerging trends, such as personalized nutrition and advanced delivery systems, highlight the potential of these metabolites for addressing aging.

KEYWORDS

aging, nutrients, natural products, pharmaceuticals, healthspan

1 Introduction

The increasing number of aged people in the world is causing more problems, especially diseases related to age (Prince et al., 2015; Jaul and Barron, 2017). This situation puts a lot of pressure on healthcare systems (Organization, 2001; Evans and Stoddart, 2017). Therefore, new treatment development is needed strongly that make life longer and improve the quality (Partridge et al., 2018). The increasing prevalence of age-related diseases, such as cardiovascular disorders and neurodegeneration, underscores the need for sustainable solutions (Collaborators G. A., 2022; Collaborators G. M. D., 2022). These solutions should focus on improving both lifespan and healthspan (López-Otín et al., 2013). To solve this issue, research on nutrients and natural products is becoming an important area (Cragg and Newman, 2013; Li et al., 2016c). Natural metabolites are good alternatives to synthetic drugs because synthetic drugs are effective but often cause side effects (Newman et al., 2003; Atanasov et al., 2015). Natural products from food and traditional medicine are safer to use because they do not cause side effects and can still improve health (Veeresham, 2012; Ekor, 2014).

To address these challenges, this review investigates the role of nutrients and natural products in aging. Researchers are studying natural metabolite functions that protect and repair aging cells at the molecular level (Choi and Friso, 2010; Yuan et al., 2016). These metabolites work to reduce oxidative stress, improve metabolism, and regulate aging-related genes (Harman, 1992; Chen et al., 2016). Recently aging studies aim to delay age-related conditions and reverse some cellular changes (López-Otín et al., 2013; Ristow and Schmeisser, 2014).

In this context, the paper focuses on how vitamins, minerals, antioxidants, and botanical drugs can prevent or reverse cellular damage caused by aging (Calder, 2017). These approaches represent a shift in healthcare, where the focus is moving from treating symptoms to preventing diseases (Kaeberlein et al., 2015). This review examines the increasing interest in using nutrients and natural products as potential drugs (Cragg and Newman, 2013; Atanasov et al., 2015). It also explains their important role in healthcare by analyzing recent research trends (Li et al., 2016c; Yuan et al., 2016).

This review focuses on vitamins and minerals with well-documented roles in aging-related pathways, particularly their effects on oxidative stress regulation, immune modulation, mitochondrial function, and neuroprotection. Metabolites were selected based on their mechanistic insights and clinical relevance, with priority given to randomized controlled trials (RCTs), preclinical studies, and systematic reviews/meta-analyses that provided mechanistic or clinical insights into aging interventions. Case reports, narrative reviews, and studies with insufficient methodological details were excluded.

Moreover, this review explores how natural products can be integrated into mainstream medical practices, while also addressing key challenges such as standardization, clinical validation, and regulatory approval. By adopting this approach, we aimed to provide a balanced evaluation of their therapeutic potential in aging-related pathways while discussing their translational challenges.

2 The role of nutrients in anti-aging drug development

Nutrients, the building blocks of life, play a crucial role in maintaining cellular health and preventing age-related decline (Kennedy et al., 2014; Ames, 2018). This section explores various vitamins, minerals, and antioxidants that have shown promise in slowing aging processes. Key nutrients like Vitamin C, Vitamin E, selenium, and omega-3 fatty acids are highlighted for their antioxidative and anti-inflammatory properties, which contribute to cellular longevity and resilience (Rayman, 2012; Li et al., 2016c; Calder, 2017). Recent studies illustrate how these nutrients not only prevent cellular damage but also enhance the efficacy of other therapeutic products, offering a dual advantage in anti-aging therapy (Traber and Atkinson, 2007; Ames, 2018). The discussion extends to the mechanisms through which these nutrients influence genetic pathways associated with aging, such as DNA repair and metabolic regulation (Harman, 2003; López-Otín et al., 2013). These nutrients and their effects and mechanisms are summarized in Table 1.

2.1 Omega-3 fatty acids

Omega-3 fatty acids are essential polyunsaturated fats primarily found in fish oil, flaxseeds, and walnuts (Calder, 2013). These fatty acids are known for their ability to reduce inflammation, support cardiovascular health, and enhance brain function, making them critical for healthy aging (Swanson et al., 2012; Calder, 2017). Their benefits arise mainly from their active forms, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Das, 2006). EPA serves as a precursor for anti-inflammatory eicosanoids, which inhibit pro-inflammatory mediators such as prostaglandins and leukotrienes (Serhan and Levy, 2018). DHA, as a structural metabolite of neuronal membranes, enhances membrane fluidity and modulates synaptic signaling, contributing to cognitive health and neuroprotection (Bazan, 2006). In the cardiovascular system, omega-3 fatty acids improve endothelial function by enhancing eNOS activity, which increases nitric oxide production and promotes vasodilation (Zgheel et al., 2014). They lower blood triglycerides, reduce low-density lipoprotein (LDL) oxidation, and suppress NF-κB signaling, contributing to cardiovascular health (Mozaffarian and Wu, 2011; Calder, 2017). In the brain, omega-3 fatty acids mitigate oxidative stress and neuroinflammation by reducing reactive oxygen species (ROS) and suppressing pro-inflammatory cytokines like IL-6 and TNF-α (Bazan, 2007). DHA activates PI3K/Akt signaling, supporting neuronal survival and reducing the risk of neurodegenerative conditions such as Alzheimer's disease (Akbar et al., 2005; Bazan, 2006). Furthermore, omega-3 fatty acids influence brain-derived neurotrophic factor (BDNF) levels, promoting neurogenesis and cognitive (Cardoso et al., 2014; Dyall, 2015). These combined properties highlight their versatile role in promoting overall health and longevity (Simopoulos, 2008).

2.2 Vitamin A

Vitamin A (Retinoids), a fat-soluble vitamin found in foods like liver, carrots, and leafy green vegetables, plays a crucial role in vision, immune function, and cellular regulation (Blomhoff, 1994; Tanumihardjo, 2011). In vision, vitamin A is indispensable for forming rhodopsin, a light-sensitive pigment in the retina essential for low-light and color vision (Saari, 2012). It also maintains the health of the corneal epithelium and conjunctiva, reducing the risk of conditions like xerophthalmia and age-related macular degeneration (AMD) (Sommer, 2008). Retinoic acid, an active metabolite of vitamin A, regulates genes involved in photoreceptor differentiation and repair, preserving visual function with age (Saari, 2012). Vitamin A supports the immune system by strengthening epithelial barriers in the skin and mucous membranes, which act as the first line of defense against pathogens (Ross, 2012). It enhances the activity of T-cells and B-cells while promoting cytokine production essential for adaptive immunity (Stephensen, 2001; Ross, 2012). This is particularly vital in older populations, where weakened immunity heightens susceptibility to infections (Michaud et al., 2013). Additionally, vitamin A's anti-inflammatory properties modulate NF-κB signaling, helping to reduce chronic inflammation associated with aging (Mora et al., 2008). At the cellular level, retinoic acid governs cell proliferation, differentiation, and apoptosis by activating nuclear receptors such as

TABLE 1 Role of nutrients.

Nutrient	Effect	Mechanism
Omega-3 Fatty Acids	Cardiovascular Health	Improves endothelial function, reduces LDL oxidation
	Cognitive Health	Activates PI3K/Akt signaling, increases BDNF levels
	Anti-Inflammation	Produces resolvins, inhibits NF-κB
Vitamin A	Vision Support	Forms rhodopsin, maintains retinal health
	Immune Function	Strengthens epithelial barriers, enhances T-cell activity
	Cellular Regulation	Activates RARs/RXRs, regulates cell differentiation
	Anti-Inflammation	Modulates NF-κB, reduces chronic inflammation
Vitamin C	Antioxidant	Scavenges ROS, regenerates Vitamin E
	Immune Support	Enhances T-cell activity, boosts interferon production
	Cardiovascular Health	Activates eNOS, prevents LDL oxidation
Vitamin D	Bone Health	Enhances calcium absorption, strengthens bones
	Immune Regulation	Produces antimicrobial peptides, reduces inflammation
	Cognitive Support	Promotes neuronal repair, reduces neurodegeneration risk
Vitamin E	Antioxidant	Neutralizes ROS, prevents lipid peroxidation
	Cardiovascular Benefits	Improves endothelial function, reduces LDL oxidation
	Immune Support	Enhances T-cell function, reduces inflammatory cytokines
Vitamin K2	Bone Mineralization	Activates osteocalcin, promotes calcium binding in bone matrix
	Cardiovascular Health	Activates MGP to prevent arterial calcification, improves arterial elasticity
	Calcium Homeostasis	Regulates calcium distribution between bones and arteries
Coenzyme Q10	Energy Production	Facilitates ATP synthesis by supporting electron transport
	Antioxidant	Neutralizes ROS, protects mitochondrial membranes
	Cardiovascular Health	Enhances nitric oxide production, reduces lipid peroxidation
	Neuroprotection	Stabilizes mitochondria, reduces oxidative neuronal damage
Selenium	Antioxidant	Activates GPx, reduces ROS and oxidative stress
	Immune Support	Enhances T-cell proliferation, balances cytokine levels
	Cognitive Protection	Shields neurons, mitigates neuroinflammation
	Mitochondrial Health	Promotes fusion, prevents protein misfolding
Zinc	Immune Support	Enhances T-cell activity, balances cytokine production
	DNA Repair	Acts as a cofactor for DNA synthesis and repair enzymes
	Antioxidant	Stabilizes cell membranes, supports SOD activity
	Tissue Integrity	Regulates MMPs, prevents tissue stiffness and fibrosis

RARs (Retinoic Acid Receptors) and RXRs (Retinoid X Receptors) (Altucci and Gronemeyer, 2001). Retinoic acid also influences Wnt/β-catenin signaling, playing a key role in tissue regeneration and repair (Blum and Begemann, 2012).

2.3 Vitamin C

Vitamin C (Ascorbic acid), also known as ascorbic acid, is a water-soluble vitamin found in fruits and vegetables such as citrus fruits, strawberries, and bell peppers (Levine et al., 1996; Carr A. C. and Frei B., 1999). It plays a critical role in healthy aging through its antioxidant properties, immune support, and cardiovascular benefits (Carr and Maggini, 2017). As a potent antioxidant, vitamin C scavenges ROS and regenerates other antioxidants like vitamin E, preventing lipid peroxidation and DNA damage, which are key contributors to aging (Frei et al., 1989; Carr A. and Frei B., 1999). It also modulates inflammatory pathways by inhibiting NF-κB activation, reducing chronic inflammation in aging populations (Monacelli et al., 2017). In immune function, vitamin C enhances

T-cell and macrophage activity, promoting pathogen clearance and adaptive immunity (Carr and Maggini, 2017). Additionally, it boosts interferon production, improving antiviral responses and reducing the severity of infections, particularly in older adults (Hemilä, 2017). Vitamin C supports cardiovascular health by activating eNOS to increase nitric oxide (NO) production, improving blood vessel dilation and reducing arterial stiffness (May and Harrison, 2013). Vitamin C prevents LDL cholesterol oxidation, reducing the risk of atherosclerosis (Frei, 1991). Additionally, clinical studies have shown its blood pressure-lowering effects, contributing to cardiovascular health (Juraschek et al., 2012).

2.4 Vitamin D

Vitamin D (Secosteroids), a fat-soluble vitamin, is synthesized in the skin through sunlight exposure and obtained from fatty fish, fortified foods, and supplements (Holick, 2007). It is crucial for bone health, immune regulation, and overall wellbeing, particularly in aging populations (Pludowski et al., 2013). In the immune system, vitamin D enhances innate immunity by stimulating antimicrobial peptides like cathelicidins to combat infections (Wang et al., 2004; Gombart et al., 2005). It modulates adaptive immunity by reducing excessive inflammation via NF-κB signaling, which is vital for preventing respiratory infections in older adults (Wang et al., 2004; Zhang et al., 2014). Vitamin D supports cardiovascular health by improving endothelial function and regulating the renin-angiotensin-aldosterone system (RAAS), lowering blood pressure and reducing arterial stiffness (Carrara et al., 2016; Al-Ishaq et al., 2021). Its anti-inflammatory effects further protect against vascular inflammation, a key factor in cardiovascular diseases (Norman and Powell, 2014; Yin and Agrawal, 2014). In the brain, vitamin D promotes neuronal survival and repair through neurotrophic factors (Khairy and Attia, 2021). Deficiency is linked to increased risks of neurodegenerative diseases and cognitive decline (Berridge, 2017; Feart et al., 2017). Supplementation may improve memory and in deficient individuals (Gowda et al., 2015; Pettersen, 2017).

2.5 Vitamin E

Vitamin E (Tocopherols) is a fat-soluble antioxidant primarily found in nuts, seeds, and vegetable oils (Bramley et al., 2000). It plays a crucial role in protecting cellular metabolites from oxidative damage and supports immune function and cardiovascular health, particularly during aging (Gombart et al., 2005; Traber and Stevens, 2011). Its primary function is to neutralize ROS and prevent lipid peroxidation, thereby safeguarding cell membranes from oxidative damage—a key contributor to aging and chronic diseases (Traber and Atkinson, 2007). Vitamin E also regenerates other antioxidants, such as vitamin C, to their active forms (Niki, 2014). In the immune system, Vitamin E enhances T-cell and natural killer (NK) cell function by stabilizing membranes and improving pathogen responsiveness. It reduces pro-inflammatory cytokines by inhibiting NF-κB signaling, mitigating chronic inflammation commonly seen in aging (Wu and Meydani, 2008). Vitamin E supports cardiovascular health by preventing LDL cholesterol oxidation, improving endothelial function, and reducing platelet aggregation (Freedman and Keaney, 2001; Meydani, 2001). These actions lower the risk of atherosclerosis, heart attacks, and strokes, particularly in individuals with low baseline levels of Vitamin E (Rimm et al., 1993). It also interacts with membrane lipids to optimize fluidity, promoting effective signal transduction for cellular communication (Traber and Packer, 1995; Zingg, 2019).

2.6 Vitamin K2

Vitamin K2 (Menaquinones) is a fat-soluble vitamin found in fermented foods, egg yolks, and dairy products (Khalil et al., 2021). It regulates calcium distribution to support bone and cardiovascular health, especially in aging populations (Hariri et al., 2021; Khalil et al., 2021). It activates osteocalcin, a protein responsible for binding calcium to the bone matrix, thereby improving bone mineral density and reducing the risk of fractures and osteoporosis (Na et al., 2022; Aaseth et al., 2024). Vitamin K2 also synergizes with vitamin D to support bone integrity and mitigate bone loss associated with aging (Kidd, 2010; Capozzi et al., 2020; Singh et al., 2022). In the cardiovascular system, vitamin K2 activates Matrix Gla Protein (MGP), which inhibits arterial calcium deposition (Shioi et al., 2020; Hariri et al., 2021). Vitamin K2 reduces vascular calcification and decreases the risk of atherosclerosis and cardiovascular diseases (Kurnatowska et al., 2015; Shioi et al., 2020; Hariri et al., 2021).

2.7 Coenzyme Q10 (CoQ10)

Coenzyme Q10 (CoQ10, Ubiquinone-10) is a lipid-soluble metabolite found in mitochondria, essential for cellular energy production and antioxidant defense (Turunen et al., 2004; Hidalgo-Gutiérrez et al., 2021). It is abundant in energy-demanding tissues like the heart, brain, and muscles but decreases with age, making it significant in aging research (López-Lluch, 2019; Gutierrez-Mariscal et al., 2021). CoQ10 supports ATP production by facilitating electron transfer in the mitochondrial electron transport chain (ETC) (Mantle and Dybring, 2020; Elgar, 2021). This ensures sufficient energy supply for tissues vulnerable to mitochondrial dysfunction during aging (Barcelos and Haas, 2019; Fišar and Hroudová, 2024). As a potent antioxidant, CoQ10 neutralizes ROS and protects mitochondrial membranes, proteins, and DNA from oxidative damage (Littarru and Tiano, 2007; Akbari et al., 2020). It also regenerates other antioxidants like vitamin E, enhancing overall antioxidant defense (Kagan et al., 2000). In the cardiovascular system, CoQ10 improves endothelial function by boosting nitric oxide (NO) production, promoting vasodilation, and reducing arterial stiffness (Rabanal-Ruiz et al., 2021). It inhibits lipid peroxidation, lowering the risk of atherosclerosis, and has been shown to improve heart function in heart failure patients (Rabanal-Ruiz et al., 2021). CoQ10 contributes to neuroprotection by reducing oxidative damage and improving mitochondrial efficiency in neurons (Young et al., 2007; Pradhan et al., 2021). These effects help maintain synaptic integrity and reduce the risk of neurodegenerative diseases such as Alzheimer's and Parkinson's (Kadian et al., 2022; Bagheri et al., 2023).

2.8 Selenium

Selenium is an essential trace element found in foods like Brazil nuts, seafood, and whole grains (Rayman, 2008). It acts as a cofactor for antioxidant enzymes, including glutathione peroxidase (GPx), and plays a crucial role in protecting cells from oxidative stress while supporting immune function (Beck et al., 2003; Santi et al., 2013). In the antioxidant system, selenium-dependent enzymes such as GPx reduce hydrogen peroxide and lipid hydroperoxides, preventing oxidative damage to lipids, proteins, and DNA (Ingold and Conrad, 2018; Weaver and Skouta, 2022). This action helps maintain cellular integrity and lowers the risk of age-related diseases, including cardiovascular and neurodegenerative disorders (Cai et al., 2019; Bjørklund et al., 2022a). Selenium also enhances the proliferation and function of T-cells and natural killer (NK) cells, modulating cytokine production to balance pro-inflammatory and anti-inflammatory responses (Hoffmann and Berry, 2008; Avery and Hoffmann, 2018). Emerging research suggests that selenium contributes to cognitive health by protecting neurons from oxidative damage and reducing neuroinflammation (Bai et al., 2024). Selenium-dependent enzymes like GPx and thioredoxin reductase help mitigate inflammation linked to neurodegenerative diseases such as Alzheimer's and Parkinson's (Bjørklund et al., 2022c; Oliveira et al., 2023). Beyond its antioxidant role, selenium influences mitochondrial dynamics by promoting mitochondrial fusion and preventing excessive fission, maintaining cellular energy balance (Kumari et al., 2012). Additionally, selenium-dependent enzymes help prevent protein misfolding, a key factor in neurodegenerative diseases (Cardoso et al., 2015; Zhang and Song, 2021).

2.9 Zinc

Zinc is an essential trace mineral found in foods such as meat, fish, and legumes (Fairweather-Tait, 1988). It is involved in various cellular processes, including enzymatic activity, DNA repair, and immune regulation, making it vital for healthy aging (Haase and Rink, 2009; Costa et al., 2023). In the immune system, zinc enhances the function of T-cells and natural killer (NK) cells, which are essential for adaptive and innate immunity (Shankar and Prasad, 1998). It also modulates cytokine production and prevents excessive inflammatory responses by inhibiting NF-κB signaling (Jarosz et al., 2017). This helps maintain immune balance and reduces chronic inflammation, a key contributor to aging-related diseases (Vasto et al., 2006; Wong et al., 2021). Zinc plays a crucial role in DNA repair and genomic stability by serving as a cofactor for enzymes such as DNA polymerase (Wu and Wu, 2023). By supporting DNA synthesis and repair, zinc helps prevent genomic instability, a key factor in cellular aging and age-related diseases (Dreosti, 2001; Costa et al., 2023). Zinc-finger transcription factors contribute to chromatin remodeling and gene regulation, further supporting cellular health during aging (Powell et al., 2019; Kamaliyan and Clarke, 2024). As an antioxidant, zinc stabilizes cell membranes and neutralizes ROS, preventing oxidative damage (Marreiro et al., 2017; Lee, 2018). It also supports the function of antioxidant enzymes like superoxide dismutase (SOD), which protects cells from oxidative stress (Mondola et al., 2016). This antioxidant role helps delay aging and lowers the risk of neurodegenerative and cardiovascular diseases (Prasad, 2014). Zinc regulates cellular senescence by modulating matrix metalloproteinases (MMPs), which remodel extracellular matrix metabolites and prevent tissue stiffness and fibrosis, common in aging tissues (Cabral-Pacheco et al., 2020).

3 Natural products as innovators in aging therapeutics

Natural products, including botanical drugs and phytochemicals, have been used for centuries in traditional medicine, but now gaining prominence in modern pharmacology for their anti-aging properties (Bjørklund et al., 2022b). This paragraph examines key natural products such as resveratrol, curcumin, and ginkgo biloba, focusing on their roles in modulating age-related pathways (Pyo et al., 2020; Assi et al., 2023). Research findings are discussed that demonstrate how these products not only mitigate oxidative stress and inflammation but also activate longevity genes such as sirtuins and AMP-activated protein kinase (AMPK) (Shehzad et al., 2011; Chung et al., 2012; Li X. et al., 2020). This review also explores the potential of these natural products to reverse or delay aging symptoms. These natural products and their effects and mechanisms are summarized in Table 2.

3.1 Resveratrol

Resveratrol (3,5′,4′-trihydroxystilbene, [Polygonaceae]) is a polyphenolic metabolite found in red wine, grapes, and berries (Bonnefont-Rousselot, 2016). It is widely recognized for its antioxidant and anti-inflammatory properties and has been extensively studied for its role in promoting longevity and preventing age-related diseases (Bonnefont-Rousselot, 2016; Pyo et al., 2020). Resveratrol exerts its effects by scavenging ROS, thereby reducing oxidative stress and protecting lipids, proteins, and DNA from oxidative damage (Truong et al., 2018; Koushki et al., 2020). Since oxidative stress is a key contributor to aging and chronic diseases such as cardiovascular and neurodegenerative disorders, resveratrol's antioxidant function plays a critical role in maintaining cellular health (Meng et al., 2020; Leyane et al., 2022). At the molecular level, resveratrol activates SIRT1 (Sirtuin 1), a key regulator of cellular metabolism, DNA repair, and cell survival (Borra et al., 2005; Jeong et al., 2007). By stimulating SIRT1, resveratrol mimics the effects of calorie restriction, enhancing mitochondrial function and promoting longevity (Rogina and Tissenbaum, 2024). Additionally, resveratrol increases mitochondrial biogenesis through the PGC-1α pathway, improving energy efficiency and reducing oxidative damage in aging cells (Higashida et al., 2013; Zhou et al., 2021). It also modulates the NAD+/NADH ratio, an essential factor in cellular metabolism, promoting adaptive stress responses and delaying cellular senescence (Jang et al., 2012; Desquiret-Dumas et al., 2013). Beyond its role in longevity, resveratrol has significant cardiovascular benefits. It improves endothelial function, enhances nitric oxide (NO) production, and increases blood flow,

TABLE 2 Role of natural products.

Natural products	Effect	Mechanism
Resveratrol	Longevity Support	Activates SIRT1, enhances mitochondrial function
	Cardiovascular Health	Improves endothelial function, reduces LDL oxidation
	Neuroprotection	Reduces oxidative stress, protects neurons from inflammation
	Anti-Inflammatory Action	Inhibits NF-κB, lowers pro-inflammatory cytokines
Curcumin	Neuroprotection	Promotes amyloid clearance, reduces oxidative stress
	Anti-Inflammation	Inhibits NF-κB and COX-2 pathways, lowers cytokine levels
	Antioxidant	Scavenges ROS, boosts antioxidant enzyme activity
	Mitochondrial Health	Induces mitophagy through PINK1/Parkin pathway
Quercetin	Antioxidant	Scavenges ROS, prevents oxidative damage to lipids and DNA.
	Anti-Inflammation	Inhibits NF-κB, reduces pro-inflammatory cytokines
	Cardiovascular Health	Enhances endothelial function, reduces LDL oxidation
	Neuroprotection	Protects neurons, reduces oxidative stress and inflammation
Ginseng	Stress Reduction	Modulates HPA axis, lowers cortisol levels
	Cognitive Enhancement	Protects neurons, improves synaptic plasticity
	Antioxidant	Reduces ROS, enhances cellular resilience
	Immune Support	Enhances NK cell activity, strengthens immune defenses
Kaempferol	Antioxidant	Scavenges ROS, protects cells from oxidative damage
	Anti-Inflammation	Inhibits NF-κB and MAPK pathways, reduces cytokine levels
	Neuroprotection	Enhances BDNF expression, supports synaptic plasticity
Apigenin	Antioxidant	Neutralizes ROS, prevents lipid and DNA oxidation
	Anti-Inflammation	Inhibits NF-κB, reduces pro-inflammatory cytokines
	Neuroprotection	Enhances BDNF expression, supports neuronal survival
Green Tea Extract	Antioxidant	Neutralizes ROS, protects DNA and lipids from oxidative damage
	Anti-Inflammation	Inhibits NF-κB, reduces pro-inflammatory cytokines
	Cardiovascular Health	Enhances endothelial function, reduces LDL oxidation
	Neuroprotection	Protects neurons, reduces oxidative stress and neuroinflammation
Alpha-Lipoic Acid	Metabolic Regulations	Activates AMPK, enhances glucose uptake and insulin sensitivity
	Antioxidant	Scavenges free radicals, regenerates antioxidants
	Anti-Inflammation	Modulates NF-κB signalin, reduces inflammation, protects tissues
Astaxanthin	Antioxidant	Neutralizes ROS, protects lipids, proteins, and DNA.
	Neuroprotection	Reduces neuroinflammation, supports neuronal survival
	Cardiovascular Health	Prevents LDL oxidation, enhances endothelial function
	Skin Health	Reduces UV-induced damage, improves skin elasticity

reducing the risk of heart disease (Xia et al., 2014). Additionally, it lowers LDL cholesterol oxidation, a critical factor in atherosclerosis, and improves blood vessel elasticity (Frémont et al., 1999; Cherniack and Troen, 2013; Breuss et al., 2019). Moreover, resveratrol exhibits anti-inflammatory properties by inhibiting NF-κB signaling, a central mediator of chronic inflammation (Ma et al., 2015; Meng et al., 2021).

3.2 Curcumin

Curcumin (*Curcuma longa* L., [Zingiberaceae]), the active metabolite in turmeric (Curcuma longa), is well known for its anti-inflammatory, antioxidant, and neuroprotective properties (Silvestro et al., 2021; Genchi et al., 2024). It has been extensively studied for its potential therapeutic effects in aging and age-related

diseases (Sikora et al., 2010; Nunes et al., 2024). Curcumin exerts its anti-inflammatory effects primarily by inhibiting key inflammatory pathways such as NF-κB and COX-2, which regulate the production of pro-inflammatory cytokines and mediators involved in chronic inflammation (Yuan et al., 2018; Fu et al., 2022). By reducing inflammation, curcumin helps lower the risk of age-related conditions, including arthritis and cardiovascular diseases (He et al., 2015). As an antioxidant, curcumin neutralizes ROS and enhances cellular defense mechanisms (Sathyabhama et al., 2022). It stimulates antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase, which protect cells from oxidative stress, a key driver of aging and neurodegenerative diseases (Khayatan et al., 2024). In the brain, curcumin's antioxidant and anti-inflammatory properties contribute to its neuroprotective effects (Genchi et al., 2024). It mitigates oxidative damage and neuroinflammation, both implicated in Alzheimer's disease, and promotes the clearance of amyloid plaques by activating autophagy and modulating microglial activity (Azzini et al., 2024). Curcumin also plays a crucial role in mitochondrial quality control by inducing mitophagy through the PINK1/Parkin pathway, thereby preventing age-related cellular decline (Cao et al., 2020; Jin et al., 2022). Additionally, it activates the Nrf2-Keap1 pathway, enhancing the production of endogenous antioxidant enzymes to counteract oxidative stress (Soetikno et al., 2013; Lin et al., 2019).

3.3 Querectin

Quercetin (3,3′,4′,5,7-Pentahydroxyflavone, [Fagaceae/Rutaceae]) is a flavonoid found in apples, onions, and green tea. It has strong antioxidant and anti-inflammatory properties, making it useful for aging and age-related diseases (Li et al., 2016b; Deepika and Maurya, 2022). Quercetin neutralizes ROS and reduces oxidative stress in cells, protecting them from damage (Costa et al., 2016). In the immune system, quercetin reduces inflammation by inhibiting the NF-κB pathway, which regulates pro-inflammatory cytokine production (Comalada et al., 2005). This makes quercetin useful in controlling chronic inflammation, especially in aging populations (David et al., 2016). Quercetin improves vascular health by enhancing blood vessel function and reducing LDL cholesterol oxidation, which helps prevent atherosclerosis (Jiang et al., 2020). It also improves circulation and lowers blood pressure (Serban et al., 2016). Studies show that quercetin may protect the brain from oxidative damage and neuroinflammation, both of which contribute to neurodegenerative diseases like Alzheimer's (Khan et al., 2019). By crossing the blood-brain barrier, it helps sustain brain health throughout aging (Grewal et al., 2021). Quercetin also exerts its anti-aging effects by inhibiting the PI3K/AKT/mTOR pathway, which regulates cellular growth and aging (Granato et al., 2017; Li et al., 2018). By modulating this pathway, quercetin promotes autophagy, prevents cellular senescence, and strengthens intercellular junctions to enhance tissue integrity while reducing inflammation-induced damage (Rather and Bhagat, 2020).

3.4 Ginseng

Ginseng (*panax ginseng*, C.A.Mey., [Araliaceae]) is a well-known botanical drug used in traditional medicine for its adaptogenic properties, modulating immune functions and enhancing resilience to various stressors (Ratan et al., 2021). The active metabolites in ginseng, particularly ginsenosides, are responsible for its medicinal effects (Leung and Wong, 2010). Ginseng modulates the hypothalamic-pituitary-adrenal (HPA) axis, which regulates the body's stress response (Lee and Rhee, 2017). By reducing the secretion of cortisol, a stress hormone, ginseng helps lower chronic inflammation and balance hormonal levels (Liao et al., 2018). In the immune system, ginseng enhances the activity of macrophages, T-cells, and natural killer (NK) cells, all of which are crucial for fighting infections and maintaining immune homeostasis (He et al., 2017). Ginsenosides activate pathways such as NF-κB and MAPK, which are involved in immune response and inflammation regulation, helping to strengthen the body's defense mechanisms (Kim et al., 2017). Ginseng also supports cognitive function by protecting neurons from oxidative stress and improving synaptic plasticity (Shin et al., 2024). Ginseng's active metabolites, ginsenosides, modulate AMPK signaling to enhance cellular energy sensing and lipid metabolism (Wang et al., 2022). This mechanism not only combats age-related metabolic disorders but also reduces chronic low-grade inflammation (Fan et al., 2020). Ginseng has also been shown to promote angiogenesis through VEGF signaling, aiding tissue repair and regeneration (Song et al., 2023).

3.5 Kaempferol

Kaempferol (3,4′,5,7-Tetrahydroxyflavone [Fabaceae/Brassicaceae]) is a flavonoid found in a variety of fruits, vegetables, and beverages, including broccoli, kale, beans, and tomato (Calderon-Montano et al., 2011). It has been widely studied for its antioxidant, anti-inflammatory, and anti-cancer properties, making it a promising metabolite for promoting health, particularly in aging (Gutiérrez-del-Río et al., 2016; Shahbaz et al., 2023). Kaempferol acts as a potent antioxidant by neutralizing ROS and preventing oxidative damage to cells (Wu et al., 2018). By scavenging free radicals, kaempferol reduces oxidative stress, which is a major contributor to aging and the development of chronic diseases, such as cardiovascular disease, cancer, and neurodegenerative disorders (Liao et al., 2016; Rahul and Siddique, 2021; Kamisah et al., 2023). In terms of inflammation, kaempferol modulates inflammatory pathways, particularly by inhibiting NF-κB and MAPK signaling, which are involved in the production of pro-inflammatory cytokines (Wang et al., 2020). In neuroprotection, kaempferol has been shown to protect neurons from oxidative stress and reduce neuroinflammation (Nezhad Salari et al., 2024). It may help in maintaining cognitive function by promoting the production of BDNF, which is involved in neuronal survival and synaptic plasticity (Silva dos Santos et al., 2021). Kaempferol inhibits the senescence-associated secretory phenotype (SASP) by reducing the production of inflammatory cytokines such as IL-6 and TNF-α (Lim et al., 2015; Hussain et al., 2024).

3.6 Apigenin

Apigenin (4′,5,7-Trihydroxyflavone) [Asteraceae] is a flavonoid found in a variety of plants, including parsley, celery, chamomile, and basil (Salehi et al., 2019b). Known for its antioxidant, anti-inflammatory, and anti-cancer properties, apigenin has been studied for its potential health benefits, particularly in aging

(Madunić et al., 2018). Apigenin acts as a potent antioxidant by scavenging ROS and reducing oxidative stress in cells (Jung, 2014). In terms of inflammation, apigenin inhibits the NF-κB pathway, which is involved in the production of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α (Patil et al., 2016). By modulating this pathway, apigenin helps reduce chronic inflammation, a common contributor to diseases like arthritis, heart disease, and neurodegenerative disorders in aging populations (Ali et al., 2017). In neuroprotection, apigenin has been shown to reduce oxidative stress and inflammation in the brain (Li R. et al., 2016). It increases the expression of BDNF, which is important for neuronal survival and synaptic plasticity (Sharma et al., 2020; Gao et al., 2023). These effects help maintain cognitive function and may reduce the risk of neurodegenerative diseases like Alzheimer's (Zhao et al., 2013). Apigenin enhances mitochondrial function through increased expression of SOD2, reducing mitochondrial-derived oxidative stress (Jung, 2014; Pal et al., 2017). Apigenin's ability to stabilize transcription factors like FOXO3 further contributes to its longevity-enhancing effects (Kawasaki et al., 2010; Lin et al., 2015).

3.7 Green tea extract

Green Tea Extract (*Camellia sinensis* (L.) Kuntze) [Theaceae] is derived from the leaves of Camellia sinensis and is rich in polyphenols, particularly epigallocatechin gallate (EGCG), which is the main natural products (Komes et al., 2010; Du et al., 2012). It is well-known for its antioxidant, anti-inflammatory, and metabolism-enhancing properties, making it a popular natural product for promoting healthy aging (Chen et al., 2009; Senanayake, 2013; Ohishi et al., 2016). Green tea extract exhibits strong antioxidant activity by neutralizing ROS and enhancing the activity of endogenous antioxidant enzymes (Elbling et al., 2005). The extract also exerts anti-inflammatory effects by inhibiting pathways like NF-κB and reducing levels of pro-inflammatory cytokines such as IL-6 and TNF-α (Ohishi et al., 2016). In addition, EGCG from green tea extract improves mitochondrial function and enhances energy metabolism by activating pathways like AMPK, which promotes cellular energy balance (Pournourmohammadi et al., 2017; Ha et al., 2018). Green tea extract also supports cognitive health by protecting neurons from oxidative stress and reducing neuroinflammation (Prasanth et al., 2019; Valverde-Salazar et al., 2023). Studies have shown its ability to enhance memory and learning in aging populations (Mancini et al., 2017). Green tea extract, rich in EGCG, enhances the DNA damage response (DDR) by upregulating ATM and ATR kinases, which repair double-strand breaks (Kuo et al., 2016; Majidinia et al., 2019). Additionally, EGCG modulates circadian clock genes, improving metabolic and cognitive functions in aging individuals (Liu et al., 2023).

3.8 Alpha-Lipoic Acid (ALA)

Alpha-Lipoic Acid (1,2-Dithiolane-3-pentanoic acid) (ALA) is a natural metabolite with strong antioxidant properties, crucial for energy metabolism and cellular protection (Tibullo et al., 2017; Superti and Russo, 2024). It is found in small amounts in foods like vegetables, futis and red meat (Salehi et al., 2019a). ALA's water- and fat-solubility allows it to act in various cellular compartments, where it scavenges free radicals and regenerates other antioxidants, contributing to healthy aging (Shay et al., 2009; Nobakht-Haghighi et al., 2018). Additionally, ALA modulates NF-κB signaling, reducing inflammation and protecting tissues from chronic damage (Chang et al., 2017; Ishii et al., 2017). In metabolic health, ALA enhances insulin sensitivity by activating AMPK (AMP-activated protein kinase), which improves glucose uptake in muscle cells and lowers blood sugar levels (Lee et al., 2005; Targonsky et al., 2006; Shen et al., 2007).

3.9 Astaxanthin

Astaxanthin (3,3′-Dihydroxy-β,β-carotene-4,4′-dione) [Haematococcaceae] is a carotenoid pigment primarily found in microalgae, salmon, and krill (Johnson and An, 1991). It is known for its powerful antioxidant and anti-inflammatory properties, which contribute to its benefits for aging, skin health, and overall wellbeing (Pereira et al., 2021; Chae et al., 2022). As a powerful antioxidant, astaxanthin is significantly stronger than other carotenoids such as β-carotene and lutein (Naguib, 2000; Yuan et al., 2011; Ambati et al., 2014). It neutralizes ROS and protects cells from oxidative damage by scavenging free radicals (Mohammadi et al., 2021). Its unique structure enables it to safeguard both the lipid and aqueous phases of cells, effectively protecting various cellular metabolites, including cell membranes, proteins, and DNA (Ambati et al., 2014; Brotosudarmo et al., 2020). In addition to its antioxidant effects, astaxanthin exhibits anti-inflammatory properties by inhibiting key signaling pathways, including NF-κB and COX-2 (Choi et al., 2008; Farruggia et al., 2018). These pathways are involved in the production of pro-inflammatory cytokines and mediators, which contribute to chronic inflammation (Lee et al., 2003). By modulating these pathways, astaxanthin helps prevent the progression of inflammation-related diseases such as arthritis, cardiovascular disease, and neurodegeneration (Chang and Xiong, 2020). Astaxanthin also supports eye health by reducing oxidative stress and inflammation in the retina (Yeh et al., 2016; Giannaccare et al., 2020). It protects retinal cells from damage caused by light exposure and helps prevent AMD, a leading cause of vision loss in older adults (Otsuka et al., 2013; Giannaccare et al., 2020; Alugoju et al., 2023). Additionally, astaxanthin improves blood flow to the eyes, further enhancing visual function (Giannaccare et al., 2020).

4 Upcoming trends and challenges

As research continues to uncover the vast potential of nutrients and natural products in promoting health and longevity, several emerging trends and challenges are shaping the future of anti-aging therapies (Ros and Carrascosa, 2020; Bjørklund et al., 2022b). These developments highlight the importance of a holistic and evidence-based approach to leveraging natural metabolites for sustainable health benefits.

4.1 Personalized nutrition and precision medicine

One of the most promising trends is the move towards personalized nutrition and precision medicine. Advances in

genomics and metabolomics are enabling a deeper understanding of individual differences in nutrient metabolism and response (Kaput and Rodriguez, 2006; Hadi, 2023). This personalized approach allows for tailored dietary and supplementation strategies that align with a person's genetic profile, lifestyle, and health status, optimizing the efficacy of natural anti-aging interventions (Biesalski et al., 2009; Afshin et al., 2019). The integration of artificial intelligence and big data analytics further enhances the ability to develop customized health plans that promote longevity and mitigate age-related conditions (Topol, 2019).

Several clinical applications of precision medicine in nutrition are already in use (Wishart, 2016). Nutrigenomics-based dietary interventions enable personalized dietary recommendations based on genetic variants affecting nutrient metabolism, such as MTHFR polymorphisms influencing folate metabolism (Kiani et al., 2022; Andrade et al., 2025). Metabolomics-guided supplementation tailors nutrient intake by analyzing an individual's metabolic profile to identify micronutrient deficiencies and optimize supplementation strategies (Rigamonti et al., 2023). Additionally, biomarker-driven antioxidant therapy assesses oxidative stress markers, such as the glutathione (GSH/GSSG) ratio, to guide personalized antioxidant interventions aimed at reducing cellular damage and improving metabolic health (Yagishita et al., 2020; Pan et al., 2024). These advancements illustrate how precision medicine strategies are being integrated into clinical practice to enhance the effectiveness of nutrient-based interventions (Bailey and Stover, 2023; Keijer et al., 2024).

4.2 Synergistic formulations

Another trend is the development of synergistic formulations that combine multiple nutrients and natural products to enhance their collective benefits (Ebrahimi et al., 2023). Research is increasingly focusing on the synergistic effects of metabolites such as vitamins, polyphenols, and omega-3 fatty acids (Liu, 2003; Li et al., 2016c; Calder, 2017; Ebrahimi et al., 2023). These combinations can potentially offer more significant health benefits than individual metabolites by targeting multiple pathways involved in aging (Liu, 2003). Formulating these combinations into easily accessible supplements and functional foods can provide practical solutions for aging populations seeking to maintain their health (Cencic and Chingwaru, 2010).

4.3 Advances in delivery systems

Many natural metabolites suffer from poor bioavailability due to low solubility, rapid metabolism, and degradation, limiting their therapeutic efficacy. Addressing these challenges, improving the bioavailability and efficacy of natural metabolites through advanced delivery systems is a crucial area of innovation (Obeid et al., 2017).

In vivo bioavailability is often restricted by multiple factors, including poor water solubility, instability in the gastrointestinal (GI) tract, extensive first-pass metabolism, and rapid systemic clearance. For instance, curcumin, a well-known polyphenol, has poor absorption due to its hydrophobic nature and rapid hepatic metabolism (Nelson et al., 2017). Coenzyme Q10 (CoQ10) also exhibits poor bioavailability due to its high molecular weight and low water solubility, which limit its intestinal absorption and systemic circulation (Zhang et al., 2022). Polyphenols such as quercetin and resveratrol also face challenges related to instability and rapid conjugation, leading to reduced systemic bioavailability (El Monfalouti and Kartah, 2024).

To overcome these limitations, several advanced drug delivery technologies are being explored. Nanoencapsulation improves the solubility and stability of hydrophobic metabolites, facilitating better absorption (Livney, 2016). Liposomal delivery systems, composed of phospholipid bilayers, protect active metabolites from enzymatic degradation and enhance their permeability across biological membranes (Singh et al., 2019). Solid lipid nanoparticles and nanostructured lipid carriers further enhance drug stability and prolong systemic circulation, improving therapeutic efficacy (Safta et al., 2024).

Another promising approach is the use of cyclodextrin complexes, which form inclusion complexes with poorly soluble metabolites, increasing their water solubility and enhancing bioavailability (Loftsson and Brewster, 2012; Kurkov and Loftsson, 2013). Prodrug strategies, which involve modifying the chemical structure of a metabolite to improve its absorption and metabolic stability, are also gaining attention in the field of natural product-based therapeutics (Rautio et al., 2008).

These technologies can overcome the limitations of traditional supplementation, ensuring that active metabolites reach their target tissues at therapeutic levels. Further research is needed to refine these delivery strategies and optimize their application in clinical settings to maximize the therapeutic potential of natural metabolites.

4.4 Regulatory and quality assurance challenges

Despite the potential benefits, the widespread adoption of nutrient and natural product-based therapies faces significant regulatory and quality assurance challenges (Yadav et al., 2024). Ensuring the safety, efficacy, and consistency of these products requires stringent regulatory oversight and robust quality control measures (Bailey et al., 2013). The variability in the composition of natural products, influenced by factors such as sourcing, processing, and storage, necessitates standardized practices to guarantee their therapeutic potential (Kunle et al., 2012).

In addition, the interaction between natural metabolites and conventional medications remains a critical concern. Some nutrients and phytochemicals can influence drug metabolism by modulating cytochrome P450 enzymes, leading to altered drug efficacy or toxicity (Holst and Williamson, 2008; Johnson, 2008). For instance, St. John's Wort induces CYP3A4, reducing the plasma concentration of certain drugs, whereas grapefruit juice inhibits the same enzyme, leading to potential drug accumulation and adverse effects (Dürr et al., 2000; Dahan and Altman, 2004; Rahimi and Abdollahi, 2012). Moreover, excessive intake of fat-soluble vitamins, iron, or polyphenols may lead to toxicity or interfere with drug absorption (Martin and Appel, 2009; Awuchi et al., 2020). Addressing these risks requires comprehensive safety assessments and clearer guidelines on the co-administration of natural products with pharmaceuticals (Colalto, 2010).

Furthermore, the lack of standardized labeling and dosage recommendations for natural supplements increases the risk of misuse (Avigan et al., 2016; Bailey, 2020). Unlike pharmaceuticals, where dosing is strictly regulated, natural metabolites often exhibit dose-dependent effects, making it challenging to establish universally safe and effective intake levels (Hasler, 2002; Wen et al., 2021). Certain natural products may also be contraindicated for individuals with specific health conditions. For example, high doses of omega-3 fatty acids may increase bleeding risk in individuals taking anticoagulants (Kapoor et al., 2021). Regulatory agencies must work towards harmonized guidelines that define safe consumption limits and potential contraindications, ensuring that natural product-based therapies are both effective and safe for consumers (Bast et al., 2002).

4.5 Ethical and accessibility considerations

As the popularity of anti-aging therapies grows, ethical and accessibility considerations must be addressed (Trothen, 2024). Ensuring that advancements in personalized nutrition and natural therapies are accessible to diverse populations, including underserved communities, is vital for promoting health equity (Petersen and Kwan, 2011). Additionally, ethical considerations regarding the use of genetic information and personalized health data must be carefully managed to protect individual privacy and autonomy (Vayena et al., 2018).

Furthermore, economic and environmental challenges must be considered when evaluating the scalability of natural metabolite-based interventions. Certain high-value natural metabolites, such as fish-derived omega-3 fatty acids and rare plant extracts, pose challenges in terms of cost, availability, and sustainability (Venegas-Calerón et al., 2010). The extraction and production of these metabolites often require significant resources, leading to high consumer prices and potential supply chain limitations (Palma et al., 2016). Additionally, overharvesting of rare botanical sources and intensive aquaculture practices for omega-3 production can contribute to ecological imbalances and biodiversity loss (Falkenberg et al., 2020; Zhang et al., 2024). To mitigate these challenges, research is increasingly focusing on alternative sources, such as microalgae-derived omega-3 and biotechnologically synthesized plant metabolites, which offer more sustainable and scalable solutions (Liu et al., 2022; Hassan Zadeh, 2024). Addressing these economic and environmental barriers is crucial for ensuring that natural therapies remain viable and accessible to a broader population.

4.6 Clinical trials investigating nutrients and natural products for age-related diseases

Several clinical trials have investigated the efficacy of nutrients and natural products in treating age-related diseases, including ocular, neurological, and muscular disorders (Dai et al., 2010; Suzuki et al., 2013; Dysken et al., 2014; Giannaccare et al., 2019; Voulgaropoulou et al., 2019). These studies provide critical insights into the therapeutic potential and limitations of natural metabolites in clinical settings. Table 3 summarizes key trials assessing the impact of these products on human health. While many studies demonstrate promising effects of natural products in *in vitro* models, these findings require further validation through well-designed *in vivo* and clinical studies to establish translational relevance (Denayer et al., 2014; Sorkin et al., 2020). While some studies demonstrate promising benefits, such as improved cognitive function and reduced oxidative stress, further large-scale, long-term trials are needed to confirm their effectiveness and establish optimal dosing strategies.

4.7 Essential amino acids in aging

Beyond vitamins, minerals, and natural products, essential amino acids also contribute to aging-related processes, particularly in muscle maintenance, metabolic regulation, and immune function (Roth, 2007). Branched-chain amino acids (BCAAs) such as leucine, isoleucine, and valine have been shown to support protein synthesis and mitochondrial function, while other amino acids like arginine and methionine play roles in oxidative stress modulation and immune response (Nie et al., 2018; Dai et al., 2020). While this review primarily focuses on non-protein nutrients, future research should explore the potential synergistic effects of essential amino acids with vitamins and natural metabolites in aging interventions.

4.8 Study limitations and research challenges

While numerous studies support the beneficial effects of nutrients and natural products in aging-related processes, many face limitations such as small sample sizes, lack of long-term follow-up, and variability in study designs, including differences in formulations, treatment duration, and administration routes (Ziegler et al., 2009; Puri et al., 2022; Li and Wang, 2024; Wimalawansa, 2025). Additionally, heterogeneity in study populations, inconsistent methodologies, and potential biases in self-reported dietary intake challenge the generalizability of findings (Behmer et al., 2002; Carocho and Ferreira, 2013; Mirmiran et al., 2021). Some clinical trials report positive outcomes, but confounding factors such as lifestyle, genetic variability, and concurrent interventions may influence results (Davey Smith and Ebrahim, 2003; Welch et al., 2011). Furthermore, preclinical studies, including In silico and *In vitro* models, provide valuable mechanistic insights but have inherent limitations in predicting clinical efficacy (Wu et al., 2020; Vashishat et al., 2024). Computational models rely on algorithm-based predictions that may not fully capture the complex biochemical interactions occurring in human physiology (Southern et al., 2008; Lee and Hu, 2019). Likewise, *In vitro* studies often utilize simplified cellular systems that lack systemic metabolic interactions, immune responses, and tissue-specific effects, making it difficult to extrapolate findings to whole-body physiology (Cook et al., 2012; McGonigle and Ruggeri, 2014).

A major controversy in the field is the lack of consensus on the effective doses of natural metabolites, which complicates their clinical translation (Singh et al., 2015). Unlike pharmaceuticals with well-defined dosing parameters, natural products often exhibit dose-dependent effects influenced by formulation, bioavailability, and individual metabolic differences (Boullata, 2005). For instance, curcumin requires high doses to achieve

TABLE 3 Clinical trials on nutrients and natural products for age-related diseases.

Nutrient/Natural product	Disease	Key findings	Model	Dose	References
Omega-3 Fatty Acids	Dry Eye Disease	Improves dry eye symptoms, tear film stability, and tear production	Clinical (RCTs)	Eicosapentaenoic acid (EPA) 128~1,440 mg Docosahexaenoic acid (DHA) 99~1,050 mg	Brignole-Baudouin et al. (2011), Bhargava et al. (2013), Bhargava and Kumar (2015), Bhargava et al. (2016a), Bhargava et al. (2016b), Giannaccare et al. (2019)
Vitamin D	Alzheimer's disease, Parkinson's disease	Enhanced cognitive function, Stabilized Parkinson's disease	Clinical (RCTs)	1200 IU/d, 2000 IU/d	Annweiler and Beauchet (2011), Annweiler et al. (2012), Suzuki et al. (2013)
Vitamin E	Alzheimer's Disease	Slowed functional decline in Alzheimer's disease	Clinical (RCTs)	1000 IU twice a day	Sano et al. (1997), Grundman (2000), Dysken et al. (2014)
Coenzyme Q10	Cardiovascular disease, Parkinson's disease	Improved mitochondrial function and vascular health in heart failure Slowed functional decline in early Parkinson's with 1,200 mg/day CoQ10	Clinical (RCTs)	200~1,200 mg/day	Shults et al. (2002), Mortensen et al. (2014)
Curcumin	Alzheimer's disease	Enhanced cognitive function	Clinical (RCTs)	300~1,500 mg/day	Hishikawa et al. (2012), Cox et al. (2015), Rainey-Smith et al. (2016), Voulgaropoulou et al. (2019)

therapeutic effects due to its poor bioavailability, whereas lower doses may be sufficient for other polyphenols (Mahran et al., 2017; Abd El-Hack et al., 2021). Additionally, discrepancies in study methodologies, including variability in outcome measurements and data analysis approaches, contribute to inconsistent conclusions across studies (Kuhl, 2005; Argyropoulou et al., 2013; Sorkin et al., 2016). Many preclinical studies rely on *in vitro* models or animal experiments that do not fully replicate human physiological conditions, limiting their direct applicability (Henderson et al., 2013). While *In vitro* assays can provide initial screening for bioactivity, they lack the systemic complexity required to assess pharmacokinetics, organ-specific metabolism, and potential toxicity (Lipscomb and Poet, 2008; Astashkina et al., 2012). Therefore, findings from these models should be interpreted cautiously and validated through well-designed *In vivo* and clinical studies (Denayer et al., 2014).

In clinical trials, differences in intervention protocols, inconsistencies in sample selection, and inadequate control groups often result in conflicting findings (Jüni et al., 2001). Moreover, a lack of standardization in study endpoints and assessment methods further complicates comparative analysis, making it difficult to derive definitive conclusions about the efficacy of natural metabolites (Ziegelmann et al., 2020).

Another critical limitation is the insufficient understanding of the pharmacokinetics and bioavailability of many natural products (Hao et al., 2014; Pathak and Raghuvanshi, 2015). Variability in formulation types (e.g., whole extracts vs isolated metabolites), administration routes, and metabolic differences among individuals can significantly alter their biological activity (Possemiers et al., 2011; Sova and Saso, 2020). Without standardized dosing guidelines, it remains challenging to translate preclinical findings into effective therapeutic applications (Ioannidis et al., 2018).

Addressing these challenges requires standardized pharmacokinetic profiling and dose-response studies to establish optimal therapeutic windows for natural metabolites (Zeng et al., 2022). Future studies should also prioritize multicenter RCTs with larger and more diverse populations to improve the robustness of findings (Li W. et al., 2020). Furthermore, long-term clinical trials are necessary to determine the sustained effects and safety profiles of these metabolites over extended periods (Reginster et al., 2001; Zhou Y. et al., 2016). Given the variability in bioavailability and metabolic responses, future research should explore personalized approaches integrating metabolomics and nutrigenomics to tailor interventions to individual needs (Vyas et al., 2018; Lagoumintzis and Patrinos, 2023). Additionally, studies should focus on the synergistic effects of multiple natural metabolites, as combination therapies may enhance efficacy through complementary mechanisms (HemaIswarya and Doble, 2006; Zhou X. et al., 2016). Advanced drug delivery systems, such as nanoformulations and liposomal encapsulation, should also be investigated to improve bioavailability and therapeutic potential (Allen and Cullis, 2013; Khan et al., 2013). Additionally, *In silico*/*In vitro* methodologies should be supplemented with well-structured translational research strategies, ensuring that mechanistic findings can be effectively validated in physiologically relevant models (Bai et al., 2018; Rowland et al., 2018). Emerging technologies such as organ-on-a-chip systems and advanced 3D cell cultures may provide more predictive data, bridging the gap between early-stage research and clinical applications (Zhang and Radisic, 2017; Cavero et al., 2019). To establish more definitive conclusions, well-designed, large-scale, long-term clinical trials are needed (Piantadosi, 2024).

4.9 As the popularity future research directions

Ongoing research is critical to fully elucidate the mechanisms underlying the anti-aging effects of nutrients and natural products (Wu et al., 2024). Large-scale, long-term clinical trials are needed to confirm their efficacy and safety (Ahmed et al., 2023).

Interdisciplinary collaborations among researchers, healthcare professionals, and industry stakeholders will drive innovation and translate scientific findings into practical applications (Bhavnani et al., 2017). Exploring new natural metabolites with anti-aging potential and understanding their interactions with existing therapies will continue to expand the repertoire of effective interventions (Wan et al., 2024).

5 Conclusion

The exploration of nutrients and natural products for sustainable drug development against offers significant health benefits. Unlike conventional pharmaceuticals, these natural metabolites often have fewer side effects while maintaining both efficacy and safety. By focusing on the therapeutic potential of metabolites derived from food and natural sources, researchers are uncovering new pathways to enhance health and longevity. Nutrients and natural products have demonstrated their capacity to modulate key biological pathways related to immune function, inflammation, and neuroprotection. Recent studies have highlighted the importance of targeting aging-related processes such as mitochondrial dysfunction, genomic instability, and cellular senescence.

In conclusion, leveraging the potential of nutrients and natural products provides a sustainable, effective approach to aging-related therapies. While this review primarily focuses on vitamins, minerals, and natural products, future research should also consider the role of essential amino acids in aging-related interventions. These metabolites address aging symptoms and target its underlying causes, offering the potential to significantly improve healthspan and quality of life. Anti-aging strategies could be transformed through continued exploration and advancements in delivery technologies and personalized medicine, benefiting individuals and healthcare systems worldwide.

Author contributions

FD: Conceptualization, Writing – original draft. YY: Data curation, Writing – original draft. YZ: Data curation, Writing – original draft. SW: Data curation, Writing – original draft. JH: Writing – original draft, Writing – review and editing. ZL: Conceptualization, Investigation, Writing – original draft. H-BJ: Investigation, Writing – original draft. DR: Writing – review and editing. WP: Funding acquisition, Writing – review and editing. K-TH: Funding acquisition, Writing – review and editing. LG: Conceptualization, Supervision, Writing – review and editing.

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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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BCAA metabolism in cancer progression and therapy resistance: The balance between fuel and cell signaling

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Branched-chain amino acids (BCAAs), including leucine, isoleucine, and valine, play a crucial role in cellular metabolism and signaling. Recent studies have demonstrated that BCAA metabolic reprogramming is a key driver of tumor progression and treatment resistance in various cancers. BCAA metabolism supports cancer cell growth, survival, and proliferation by modulating pathways such as mTOR signaling and oxidative stress responses. By promoting immunosuppressive conditions and increasing the survival rate of cancer stem cells (CSCs), BCAAs contribute to immune evasion and resistance to therapies such as chemotherapy and immune checkpoint inhibitors. This article explores the different metabolic reprogramming patterns of BCAAs in various tumors and introduces BCAA-related metabolic targets for overcoming tumor resistance, offering new directions for precision cancer treatment, reducing resistance, and improving patient outcomes.

KEYWORDS

branched-chain amino acids (BCAAs), tumor progression, metabolic reprogramming, therapy resistance, the tumor microenvironment (TME)

1 Introduction

Cell metabolism is a fundamental characteristic for maintaining life activities. The growth, differentiation, death, and stress responses of cells require regulation centered around metabolites and metabolic enzymes (Pavlova et al., 2022; Martínez-Reyes and Chandel, 2021; Stine et al., 2022). Recent studies have shown that metabolites can act as signaling molecules to regulate cellular signal transduction and participate in various biological processes such as intercellular communication and epigenetic regulation (Bergers and Fendt, 2021; Wu et al., 2023; Zanotelli et al., 2021). Compared to normal physiological activities, tumor metabolism is a highly complex process that involves an imbalance of multiple metabolites and the reshaping of metabolic pathways during tumor development. Tumor cell metabolism requires the support of various nutrients, including glucose, amino acids, and fatty acids. For example, under normal oxygen conditions, tumor cells, unlike normal cells, still rely heavily on glycolysis to consume large amounts of glucose and produce lactic acid—a phenomenon known as the "Warburg effect" (Zhong et al., 2022). Tumors are widely recognized as metabolic diseases, and metabolic reprogramming is one of the key characteristics of tumors (Xia et al., 2021). Metabolic reprogramming enables tumor cells to adjust their metabolic patterns in response to various stimuli and stressors in

the microenvironment, thereby enhancing their survival and proliferation (Martínez-Reyes and Chandel, 2021). The occurrence of tumor metabolic reprogramming may result from the activation or mutation of oncogenes and tumor suppressor genes, which alters the expression and activity of key metabolic enzymes in metabolic signaling pathways, leading to metabolic reprogramming and tumor progression (Xu D. et al., 2021).

Branched-chain amino acids (BCAAs) are essential amino acids for human nutrition and include three amino acids with branched side chains: leucine, isoleucine, and valine. These three amino acids not only form the basic building blocks of proteins but also play critical roles in cellular signaling pathways, energy metabolism, and immune regulation, influencing tumor development and progression (Sivanand and Vander Heiden, 2020; Qian et al., 2023; Ma et al., 2024).

The tumor microenvironment (TME) is a complex cellular environment in which tumor cells reside, composed of various cell types and extracellular components surrounding the tumor cells (de Visser and Joyce, 2023). Cells and extracellular components in the TME interact with tumor cells, promoting their proliferation and invasion while reducing drug permeability. Immune cells are a key component of the TME, and amino acids are essential for protein synthesis and play a role in various physiological activities and immune system regulation (Kao et al., 2022; Leone and Powell, 2020). The reshaping of amino acid metabolism provides energy and raw materials for tumor growth and acts as signaling molecules regulating tumor development (Muthusamy et al., 2020). They also function in maintaining cellular redox balance, driving nucleotide synthesis, and generating energy (Raffel et al., 2017). Particularly, in different types of tumors, selectively inhibiting tumor progression can be achieved by limiting specific amino acid metabolism (Ericksen et al., 2019). Tumor resistance remains a major challenge in cancer treatment, leading to treatment failure and disease progression (O'Donnell et al., 2019). Metabolic reprogramming-induced tumor resistance mediated by the tumor microenvironment can also serve as a new therapeutic target. This review explores the mechanisms by which BCAAs metabolic reprogramming promotes cancer immune evasion and immune suppression. Additionally, it discusses the potential of targeting BCAA metabolism as a therapeutic strategy to inhibit tumor growth, enhance anti-tumor immune responses, and overcome drug resistance.

2 Metabolism of BCAAs in the body and metabolic reprogramming of BCAAs in tumors

Branched-chain amino acids (BCAAs), including leucine, valine, and isoleucine, can only be supplemented through diet and account for approximately 35% of essential amino acids in proteins and 18% of all amino acids. Under normal conditions, there is a dynamic balance between the intake and consumption of BCAAs (Blair et al., 2021). The most common dietary sources of BCAAs are high-fat dairy products, meat, and synthetic fitness supplements, making them important nutrients. Generally, supplementing BCAAs or a diet rich in BCAAs is beneficial for maintaining metabolic balance in the body. However, long-term elevated circulating BCAA levels (20%–50% higher than normal physiological concentrations) (Wang et al., 2011) have been associated with obesity, type 2 diabetes mellitus (T2DM), cardiovascular diseases, and certain tumors (White and Newgard, 2019; Siddik and Shin, 2019; Zheng et al., 2024).

BCAAs are ingested through food (mainly from proteins) and are broken down by proteolytic enzymes in the gastrointestinal tract into individual amino acids, which are then absorbed into the bloodstream *via* the small intestine. Notably, gut microbiota, contribute approximately 12% of circulating BCAAs through proteolytic activity (Zhang et al., 2017; Pedersen et al., 2016). The gut microbiome, particularly *Prevotella copri* and *Bacteroides vulgatus*, promotes insulin resistance by increasing circulating branched-chain amino acids through enhanced microbial biosynthesis and reduced bacterial uptake (Pedersen et al., 2016). Once absorbed, BCAAs enter the bloodstream and are predominantly taken up by muscle tissue, which is rich in enzymes necessary for BCAA metabolism. Extracellular BCAAs utilize L-type amino acid transporters (LATs) to shuttle the cytoplasmic membrane into the cytoplasm (Peng et al., 2020) and the transport protein SLC25A44 assists BCAAs in entering mitochondria (Yoneshiro et al., 2019). These processes can all influence the levels of branched-chain amino acids (BCAAs) in plasma. The metabolism of BCAAs involves several steps (Figure 1): **Step 1:** In muscle and other tissues, BCAAs first undergo transamination catalyzed by branched-chain amino acid aminotransferase (BCAT), generating the corresponding branched-chain keto acids (BCKAs) (Dimou et al., 2022). This step converts BCAAs into α-keto acids, releasing amino groups that are used for amino acid synthesis or the urea cycle. **Step 2:** Oxidative decarboxylation occurs, where the generated branched-chain keto acids undergo further oxidative decarboxylation by the branched-chain keto acid dehydrogenase complex (BCKDH), producing their respective acyl-CoA derivatives (e.g., isovaleryl-CoA) (Peng et al., 2020). This process is the rate-limiting step of BCAA metabolism and mainly occurs in the liver and muscle (Du et al., 2022). **Step 3:** These acyl-CoA intermediates are further metabolized in the tricarboxylic acid (TCA) cycle, producing carbon dioxide, water, and energy. Leucine metabolism generates acetyl-CoA and acetoacetate, while isoleucine produces succinyl-CoA, and valine yields propionyl-CoA (Mann et al., 2021).

The metabolism of BCAAs is governed by multilayered regulatory networks involving enzymatic, hormonal, and microbial components. For instance, the activity of the BCKDH complex is regulated by phosphorylation and dephosphorylation (White et al., 2018). Changes in insulin and amino acid levels can also affect BCAA metabolism by modulating the activity of these key enzymes (Neinast et al., 2019). Tumor cells enhance BCAA synthesis through multiple pathways, including upregulating biosynthetic enzymes, metabolic reprogramming, nutrient scavenging, reductive carboxylation in hypoxia, and crosstalk with microenvironment. Studies have shown that elevated plasma BCAAs levels are associated with lung cancer and pancreatic cancer (Xu H. et al., 2023; Zhu et al., 2020; Katagiri et al., 2018). In these tumors, BCAA metabolism may be reprogrammed to fulfill the specific metabolic needs of tumor cells.

The occurrence of different tumors is associated with distinct genetic backgrounds, and cellular studies have shown that specific

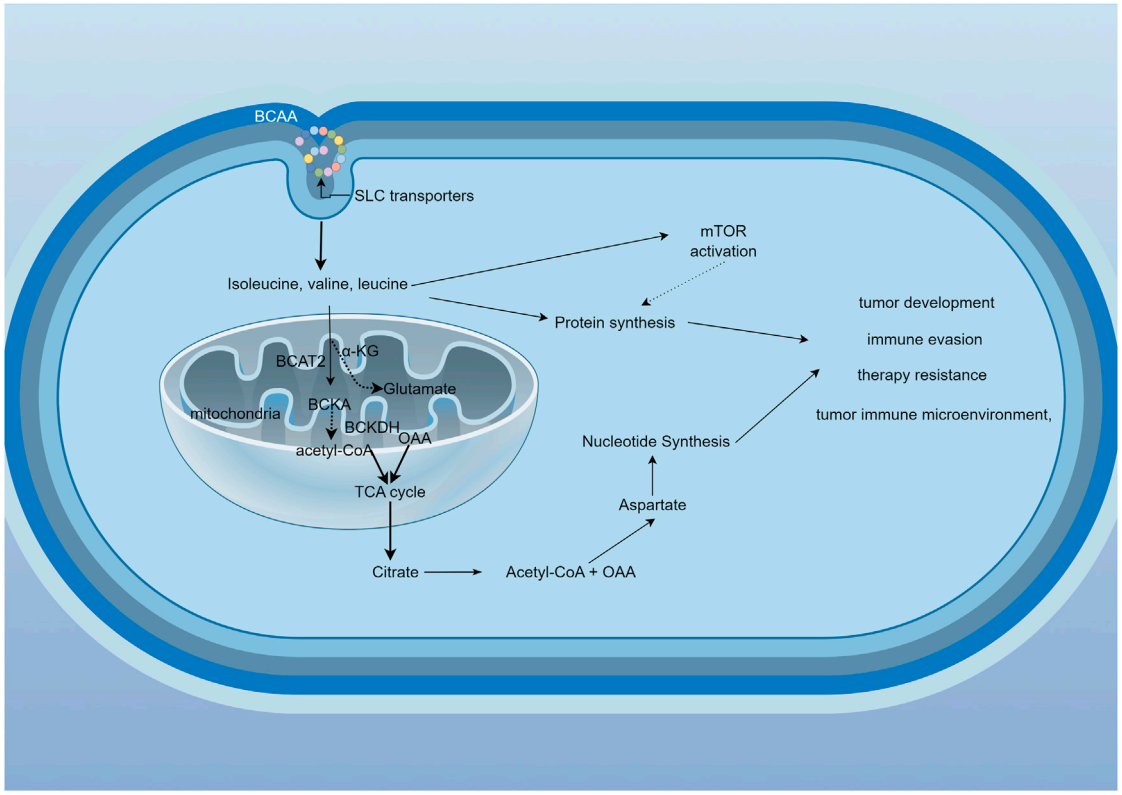

FIGURE 1
BCAAs metabolic cycle and related signaling pathways. BCAAs enter the cell *via* transporters and are metabolized by BCAT1 or BCAT2, generating branched-chain keto acids (BCKAs). These BCKAs are then processed by the BCKDH complex, which consists of three subunits: E1, E2, and E3. The catalytic efficiency of BCKDH depends on its phosphorylation status, regulated by BCKDK and PPM1K. BCKDK phosphorylates E1α to inhibit BCKDH, while PPM1K dephosphorylates the same site to activate BCKDH. Ultimately, acetyl-CoA is produced, entering the TCA cycle. Metabolites generated during this process may be associated with tumor development, immune evasion, therapeutic resistance, and the tumor microenvironment.

gene mutations can promote diverse metabolic phenotypes (Garraway, 2013) (Table 1). However, it remains unclear whether the genetic background of the entire tumor tissue determines the metabolic pathways of different cancers. The metabolic differences between tumor types may also be attributed to cell-autonomous effects, with tumor metabolic gene expression being more similar to that of their tissue of origin compared to other tumors (Martínez-Jiménez et al., 2020). The same oncogenic drivers may also result in distinct metabolic phenotypes in lung and liver tumors (Yuneva et al., 2012). For example, in mouse and human tumor tissues, *Kras* activation and *Trp53* mutation deletion in the pancreas or lungs lead to pancreatic ductal adenocarcinoma (PDAC) or non-small-cell lung cancer (NSCLC). Although these tumors are caused by the same genetic mutations, they utilize BCAAs differently. NSCLC tumors incorporate free BCAAs into tissue proteins and use BCAAs as a nitrogen source, thereby increasing BCAAs uptake. In contrast, PDAC tumors show decreased BCAAs uptake. This suggests that the tissue of origin is a key determinant in how cancer meets its metabolic demands (Martínez-Jiménez et al., 2020). A striking paradox exists in pancreatic cancer: while obesity and diabetes (conditions with elevated blood BCAAs) increase PDAC risk, the tumors themselves show reduced BCAAs uptake. This likely occurs because PDAC cells downregulate BCAAs transporters and preferentially utilize other nutrients, leading to a disconnect between systemic BCAAs levels and tumor metabolic demands. The patterns of metabolic reprogramming of BCAAs are inconsistent across different tumors, with varying enzyme activities and pathway activations or inhibitions, resulting in significant metabolic alterations in various cancers (Figure 2).

2.1 Lung cancer

In lung cancer, BCAAs metabolic reprogramming is significant. Tumor cells increase the uptake and metabolism of BCAAs to meet their rapid growth demands for energy and amino acids. The loss of enzymes responsible for BCAAs utilization, Bcat1 and Bcat2, impairs NSCLC tumor formation, although these enzymes are not essential for PDAC tumor formation (Li et al., 2020). BCAAs support the growth and proliferation of tumor cells by promoting the mTOR signaling pathway. Notably, studies have found that the plasma levels of BCAAs are typically elevated in lung cancer patients, indicating a close association between altered BCAA metabolism and tumor progression. BCAT1 can enhance BCAA metabolism, thereby increasing mitochondrial respiration and biosynthesis, reducing reactive oxygen species (ROS) levels, and ultimately enhancing NF-κB pathway signaling (Yu et al., 2022), promoting lung cancer development. High levels of BCAT1 promote

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TABLE 1 Patterns of BCAAS metabolic reprogramming in human cancers.

Cancer type	BCAAS intake	BCAT1	BCAT2	BCKDH	BCKDH	Other metabolic targets	Regulatory mechanism
lung cancer	↑	↑	↑	—	↑	—	mitochondrial respiration and biosynthesis↑, ROS levels↓, EMT↑, glycolysis↓
Hepatocellular carcinoma	↓	↑	↑	↑	↑	PPM1K↑, CPT1A↓, LAT1↑	mTORC1↑, acetyl-CoA synthesis↓, epigenetic modifications
pancreatic cancer	↓	↑	↑	—	↑	PPM1K↓, LAT1↑	mTORC1↑, acetyl-CoA
Colorectal Cancer	↑	—	↓	—	↑	*C.symbiosum*↑	MAPK↑, EMT↑, MEK-ERK↑
Leukemia	↑	↑	↑	↑	—	PPM1K↑, SLC7A5↑	αKG↑, mTORC1↑
Breast Cancer	↑	↑	—	↑	↑	LARS↓, LAT1↑	mitochondrial biogenesis↑, ATP production↑, mitochondrial ROS ↓

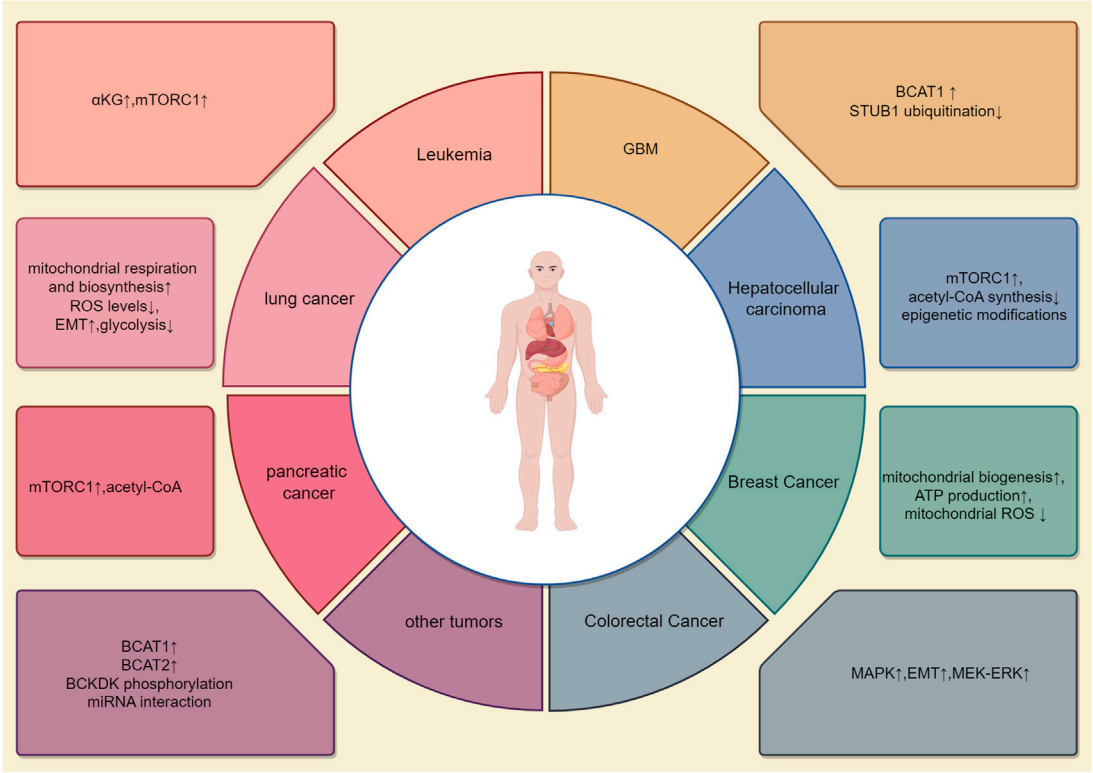

FIGURE 2
Metabolic reprogramming patterns in different tumors. The metabolism of BCAAs varies across different types of tumors. This article illustrates eight distinct BCAAs metabolic patterns, each with unique activated pathways, metabolic enzymes, and metabolic products. For example, in lung cancer, changes are seen primarily in BCAT1, while pancreatic cancer involves alterations in BCAT2. In colorectal cancer, metabolic changes focus on BCKDH-related enzymes. Additionally, BCAA metabolism in breast cancer is associated with reactive oxygen species (ROS) metabolism. BCAA metabolism interacts with other metabolic processes in a complex, interdependent manner.

the expression of SRY-box 2 (SOX2) by reducing alpha-ketoglutarate (α-KG), leading to the migration and metastasis of lung cancer cells (Mao et al., 2021). At the same time, BCAAs catabolism plays a crucial role in the metastasis of NSCLC cells, where the depletion of α-KG reduces the expression and activity of the m6A demethylase ALKBH5. As a result, the inhibition of ALKBH5 promotes the occurrence of epithelial-mesenchymal transition (EMT) in NSCLC cells and enhances their metastasis to the brain (Mao et al., 2024). Simultaneously, the upregulation of BCKDK affects the metabolism of BCAAs and citrate in NSCLC cells. Knockdown of BCKDK reduces NSCLC cell proliferation *in vitro* and induces apoptosis by inhibiting glycolysis while increasing oxidative phosphorylation and ROS levels, suggesting that BCKDK may promote NSCLC proliferation and could have clinical significance in treating NSCLC patients (Wang Y. et al., 2021). Rab1A, a small GTPase and an activator of mTORC1 as well

as an oncogene, enhances Rab1A-mTORC1 signaling and promotes tumor proliferation by inhibiting BCAA catabolism in NSCLC, making it a potential biomarker for early diagnosis and identifying metabolism-based therapeutic targets in NSCLC patients (Xue et al., 2023). Additionally, studies suggest that SAR1B, a leucine sensor, influences cell growth through amino acid levels and controls mTOR complex 1 (mTORC1) by modulating mTORC1 signaling based on intracellular leucine levels. Selectively targeting SAR1B-dependent mTORC1 signaling could have potential for lung cancer treatment (Chen J. et al., 2021). Similarly, high doses of isoleucine can also inhibit the proliferation of lung cancer cells by stabilizing nuclear PTEN (Wang et al., 2023).

2.2 Hepatocellular carcinoma

In hepatocellular carcinoma (HCC), abnormal BCAA metabolism is also very common. Liver cancer cells often break down BCAAs to generate energy, enhance their antioxidant capacity, and promote tumor growth. BCAAs levels in liver cancer patients are often reduced (Marchesini et al., 2003), especially in the late stages of liver failure, suggesting that BCAAs could serve as potential therapeutic targets or biomarkers. In human hepatocellular carcinoma and liver cancer animal models, inhibition of BCAAs catabolic enzyme expression leads to BCAAs accumulation in tumors, and the degree of enzyme inhibition is closely associated with tumor aggressiveness (Ericksen et al., 2019), making it an independent predictor of clinical outcomes. Approximately 40 enzymes are involved in BCAA catabolism, and their transcripts are widely suppressed in liver tumors.

Studies have shown that in the absence of glutamine, BCAA catabolism is activated in cancer cells, enhancing BCAAs breakdown to stimulate cell proliferation and survival. PPM1K (Protein Phosphatase, $Mg^{2+}/Mn2^{+}$ Dependent 1K) is a mitochondrial serine/threonine phosphatase that plays a key role in regulating BCAA metabolism (Yang et al., 2022). It dephosphorylates and activates the branched-chain α-keto acid dehydrogenase complex (BCKD), promoting BCAA catabolism. Stabilizing PPM1K protein leads to enhanced BCAAs and BCKDHA degradation due to increased dephosphorylation. High expression of dephosphorylated BCKDHA and PPM1K promotes tumorigenesis, making BCKDHA and PPM1K potential therapeutic targets and predictive biomarkers for liver cancer. Additionally, BCAA metabolism is linked to lipid metabolism *via* carnitine palmitoyl transferase 1 (CPT1A), the rate-limiting enzyme of fatty acid oxidation (FAO). CPT1A is widely downregulated in liver tumor tissues and is associated with poor prognosis in HCC, promoting HCC progression in both new liver tumors and xenograft tumor models. This could be due to the disruption of acetyl-CoA synthesis, reducing histone acetylation and impairing BCAAS catabolism, leading to BCAAs accumulation and excessive mTOR signaling activation (Liu et al., 2024).

PROX1 expression is reduced by glucose starvation or AMPK activation and elevated in tumors with liver kinase B1 (LKB1) deficiency. Inhibiting PROX1 activation decreases BCAAs degradation by regulating epigenetic modifications and suppressing mTOR signaling (Paput et al., 2011). The LKB1-AMPK axis in cancer cells depends on PROX1 to maintain intracellular BCAAs pools. Cancer cells lacking the LKB1-AMPK axis rely on PROX1 to maintain intracellular BCAA levels, leading to enhanced mTOR signaling, tumorigenesis, and invasiveness.

LAT1 is a transmembrane amino acid transporter responsible for transporting large neutral amino acids such as leucine, isoleucine, valine, phenylalanine, and tyrosine from outside the cell to the inside. In liver cancer, it has been found that inhibiting LAT1 can reduce BCAAs transport activity and significantly lower cell proliferation (Kim et al., 2023). LAT1 ablation results in a significant reduction in phosphorylated p70S6K, with downstream mTORC1 signaling being suppressed. Therefore, inhibiting LAT1 activity may be an effective therapeutic strategy for liver cancer.

The role of BCAAs supplementation in liver cancer treatment has been explored in numerous studies, particularly in patients with liver cirrhosis and hepatocellular carcinoma (HCC) (van Dijk et al., 2023). BCAAs have a unique role in the nutritional intervention of liver diseases. Perioperative BCAA intake has been shown to decrease postoperative infections and ascites in liver cancer patients (Yap et al., 2023) and enhance survival in cirrhotic individuals (Hanai et al., 2020). BCAAs, particularly leucine, can activate the mTOR pathway, improving the function of immune cells such as T cells and natural killer cells, thereby boosting the anti-tumor immune response (Peng et al., 2020). While BCAAs supplementation has many potential benefits, there are also some controversies (Sideris et al., 2023). Some studies suggest that excessive BCAAs supplementation may promote the growth of certain tumor cells by activating the mTOR signaling pathway (Ericksen et al., 2019), thus requiring cautious use in liver cancer patients, especially with individualized adjustments based on the patient's condition and nutritional needs. Although BCAAs supplementation is primarily used to support the nutrition and immune function of liver cancer patients, some research suggests that BCAAs may also affect tumor progression by inhibiting cancer cell proliferation and invasion. Certain BCAA metabolites may have inhibitory effects on cancer cells, particularly by modulating the mTOR signaling pathway and other metabolic pathways. However, the specific mechanisms involved still require further investigation. Additionally, studies have found that ferroptosis can regulate tumor metabolism and iron-dependent lipid peroxidation, thereby inhibiting tumor proliferation. Elevated BCAT2 expression in liver and pancreatic cancers is associated with reduced ferroptosis-related cell death. It has also been demonstrated that sorafenib and sulfasalazine have synergistic effects in inhibiting BCAT2 expression and inducing ferroptosis. Targeting BCAT2 may provide insights into overcoming resistance to sorafenib treatment (Wang K. et al., 2021).

2.3 Pancreatic cancer

Pancreatic hormone secretion is associated with obesity and insulin resistance. The development of pancreatic cancer can lead to insulin resistance and diabetes (Rossmeislová et al., 2021). However, the exact relationship between BCAA metabolism, PDAC progression, and tissue type remains unclear. Pancreatic cancer cells typically undergo metabolic reprogramming to meet the

demands of rapid growth and proliferation. Studies have also found elevated levels of BCAAs in the blood of pancreatic cancer patients, strongly correlated to tumor progression. High levels of BCAA metabolism are linked to increased aggressiveness and poor prognosis in pancreatic cancer.

BCATs, including BCAT1 and BCAT2, transfer amino groups from BCAAs to α-KG. BCAT2 levels are higher in pancreatic cancer cell lines compared to normal cell lines (Li et al., 2020), making it a potential clinical target for pancreatic cancer therapy. BCAT2 is acetylated at lysine 44 (K44), an evolutionarily conserved residue. Acetylation of BCAT2 leads to its degradation through the ubiquitin-proteasome pathway and is stimulated during BCAAS deprivation. CREB-binding protein (CBP) and Sirtuin 4(SIRT4) are BCAT2's acetyltransferase and deacetylase, respectively, controlling K44 acetylation in response to BCAAs availability (Lei et al., 2020). The K44R mutant enhances BCAAS catabolism, cell proliferation, and pancreatic tumor growth. This reveals a previously unknown regulatory mechanism of BCAT2 in PDAC and provides a potential therapeutic target for PDAC treatment.

Additionally, studies suggest that co-targeting stromal BCAT1 and the cancerous BCKDH complex impairs tumor cell proliferation and survival (Zhu et al., 2020). Cancer-associated fibroblasts (CAFs) take up extracellular matrix (ECM) components under nutrient-restricted conditions, with fibroblasts upregulating the Urokinase-type plasminogen activator receptor-associated protein (uPARAP) receptor for ECM uptake. CAFs can secrete ECM and induce a fibrotic environment within tumors. Enzymes or transporters related to BCAA metabolism, such as LAT1, may serve as potential therapeutic targets. Inhibiting BCAA metabolism can reduce tumor cells' access to BCAAs, decreasing mTOR pathway activity, thereby inhibiting tumor growth and metastasis. A BCAAS-rich diet promotes pancreatic cancer development through USP1-mediated BCAT2 stabilization, and BCAAS intake is positively correlated with pancreatic cancer risk (Li J. T. et al., 2022; Rossi et al., 2022).

2.4 Colorectal cancer

In colorectal cancer (CRC), BCAA metabolic reprogramming enables tumor cells to adapt to nutritional stress in the microenvironment, enhancing their survival capacity. The accumulation of BCAAs caused by BCAT2 deficiency promotes chronic activation of mTORC1, mediating the carcinogenic effect of BCAAs (Kang Z. R. et al., 2024). BCKDK can also promote CRC development by upregulating the MEK-ERK signaling pathway. BCKDK is upregulated in CRC tissues, and increased BCKDK expression is associated with metastasis and poor clinical prognosis in CRC patients. Knockdown of BCKDK reduces CRC cell migration and invasion *in vitro* and lung metastasis *in vivo*. BCKDK promotes EMT by decreasing the expression of the epithelial marker E-cadherin and increasing the expression of mesenchymal markers N-cadherin and vimentin (Tian et al., 2020). Src phosphorylates BCKDK, enhancing its activity and stability, thereby promoting CRC cell migration, invasion, and EMT. Additionally, studies have shown that BCKDK enhances the MAPK signaling pathway by directly phosphorylating MEK, rather than through branched-chain amino acid catabolism, thereby promoting colorectal cancer progression (Xue et al., 2017). BCKDK may serve as a novel therapeutic target for colorectal cancer. BCAAs are also involved in maintaining redox balance, which plays an important role in the growth of colorectal cancer cells.

Recent studies have demonstrated that **C. symbiosum** selectively enriches in tumor tissues of colorectal cancer (CRC) patients and is associated with higher recurrence of colorectal adenomas after endoscopic polypectomy. The tumorigenic effect of **Clostridium symbiosum** has been observed in various mouse models (Ren et al., 2024). The mechanism involves **C. symbiosum** enhancing cellular cholesterol synthesis through BCAAs production, which in turn activates the Sonic Hedgehog signaling pathway. **C. symbiosum** has been identified as a bacterial driver of colorectal tumorigenesis, providing a potential target for CRC prediction, prevention, and treatment. Dietary supplementation with BCAAs may improve insulin resistance and inhibit the activation of the IGF/IGF-IR axis, thereby preventing the development of obesity-related colorectal cancer precursors. BCAAs may be an effective strategy for preventing colorectal cancer in obese individuals (Rossi et al., 2021). However, whether BCAAs intake affects the prognosis of colorectal cancer patients remains controversial (Shimizu et al., 2009; Long et al., 2021), and further research is needed to explore its role and mechanism in colorectal cancer.

2.5 Metabolic reprogramming of BCAAs in leukemia

Similar to many other tumor cells, leukemia cells reprogram the metabolism of BCAAs to meet the demands of rapid proliferation. Studies have shown that BCAAs are highly absorbed and quickly broken down in leukemia cells, providing energy and generating key metabolites such as nucleotides and lipids, which are essential for tumor cell proliferation (Neinast et al., 2019). In primary leukemia cells, BCAT1 actively breaks down BCAAs into branched-chain α-keto acids using α-KG, supplying key substrates for the tricarboxylic acid cycle and the synthesis of non-essential amino acids. Both processes help maintain α-KG levels, which are crucial for sustaining leukemia stem cell function (Kikushige et al., 2023). Research has found that BCAT1 is abnormally activated in chronic myeloid leukemia (CML) in both humans and mice, promoting BCAAs production *via* the MSI2-BCAT1 axis, thereby driving the development of myeloid leukemia (Hattori et al., 2017). Moreover, studies have shown that BCAT1 knockout leads to an accumulation of α-KG, which is a vital cofactor for α-KG-dependent dioxygenases, such as the Egl-9 family hypoxia-inducible factor 1 (EGLN1) and the ten-eleven translocation (TET) family of DNA demethylases. This results in EGLN1-mediated degradation of HIF1α, suppressing tumor cell proliferation (Raffel et al., 2017). In AML cells with high levels of BCAT1, a DNA hypermethylation phenotype similar to cases with mutant isocitrate dehydrogenase (IDHmut) has been observed, and this is associated with poor disease prognosis.

Several genes associated with poor leukemia prognosis are also linked to BCAA metabolic pathways. EZH1, a homolog of EZH2, is essential for the initiation of leukemia in EZH2-deficient cells and contributes to epigenetic vulnerability. EZH2 inactivation leads to BCAT1 overactivation, enhancing BCAA metabolism and mTOR signaling, which together drive the transformation of

myeloproliferative neoplasms into leukemia (Gu et al., 2019). METTL16, an m6A methyltransferase and one of the most common internal modifiers of mammalian mRNAs, is abnormally overexpressed in human AML cells. Through an m6A-dependent mechanism, METTL16 promotes the expression of BCAT1 and BCAT2, reprogramming BCAA metabolism in AML and contributing to leukemogenesis (Han et al., 2023). GPRC5C, a member of the G protein-coupled receptor family group C, is a regulator of hematopoietic stem cell dormancy and is associated with poor leukemia prognosis. Elevated intracellular BCAAs levels, a tumor metabolic characteristic, are reversed after Gprc5c depletion. Targeting the BCAAs transporter SLC7A5 with JPH203 inhibited oxidative phosphorylation and exerted anti-leukemic effects, suggesting that the GPRC5C-SLC7A5-BCAAs axis may serve as a therapeutic target (Zhang Y. W. et al., 2023).

2.6 Breast cancer

In breast cancer, BCAA metabolism is also related to tumor occurrence and progression. Breast cancer cells maintain their rapid proliferation rate by increasing the uptake and utilization of BCAAs. Elevated expression of BCAT1 has been observed in breast cancer, and knocking down BCAT1 can inhibit the growth and proliferative capacity of breast cancer cells (Zhang and Han, 2017). BCAT1 promotes mitochondrial biogenesis, ATP production, and inhibits mitochondrial ROS in breast cancer cells by regulating the expression of related genes. High concentrations of BCAAs affect the migration and invasion capabilities of breast cancer cells. Elevated BCAAs inhibit tumor metastasis and cell invasion abilities and reduce the expression of N-cadherin, indicating that high BCAAs levels may suppress breast cancer tumor growth and metastasis (Tobias et al., 2021). This suggests that a high-BCAAs diet could have potential therapeutic significance in breast cancer treatment (Chi et al., 2022). Leucyl-tRNA synthetase (LARS) is inhibited in breast cell transformation and human breast cancer (Stine et al., 2022). *In vitro* experiments demonstrated that inhibition of BCKDK expression reduced the migration of human breast cancer cells, while *in vivo* it decreased lung metastasis. BCKDK inhibited the interaction between talin1 and the E3 ubiquitin ligase TRIM21, leading to reduced ubiquitination and degradation of talin1, thereby suppressing tumor cell migration (Xu C. et al., 2023). This study found that LAT1, a key amino acid transporter, plays a role in AI-resistant breast cancer by promoting leucine uptake and mTORC1 signaling. LAT1 expression increased in resistant tumors and was linked to advanced stages. The LAT1 inhibitor JPH203 reduced cell proliferation in resistant cells, suggesting LAT1 as a potential therapeutic target in AI-resistant breast cancer (Shindo et al., 2021). Additionally, studies have shown that elevated circulating BCAAs levels are associated with a reduced risk of breast cancer in premenopausal NHSII women but an increased risk in postmenopausal NHS women (Zeleznik et al., 2021).

2.7 Other tumor types

Most malignant tumors exhibit elevated levels of BCAT1, which is associated with malignant phenotypes in various cancers, such as nasopharyngeal carcinoma (NPC) (Zhou et al., 2013), gastric cancer (Qian et al., 2023), melanoma (Mao et al., 2021), and astrocytoma (Tönjes et al., 2013), as well as poor prognosis in cancer. The promoter encoding BCAT1 can interact with RNA-binding motif proteins, promoting tumorigenesis in nasopharyngeal carcinoma (Xu X. C. et al., 2021). The long non-coding RNA GAS6-AS2 has been identified as a key tumor growth driver in osteosarcoma (Wei et al., 2020) by inhibiting miR-934. Solid evidence from various cancers has demonstrated BCAT1's direct regulation in the mTOR pathway. BCAT1-mediated mTOR activation is involved in the lethal biological behaviors of gastric cancer (Shu et al., 2021)and cervical cancer (Luo et al., 2021). Similarly, the regulation of BCAT2 expression in cancer has been reported in the literature. Recent studies have shown that compared to normal tissues, BCAT2 expression is elevated in cancers such as bladder cancer (Cai et al., 2023), pancreatic cancer (Li et al., 2020; Lee et al., 2019), breast cancer (Zhang and Han, 2017), and non-small cell lung cancer (NSCLC) (Lee et al., 2019). The study found that BCAT1 is phosphorylated by BCKDK in glioblastoma, which enhances its activity and stability while inhibiting its degradation mediated by STUB1 ubiquitination, thereby promoting tumor growth. Inhibiting the BCKDK-BCAT1 axis can increase sensitivity to temozolomide (TMZ), suggesting this pathway as a potential therapeutic target (Wang W. et al., 2024).

Overexpression of BCKDH protein levels has been observed during carcinogenesis in most ovarian cancer cell lines (Ibrahim et al., 2023), oral squamous cell carcinoma (Grimm et al., 2016), osteosarcoma (Zhang and Han, 2017), and melanoma (Tian et al., 2023). Active BCKDH is tightly regulated by its phosphorylation status, which is determined by BCKDK and PPM1K levels. High expression of BCKDK and certain malignant proliferation behaviors have been confirmed in colorectal cancer (Lei et al., 2020), breast cancer (Xu C. et al., 2023), and NSCLC (Xue et al., 2023).

3 Research on mechanisms of BCAAs-induced tumor resistance

Tumor resistance refers to the phenomenon where tumor cells develop resistance to cancer therapies, rendering previously effective treatments ineffective or significantly less effective. This resistance can either be intrinsic (i.e., primary resistance) or acquired over the course of treatment (i.e., acquired resistance) (Bagchi et al., 2021).Tumor resistance typically involves a variety of complex biological mechanisms, including genetic mutations, activation of signaling pathways, drug efflux, enhanced DNA repair, and evasion of apoptosis (Vesely et al., 2022). The development of resistance makes tumors harder to control and treat, presenting a major challenge in cancer therapy.

BCAAs induce tumor resistance through multiple mechanisms. These include activation of the mTOR signaling pathway to promote tumor cell growth, regulation of oxidative stress responses to resist oxidative damage induced by treatment, modulation of glucose and lipid metabolism to support tumor cells' adaptation to changing energy demands, and shaping the immune microenvironment to suppress anti-tumor immune responses (Figure 3). Additionally, BCAAs regulate autophagy and apoptotic pathways, preventing therapy-induced cell death, thereby enhancing tumor cell

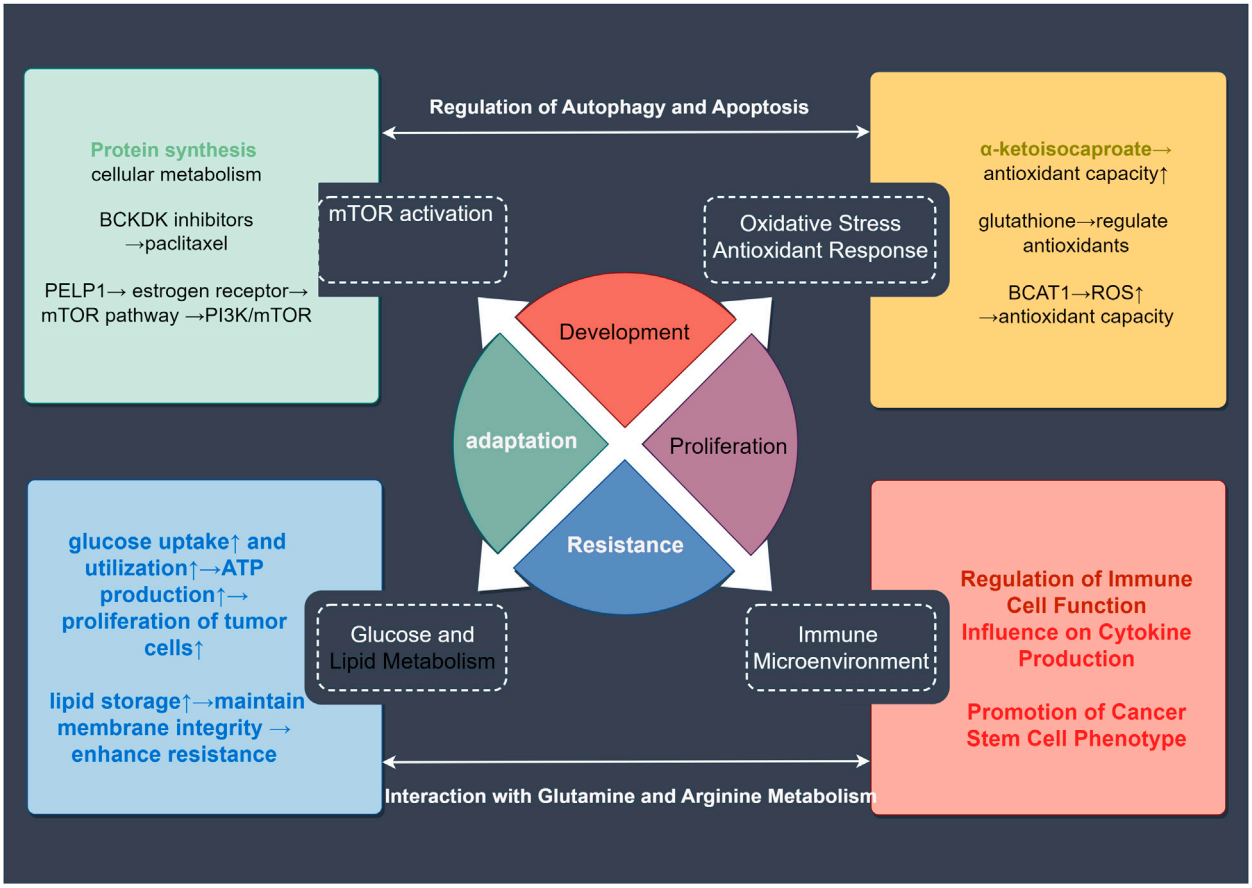

FIGURE 3
Mechanisms of BCAA-Related tumor drug resistance. Abnormal BCAA metabolism can lead to resistance in tumor-related therapies. This article covers four major aspects of this resistance: activation of the mTOR signaling pathway, oxidative stress response, alterations in glucose and lipid metabolism, and changes in the tumor microenvironment. These factors can further interact through mechanisms such as autophagy, apoptosis, and metabolic changes to enhance tumor resistance. These insights provide valuable reference points for targeting resistance in treatment.

survival. Collectively, these mechanisms contribute to the development of treatment resistance in tumor cells.

3.1 Activation of the mTOR signaling pathway

BCAAs, particularly leucine, activate the mTORC1 (mammalian target of rapamycin complex 1) pathway by directly binding to it. Persistent activation of mTOR signaling is closely linked to tumor growth, proliferation, and survival in many types of cancer (Zhan et al., 2023). By enhancing the activity of this pathway, BCAAs help tumor cells maintain survival under the stress of anticancer drugs. For instance, mTOR signaling activation can counteract the growth-inhibiting effects of chemotherapy by promoting protein synthesis and cellular metabolism. In many cancers, mTOR inhibitors have been considered as potential therapeutic drugs, but the activation of mTOR signaling by BCAAs may lead to drug resistance (Liu et al., 2022). In some cancer types, inhibiting the mTOR signaling pathway is thought to enhance drug sensitivity, suggesting that abnormal BCAA metabolism may promote resistance (Bansal and Simon, 2018).

For example, BCKDK inhibitors can disrupt the mTORC1-Aurora axis, thereby enhancing the sensitivity of breast and ovarian cancer cells to chemotherapeutic drugs. The use of BCKDK inhibitors can reverse the cell cycle arrest induced by paclitaxel. BCKDK might play an important role in increasing the sensitivity of tumor cells to paclitaxel. Certain breast cancer cells reduce sensitivity to PI3K/mTOR inhibitors by enhancing mTOR pathway activity through leucine metabolism (Ibrahim et al., 2023). Proline, Glutamate, Leucine-Rich Protein 1 (PELP1), a proto-oncogene that regulates estrogen receptor (ER) signaling, interacts with serine/threonine protein kinase mTOR and modulates mTOR signaling (Gonugunta et al., 2014). mTOR inhibitors can sensitize PELP1-expressing cells to hormone therapy.

3.2 Oxidative stress and antioxidant response

Alterations in BCAA metabolism can affect tumor cells' responses to oxidative stress. Oxidative stress is often a key cytotoxic mechanism in chemotherapy and radiotherapy,

inducing oxidative damage that leads to tumor cell death. However, products of BCAA metabolism, such as α-ketoisocaproate (produced from leucine breakdown), can enhance the antioxidant capacity of tumor cells, helping them resist the oxidative damage caused by chemotherapy and radiotherapy (Cai et al., 2022). This mechanism allows tumor cells to alleviate oxidative stress by regulating antioxidants such as glutathione, further promoting drug resistance (Zhang B. et al., 2022). These data suggest that high BCAAs concentrations may have deleterious effects on circulating blood cells, contributing to the pro-inflammatory and oxidative states observed under several pathophysiological conditions (Zhenyukh et al., 2017). Hypoxia-inducible factors (HIFs) regulate metabolic reprogramming in response to hypoxia. LAT1 is a transporter of BCAAs, and studies have found that hypoxia upregulates the mRNA and protein levels of LAT1 and BCAT1 in human glioblastoma (GBM) cell lines through the binding of HIF-1α and HIF-2α to the intron of the BCAT1 gene. However, hypoxia does not upregulate their homologs LAT2-4 and BCAT2. This allows tumor cells to continue proliferating under hypoxic conditions (Zhang et al., 2021). Moreover, studies indicate that enhanced BCAA metabolism boosts the activity of antioxidant enzymes, such as glutathione, helping tumor cells resist treatment-related accumulation of reactive oxygen species (ROS) (Pavlova et al., 2022). Studies have found that BCAT1 may possess stronger antioxidant properties compared to BCAT2. The BCAT1-CXC motif has a novel antioxidant function, with this CXXC motif proven to act as a "redox switch" in the enzymatic regulation of BCAT proteins. The BCAT1-CXC motif may help buffer ROS levels within AML cells, influencing cell proliferation, which could impact the ROS-mediated development of myeloid leukemia (Hillier et al., 2022). Low-grade gliomas and secondary glioblastomas lead to excessive production of (R)-2HG, which can effectively inhibit 2OG-dependent transaminases BCAT1 and BCAT2. By reducing glutamate levels, this inhibition sensitizes IDH-mutant glioma cells specifically to glutaminase, making them more susceptible to oxidative stress *in vitro* and to radiation both *in vivo* and *in vitro* (McBrayer et al., 2018). Chemotherapeutic agents kill tumor cells by inducing oxidative stress, and the regulation of BCAA metabolism may enhance drug resistance by mitigating this stress (Pavlova and Thompson, 2016). The C-terminal of Hsc70-interacting protein (CHIP) is an E3 ubiquitin ligase, and its coiled-coil (CC) domain interacts with BCAT1. Through the CHIP/BCAT1 axis, it enhances glioma sensitivity to temozolomide by reducing glutathione (GSH) synthesis and increasing oxidative stress (Lu et al., 2024a).

3.3 Interaction with glucose and lipid metabolism

The cross-regulation between BCAA metabolism and glucose and lipid metabolism plays a crucial role in metabolic reprogramming within tumor cells. Tumor cells often adapt to nutrient limitations and anticancer drug pressure by readjusting metabolic pathways. BCAA metabolism promotes glucose uptake and utilization, increasing ATP production, thereby helping tumor cells maintain energy supply and cope with drug pressure (Fang et al., 2022). Moreover, the interaction between BCAAs and lipid metabolism can support the rapid growth and repair of cell membranes by providing precursors for lipid synthesis, which is essential for tumor cell survival. Studies have shown that BCAAs enhance glucose uptake, increase glycolytic products, and promote tumor cell survival in harsh environments by activating the PI3K/AKT pathway (Zoncu et al., 2011). Alterations in lipid metabolism, such as increased lipid storage promoted by BCAAs, help tumor cells maintain membrane integrity and enhance resistance to chemotherapy (Bacci et al., 2021). Osimertinib, a third-generation EGFR tyrosine kinase inhibitor (TKI), has shown significant clinical efficacy in treating non-small cell lung cancer (NSCLC). Studies have found that in TKI-resistant cells, upregulated BCAT1 reprograms BCAA metabolism and promotes α-ketoglutarate (α-KG)-dependent demethylation of histone H3 at lysine 27 (H3K27), leading to the de-repression of glycolysis-related genes, thereby enhancing glycolysis and promoting tumor progression. WQQ-345, a novel BCAT1 inhibitor, has demonstrated antitumor activity in both *in vitro* and *in vivo* models of TKI-resistant lung cancer with high BCAT1 expression. BCAT1 is a promising target for treating TKI-resistant NSCLC (Zhang T. et al., 2024). PPM1K regulates glycolysis to generate hematopoietic stem cells and leukocytes through the ubiquitination of MEIS1 and p21 mediated by CDC20. Inhibition of PPM1K extended the survival time of mice in leukemia models, suggesting that PPM1K could be used in combination with chemotherapy drugs for leukemia to improve treatment efficacy (Liu et al., 2018).

3.4 Impact on the immune microenvironment

The immunosuppressive nature of the tumor microenvironment plays a crucial role in tumor drug resistance. BCAA metabolism regulates immune responses by affecting the metabolic activity of immune cells. Research shows that T cells and natural killer (NK) cells require an adequate supply of BCAAs to maintain their antitumor functions. When BCAA metabolism is disrupted, the activity of these immune cells may be suppressed, leading to enhanced immunosuppression within the tumor microenvironment. Immunotherapy relies on the host immune system's ability to recognize and eliminate cancer cells, but changes in BCAA metabolism may weaken this effect, promoting immune evasion by tumor cells.

3.4.1 Regulation of immune cell function

BCAA metabolism influences the activity and function of key immune cells, including T cells, macrophages, myeloid-derived suppressor cells (MDSCs), and NK cells. Pan-cancer biological analyses show that the infiltration levels of $CD4^+$ T cells, $CD8^+$ T cells, B cells, neutrophils, and macrophages in lung cancer, colorectal cancer, and head and neck squamous cell carcinoma are correlated with the expression of BCAT1 (Li G. S. et al., 2022). These immune cells jointly regulate the immune tumor microenvironment and play critical roles in immunotherapy. BCAA metabolism exhibits dual immunomodulatory roles in the tumor microenvironment (TME), with both pro-tumoral and anti-tumoral effects on key immune cells.

3.4.1.1 T cells

BCAAs exhibit dual roles in T cell activation, proliferation, and differentiation. In promoting tumor progression, BCAAs depletion in the tumor microenvironment can inhibit T cell function, reducing their ability to mount an effective antitumor immune response (Xia et al., 2021). While enhanced BCAA metabolism may promote the survival and function of regulatory T cells (Tregs), which suppress immune responses and help tumors evade immune detection (Ikeda et al., 2017). In suppressing tumor progression, BCAAs, particularly leucine, are indispensable amino acids for immune regulation through metabolic reprogramming. However, the molecular mechanisms underlying this phenomenon remain unclear. Many studies have shown that solute carrier (SLC) transporters play new roles in the tumor microenvironment by altering immune cell metabolism (Chen and Chen, 2022; Nachef et al., 2021; Kocher et al., 2021). SLC1A5, SLC7A5, and SLC3A2 are the most highly expressed genes encoding amino acid transport proteins in the tumor microenvironment (O'Sullivan et al., 2019). The most abundant amino acid transporter in activated T cells is SLC7A5 (Kanai, 2022; Meng et al., 2024). Studies have found that T cell receptor (TCR) activation increases the expression of BCAT1 and SLC7A5 in human $CD4^+$ T cells, promoting leucine influx and catabolism, which is particularly important for the T helper cell (Th17) response. Inhibiting SLC transporters reduces the ability of immune cells to eliminate tumor cells. SLC7A5 is involved in T cell differentiation, activation of the mTORC1 signaling pathway, and c-Myc expression, while knocking out SLC3A2 prevents T cell expansion (Ikeda et al., 2017; Najumudeen et al., 2021; Zhang C. et al., 2024). Chimeric antigen receptor (CAR)-T cells are an innovative immunotherapy where T cells are genetically engineered. Research has shown that traditional T cells or CAR-T cells can compete with tumor cells for amino acids. Artificially increasing the expression of SLC7A5 or SLC7A11 transmembrane amino acid transporters has been shown to enhance CAR-T cell proliferation and antitumor activity by upregulating intracellular arginase (Panetti et al., 2023). Additionally, BCKDK-engineered CAR T cells were designed to reprogram BCAA metabolism in the tumor microenvironment based on genotype and phenotype modifications, enhancing the ability of T cells to eliminate cancer cells (Yang et al., 2024). In an experimental autoimmune encephalomyelitis (EAE) model, blocking BCAT1-mediated leucine catabolism using BCAT1 inhibitors or LβhL treatment alleviated the severity of EAE by reducing HIF1α expression and IL-17 production in spinal cord mononuclear cells. Activated $CD4^+$ T cells induce an alternative pathway of cytosolic leucine catabolism through BCAT1 and hydroxyphenylpyruvate dioxygenase (HPD)/HPDL, producing the key metabolite β-hydroxy-β-methylbutyrate (HMB). HMB helps regulate the mTORC1-HIF1α pathway by increasing HIF1α mRNA expression, a major signaling pathway for IL-17 production. Treatment with L-β-hydroxyisoleucine (LβhL), a leucine analog and competitive inhibitor of BCAT1, can reduce IL-17 production in TCR-activated $CD4^+$ T cells, thus weakening the immune response in the tumor microenvironment (Kang Y. J. et al., 2024).

Immune checkpoint inhibitors (ICIs) have improved survival rates in patients with advanced cancer (such as bladder cancer, BLCA). However, their overall efficacy remains limited, as many patients still develop resistance to immunotherapy. Recent studies have found that LRFN2 forms a non-inflammatory tumor microenvironment (TME) in BLCA. Tumor-intrinsic leucine-rich repeat and fibronectin type III domain-containing protein LRFN2 suppresses the recruitment and functional transformation of $CD8^+$ T cells by reducing the secretion of pro-inflammatory cytokines and chemokines. LRFN2 inhibits antitumor immunity by reducing $CD8^+$ T cell infiltration, proliferation, and differentiation *in vitro*. Furthermore, spatially exclusive relationships between $LRFN2^+$ tumor cells and $CD8^+$ T cells, as well as markers such as programmed cell death-1 (PD-1) and T cell factor 1 (TCF-1), have been observed, thereby enhancing tumor resistance (Yu et al., 2023). Additionally, BCAAs promote the effector function of $CD8^+$ T cells and antitumor immunity by reprogramming glucose metabolism, which can enhance the clinical efficacy of anti-PD-1 immunotherapy against tumors (Yao et al., 2023).

3.4.1.2 Macrophages

BCAA metabolism can regulate the polarization of macrophages. A BCAAS-enriched environment may inhibit M1 macrophages, which have antitumor functions, while promoting M2 macrophages (TAM 2), which support tumor growth and immune suppression. TAM 2 infiltration is significantly elevated in the pancreatic tumor microenvironment (CME). (Zhang et al., 2023b). Increased levels of TAM two drive the tumor-promoting characteristics of cancer cells and are associated with poor disease prognosis. BCAT1, along with bone marrow stromal antigen 2 (BST 2) and the tyrosine kinase MERTK, promotes cancer progression by regulating TAM 2 polarization, offering a potential target for pancreatic cancer treatment. Another FN1-induced transcriptome network mediates immune cell infiltration in the CME of oral squamous cell carcinoma (Peng et al., 2023). Additionally, TAMs can be reprogrammed through diet or genetic modification to overcome MYC-overexpressing cancer cells *via* non-canonical phagocytosis-mediated, Rag GTPase-independent mTORC1 signaling (Zhang et al., 2023c).The regulatory role of BCAT1 in macrophage function holds therapeutic significance for inflammatory diseases. While BCAT1's role in regulating macrophage function helps reduce the infiltration of inflammatory factors and has therapeutic potential for various inflammatory diseases (Papathanassiu et al., 2017), it is still unclear whether similar mechanisms exist in the TME.

3.4.1.3 NK cells

Low concentrations of arginine can inhibit T cell proliferation and activity in the tumor microenvironment (TME), but increased expression of SLC7A5 can help NK cells in acute myeloid leukemia (AML) maintain their proliferative and activated phenotype under low arginine conditions, leading to AML cell apoptosis (Stavrou et al., 2023). In contrast, inhibiting SLC7A5 in cytokine-activated NK cells reduces c-Myc protein levels and mTORC1 signaling, thereby enhancing their antitumor effects (Loftus et al., 2018).

3.4.1.4 MDSCs

BCAAs support the immunosuppressive activity of myeloid-derived suppressor cells (MDSCs), inhibiting antitumor immune responses and promoting tumor progression. A high-fat diet (HFD) is a high-risk factor that disrupts the gut microbiome, leading to the malignant progression of cancer. Both obesity and obesity-

associated gut microbiota are linked to poor prognosis and advanced cachexia in female cancer patients. The HFD-related microbiota promotes cancer progression by generating polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs). The HFD microbiota releases an abundance of leucine, activating the mTORC1 signaling pathway in myeloid progenitor cells, thus promoting PMN-MDSC differentiation (Chen et al., 2024). Clinically, elevated leucine levels in the peripheral blood of female cancer patients, induced by the HFD microbiota, are associated with extensive tumor PMN-MDSC infiltration and poor clinical outcomes. BCAAs also affect the immunoregulatory properties of mesenchymal stem cells (MSCs). They regulate the S, G2, and M phases of the cell cycle, promoting MSC proliferation and metabolic activity (Zhang F. et al., 2022). In addition, in immune-related diseases, BCAAs modulate the immunoregulatory capacity of MSCs by increasing phosphorylated signal transducer and activator of transcription 3 (p-STAT3)/STAT3 signaling, reducing p-NF-κB/NF-κB signaling, and enhancing the production of anti-inflammatory TGF-β and prostaglandin E (Sartori et al., 2020).

3.4.2 Influence on cytokine production

BCAA metabolism can affect the production of pro-inflammatory and anti-inflammatory cytokines in the tumor immune microenvironment. For example, BCAAs can activate the NF-κB pathway (Sartori et al., 2020), leading to the production of cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), which can support tumor progression and alter immune responses. The expression of chemokines critical for $CD8^+$ T cell recruitment, such as CCL3, CCL4, CCL5, CXCL9, and CXCL10, is hindered by BCAT2. Chemotaxis experiments show that BCAT2 is negatively correlated with $CD8^+$ T cell cytotoxic INF-γ and TNF-α. More importantly, the loss of BCAT2 enhances the effectiveness of anti-PD-1 therapy (Cai et al., 2023).

3.5 Interaction with glutamine and arginine metabolism

BCAA metabolism intersects with glutamine metabolism, which is crucial for the function of rapidly proliferating cells, including tumor cells and certain immune cells. By influencing the availability and utilization of glutamine, BCAAs can regulate the immune microenvironment, affecting the balance between antitumor immune responses and immune suppression (Bader et al., 2020). Numerous studies have shown that the uptake of glutamine, arginine, and BCAAs is upregulated across various cancers and activates Th1 and $CD8^+$ T cells (Chen C. L. et al., 2021). For example, in ovarian cancer cells, $CD4^+$ and $CD8^+$ memory T cells and M0 macrophages overexpress the arginine transporter CAT1. Silencing CAT1 transporter results in decreased BCAAS levels. Arginine acts as a crucial precursor for polyamine biosynthesis, and targeting key metabolic enzymes like arginase-1 (Arg1) can effectively regulate polyamine production in the tumor microenvironment (TME) (Wetzel et al., 2023). These polyamines possess well-documented immunosuppressive properties that promote tumor growth by inhibiting cytotoxic immune responses (Lian et al., 2022). Importantly, dendritic cells frequently overexpress Arg1, establishing it as a novel metabolic checkpoint within the TME (Martí and Reith, 2021). Through Arg1-mediated arginine depletion, dendritic cells may contribute to T cell exhaustion, a key factor in tumor immune evasion and immunotherapy resistance (Lian et al., 2022). Moreover, all three of these amino acids maintain cell growth and proliferation by activating mTORC1 in tumor and immune cells (You et al., 2022). mTOR signaling is dysregulated in cancer cells, whereas T cell function requires mTOR upregulation (Kim and Guan, 2019; Waickman and Powell, 2012). mTOR sensing may occur through Rag GTPase-dependent mechanisms and can interact with various protein targets (Bodineau et al., 2022). Glutamine, along with asparagine, activates mTOR signaling *via* a Rag-GTPase-independent mechanism (Meng et al., 2020). Leucine-driven mTOR activation involves SAR1B, GATOR1-2, and Sestrin2 (Chen J. et al., 2021; Saxton et al., 2016). Under low glutamine conditions, targeting ASCT2 renders breast cancer cells more sensitive to leucine uptake inhibition, suggesting that cancer cells with reduced transporter plasticity are more vulnerable to disruptions in amino acid homeostasis (Bröer et al., 2019). In conclusion, cancer and immune cells are influenced by arginine, glutamine, and BCAAs, and these three exist in a mutually balanced and regulated relationship. Understanding their interactions may provide new therapeutic targets for the treatment of the tumor immune microenvironment.

3.6 Regulation of autophagy and apoptosis

BCAA metabolism also helps tumor cells resist therapeutic pressure by influencing autophagy and apoptosis pathways. Autophagy is a survival mechanism that cells use during nutrient deprivation or stress, maintaining energy balance by degrading damaged organelles or proteins (Debnath et al., 2023). BCAA metabolism inhibits autophagy through the mTOR signaling pathway, thereby supporting the metabolic needs of tumor cells. Additionally, BCAAs metabolic products can regulate apoptosis signaling pathways, inhibiting chemotherapy-induced programmed cell death, leading to treatment resistance in tumor cells. BCKDK inhibitors suppress protein translation, impair mitochondrial function, accelerate apoptosis, and enhance the cytotoxicity of doxorubicin in triple-negative breast cancer (Biswas et al., 2021). Inhibition of BCAA metabolism promotes glioblastoma cell apoptosis by disrupting mitochondrial dynamics mediated by mitofusin 2 (Mfn2) and inhibiting the PI3K/AKT/mTOR pathway, making it a potential novel therapeutic target for treating glioblastoma (Lu et al., 2024b). In AML, BCAT1 affects cell proliferation and regulates the cell cycle, apoptosis, and DNA damage/repair processes. BCAT1 modulates histone methylation by reducing intracellular αKG levels in AML cells. High expression of BCAT1 enhances the sensitivity of AML cells to poly (ADP-ribose) polymerase (PARP) inhibitors both *in vivo* and *in vitro*. The increased sensitivity of high-BCAT1 AML to PARP inhibitors could serve as an effective therapeutic strategy for AML patients (Pan et al., 2024). Furthermore, BCAT1 is overexpressed following NOTCH1-induced leukemic progenitor transformation and controls BCAT1 expression by binding to the BCAT1 promoter. Depletion or inhibition of BCAT1 leads to the production of 3-

TABLE 2 BCAAs resistance-related targets.

Cancer type	Inhibitor	Mechanism of drug resistance	Drug resistance target
NSCLC (Non-small cell lung cancer)	BCAT1 inhibitor WQQ-345 (Zhang et al., 2024a)	Glycolysis	Osimertinib
Liver cancer	BCAT1 inhibitor (Nishitani et al., 2013)	Inhibition of mTOR complex 2	5-FU
Glioblastoma	BCAT1 inhibitor (Bagchi et al., 2021)	BCKDK-BCAT1	Temozolomide (TMZ)
Breast cancer	LAT1 inhibitor JPH203 (Stine et al., 2022)	Leucine uptake and mTORC1 signaling	Aromatase inhibitors (AI)
Breast cancer	BCKDK inhibitor (Ibrahim et al., 2023)	mTORC1-Aurora axis	Paclitaxel
Breast cancer	PELP1 (Proline, Glutamic acid, Leucine-rich Protein 1) (Gonugunta et al., 2014)	Serine/Threonine protein kinase mTOR	Estrogen receptor (ER)
Bladder cancer	LRFN2 (Yu et al., 2023)	Anti-tumor immunity	PD-1 immunotherapy
Breast cancer	BCKDK inhibitor (Biswas et al., 2021)	Inhibition of protein translation, mitochondrial dysfunction	Doxorubicin
AML (Acute Myeloid Leukemia)	BCAT1 (Tosello et al., 2024)	Regulation of histone methylation	PARP inhibitor
Refractory T-ALL	BCAT1 inhibitor (Wang et al., 2024b)	Key transcription factor regulating autophagy	Elthrombopag targeting TFEB

hydroxybutyrate (3-HB), an endogenous histone deacetylase inhibitor, and is associated with increased sensitivity to DNA-damaging agents. The combined action of BCAT1 inhibition and etoposide can selectively eliminate tumors in human xenograft models, suggesting that BCAT1 inhibitors may play an important role in the treatment of refractory T-ALL (Tosello et al., 2024). The key transcription factor regulating autophagy, EB (TFEB), promotes the proliferation and metastasis of pancreatic cancer cells. Knockdown of TFEB inhibits PCC proliferation and metastasis by regulating BCAAS catabolism through BCAT1LAI. BCAAS deprivation, combined with the TFEB-targeting drug elthrombopag, can exert a dual effect by blocking both exogenous supply and endogenous utilization (Wang T. et al., 2024). Additionally, BCAAs suppress insulin-induced cancer cell proliferation by inducing autophagy (Wubetu et al., 2014).

3.7 Promotion of cancer stem cell phenotype

BCAA metabolism is associated with the maintenance and enhancement of cancer stem cells (CSCs). CSCs are a highly drug-resistant subpopulation of tumor cells. They typically exhibit significant metabolic plasticity, including enhanced BCAAs catabolism, enabling them to survive under adverse conditions such as chemotherapy or radiotherapy (Ma et al., 2018). By supporting the survival of CSCs, BCAA metabolism contributes to tumor recurrence and treatment resistance. In breast cancer studies, interferon-γ (IFNγ) produced by activated T cells has been shown to directly convert non-CSCs into CSCs. BCAT1 was identified as a downstream mediator of IFNγ-induced CSC plasticity, potentially contributing to immune checkpoint blockade (ICB) failure. Targeting BCAT1 has been demonstrated to improve cancer vaccination and immune checkpoint blockade by preventing IFNγ-induced CSC modification (Ma et al., 2018). In hepatocellular carcinoma (HCC) cells expressing the liver CSC marker EpCAM, inhibition of mTOR complex 2 (mTORC2) or activation of mTORC1 leads to reduced EpCAM expression, thereby decreasing the tumorigenic potential of CSCs and increasing sensitivity to the antiproliferative effects of 5-FU. BCAAs may reduce the number of CSCs through the mTOR pathway, thereby enhancing chemotherapy sensitivity (Nishitani et al., 2013).

BCAA metabolism plays a crucial role in cancer resistance to chemotherapy, targeted therapy, and immunotherapy, as illustrated in the figure. By upregulating BCAAs transaminases (such as BCAT1 and BCAT2) (Günther et al., 2022; Hutson et al., 1998; Ma et al., 2022),cancer cells enhance their metabolic activity, promoting growth and reducing sensitivity to chemotherapy drugs. BCKDH, as the rate-limiting step in BCAA metabolism, has been the focus of ongoing development for inhibitors targeting its activity (East et al., 2021; Roth Flach et al., 2023). Leucine, as an activator of the mTOR pathway, strengthens mTOR signaling, contributing to resistance against targeted therapies. Additionally, some studies have reported that miRNAs can target BCATs to exert antitumor effects (Ma et al., 2022). Furthermore, BCAA metabolism depletes resources needed by T cells, suppressing immune responses and leading to immunotherapy resistance. Inhibiting BCAA metabolism (such as using BCAT1 inhibitors) can reverse resistance and enhance cancer cells' sensitivity to chemotherapy, targeted therapy, and immunotherapy (Table 2). Combining BCAA metabolism inhibitors with existing therapies may be an effective strategy to overcome resistance and improve the efficacy of cancer treatments.

4 Summary

BCAAs play a crucial role in cellular metabolism, signaling, and energy supply. In cancer development and progression, BCAA metabolism exerts a complex regulatory function, and due to the

distinct genetic backgrounds of different tumors, BCAAs metabolic reprogramming manifests in varying patterns across different cancers. This presents a novel therapeutic approach for cancer treatment in the future. For instance, non-small cell lung cancer shows increased BCAAs uptake, while pancreatic cancer exhibits decreased BCAAs consumption, and the metabolic enzymes involved also behave differently. The dependency on the two BCAT isoenzymes, BCAT1 and BCAT2, differs across cancers. Targeting the specific metabolic reprogramming patterns of BCAAs in tumors could enable the design of precision therapies.

In the tumor immune microenvironment, immune cells and tumor cells compete for BCAAs uptake, which weakens the cytotoxic activity of immune cells and diminishes the immune microenvironment's effectiveness. Enhancing the antitumor activity of immune cells by increasing relevant BCAA metabolism without promoting tumor-associated metabolic reprogramming is a key challenge. Novel treatments like chimeric antigen receptor (CAR)-T cell therapy offer a promising approach by altering metabolic targets in T cells to improve immune cell cytotoxicity in the tumor microenvironment.

While cancer treatment strategies have become increasingly common, resistance to therapies often reduces the efficacy of these treatments. Our study explored how cancer-related resistance could be reversed by targeting BCAA metabolism, enhancing sensitivity to radiotherapy, chemotherapy, targeted therapy, and immunotherapy. Excitingly, some studies have shown that immune checkpoint inhibitors, such as PD-1/PD-L1 inhibitors, can boost T cell activity and overcome BCAA-induced immune suppression. Combining these inhibitors with BCAAs metabolic inhibitors has demonstrated stronger antitumor effects.

In advanced stages of cancer, patients often experience severe nutrient depletion, leading to symptoms like cachexia, where muscle metabolism also depends on certain BCAA-related enzymes. However, the potential side effects of BCAA metabolic enzyme inhibitors on normal tissues remain a concern. Thus, achieving tumor-specific precision targeting of BCAA metabolism is crucial. Looking ahead, the development of more precise treatments targeting BCAA metabolism holds promise for offering new directions and therapeutic models in cancer treatment.

Author contributions

YZ: Funding acquisition, Validation, Writing – original draft, Investigation, Supervision, Writing – review and editing, Software. JK: Writing – review and editing, Writing – original draft, Investigation, Software. WL: Writing – review and editing, Validation, Formal Analysis, Funding acquisition, Methodology, Investigation, Conceptualization. YW: Data curation, Writing – original draft, Investigation, Validation, Software, Writing – review and editing, Supervision, Project administration. XS: Writing – review and editing, Supervision, Writing – original draft, Software, Investigation. HZ: Writing – original draft, Resources, Writing – review and editing, Supervision, Project administration, Methodology, Validation, Investigation, Software, Visualization.

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Ginger (*Zingiber officinale*) dietary supplementation in mice regulates liver antioxidant defense systems in a dose- and age-dependent

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Introduction: Oxidative stress and impaired antioxidant defenses contribute significantly to liver dysfunction, particularly with aging. This study evaluated the dose- and age-dependent effects of dietary ginger (*Zingiber officinale*) supplementation on liver antioxidant defense systems in mice.

Methods: Male Swiss Webster mice aged 3, 6, and 12 months (n = 48 per age group) received standard feed or feed supplemented with either 0.6% or 1.8% dried ginger powder for 3 months. Liver tissue was analyzed for multiple antioxidant parameters, including DPPH radical scavenging activity, total antioxidant capacity, vitamin C levels, total phenolic content, superoxide dismutase (SOD) activity, malondialdehyde (MDA) levels, and reduced glutathione (GSH) concentrations.

Results: The results demonstrated significant age-dependent declines in several antioxidant parameters in control animals, including DPPH scavenging activity, total antioxidant capacity, vitamin C levels, total phenolic content, and SOD activity. Ginger supplementation produced differential effects based on both dose and age. While 3-month-old mice showed decreased DPPH radical scavenging with ginger supplementation, both 6- and 12-month-old mice exhibited significantly increased activity. Higher-dose (1.8%) ginger supplementation enhanced GSH levels across all age groups, with effects being most pronounced in older mice. SOD activity remained unaffected by ginger supplementation across all groups. MDA levels were significantly reduced by 1.8% ginger supplementation in 3-month-old mice, with smaller, dose-dependent but non-significant reductions in older groups.

Discussion: These findings demonstrate that ginger's effects on liver antioxidant systems are both dose- and age-dependent, with generally stronger beneficial effects observed at higher doses and in older animals. The observed dose- and age-dependent variations emphasize the importance of personalized supplementation strategies and provide a foundation for future research into the molecular mechanisms underlying ginger's antioxidant effects.

KEYWORDS

bioactivity, antioxidant, hepatoprotection, ginger, liver, aging, metabolism

1 Introduction

Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the body's ability to detoxify these reactive intermediates or repair the resultant damage (Burton and Jauniaux, 2011; Afzal et al., 2023). The liver represents a key target that is often attacked by these reactive species (Sanchez-Valle et al., 2012). Many external factors such as drinking alcohol, overusing drugs, exposure to toxins, viruses, and smoking, and internal factors like obesity and insulin resistance can increase ROS production in the liver (Manna and Jain, 2015; Muriel and Gordillo, 2016). To counteract this oxidative damage, the liver, which is the central metabolic organ for detoxification, has a sophisticated defense system. Specifically, it produces more antioxidants under stress conditions through both enzymatic and non-enzymatic mechanisms (Caetano et al., 2013; Li et al., 2015). Enzymatic antioxidants, such as superoxide dismutase (SOD), alongside non-enzymatic antioxidants like vitamin C, among others, are important in neutralizing the adverse effects of ROS and maintaining hepatic function (Cichoz-Lach and Michalak, 2014). Aside from external stressors, age is another key factor for the redox balance of the body, with the activities of key antioxidant enzymes such as SOD tending to decline with age, which is associated with increased oxidative damage due to reduced ability to neutralize ROS (Sohal et al., 1990; Tian et al., 1998). When antioxidant defenses get overwhelmed, the imbalance caused due to oxidative stress can disrupt cellular homeostasis, leading to inflammation, fibrosis, and metabolic dysfunction (Cahill et al., 2006; Kattoor et al., 2017; Masenga et al., 2023).

Dietary supplements with antioxidant properties are often used to help manage liver diseases caused by oxidative stress (Li et al., 2015). Examples include silymarin, curcumin, fenugreek seeds, vitamin C, vitamin E, and selenium, among others (Almeida et al., 2011; Tewari et al., 2020; Ahamed et al., 2022; Mohammadi et al., 2024). These supplements work by neutralizing excessive ROS and reactive nitrogen species (RNS), which, if unchecked, can severely damage cellular lipids, proteins, and DNA. By doing so, they not only preserve cellular integrity but also support critical regulatory pathways involved in maintaining the oxidative-reductive balance (Kurutas, 2016). Among natural supplements with antioxidant properties, several prior works have demonstrated that ginger (*Zingiber officinale*) exhibits promising effects in enhancing the body's antioxidant defenses and protecting against oxidative damage (Ballester et al., 2023; Mustafa and Chin, 2023; Ayustaningwarno et al., 2024). Recent works have also emphasized that ginger constituents possess potent antioxidant, anti-inflammatory, and anticancer properties, which are critical in regulating oxidative stress and protecting liver health, especially in age-related liver dysfunction (Bekkouch et al., 2022; Ozkur et al., 2022). The antioxidant potential of ginger is largely attributed to its major bioactive phenolic compounds, including [6]-gingerol, [8]-gingerol, [10]-gingerol, [6]-shogaol, and zingerone, which are abundant in fresh or dried rhizome extracts (Mao et al., 2019; Wu et al., 2023). These compounds have been shown to exert antioxidant activity by both direct radical scavenging (e.g., against DPPH, superoxide, hydroxyl, and nitric oxide radicals) and indirect modulation of cellular defense pathways (Peng et al., 2015). In particular, [6]-gingerol and [6]-shogaol can activate the Nrf2–ARE pathway, promoting transcription of cytoprotective genes like HO-1, NQO1, and GCLC, which help regulate glutathione metabolism and maintain redox homeostasis (Kim and Jang, 2016; Hong et al., 2020). Moreover, these compounds can inhibit NF-κB signaling, thereby reducing oxidative damage linked to chronic inflammation. These pleiotropic effects on redox-sensitive transcription factors make ginger a compelling candidate for dietary antioxidant intervention, particularly in age-associated liver dysfunction where these pathways are often dysregulated. Additionally, research has shown that ginger extracts can effectively scavenge superoxide, hydroxyl, and nitric oxide radicals in a dose-dependent manner (Jagetia et al., 2004). Such activity may support minimizing cellular damage and maintaining redox balance. In human cell studies, such as those conducted on C28/I2 chondrocytes, pretreatment with ginger extract reduced ROS levels, inhibited lipid peroxidation, and enhanced the expression of antioxidant enzymes, showing its role at a cellular level (Hosseinzadeh et al., 2017). Although ROS are deeply involved in liver injury, leading to the loss of its structure and function, the potential of ginger in counteracting such oxidative effects through various pathways is worth exploring (Banerjee et al., 2023). Building on *in vitro* findings, animal studies have further highlighted ginger's therapeutic potential in diverse conditions associated with oxidative stress, for example, in the context of nephrotoxicity caused by agents like gentamicin and cadmium (Hegazy et al., 2016; Gabr et al., 2019) and in Polycystic Ovary Syndrome (PCOS), among other disorders (Novakovic et al., 2024). While numerous studies have shown that ginger has strong antioxidant properties, limited research has explored dose-dependent *in vivo* effects. The significance of such studies, is for example, highlighted in a work conducted on STZ-induced diabetic rats, demonstrated that ginger not only reduces oxidative stress markers such as malondialdehyde (MDA) but also reduces genetic damage caused by oxidative stress in a dose-dependent manner (Kota et al., 2012). Moreover, recent clinical research showed that ginger supplementation improved the levels of liver stress-associated biomarkers (most consistently ALT) in patients (Rahimlou et al., 2016; Rafie et al., 2020; Matin et al., 2024b). Additionally, taking in consideration the age-dependency of the regulation of liver antioxidant defense systems it is of interest to examine how ginger's effects on liver redox balance may vary with age. While there is still a lack of conclusive research on this specific aspect, previous works have explored diverse ginger bioactivities that support putative general anti-aging action (Matin et al., 2024a).

On the background of the outlined prior research, the goal of the present study is to explore how ginger (Zingiber officinale) affects the liver's antioxidant defense system in mice, focusing on the impact of different doses and ages. While planning this research work, we hypothesized that ginger would improve the liver's antioxidant defenses at moderate to high doses, and that the effects will be stronger in older mice.

2 Materials and methods

2.1 Experimental setup

The study was carried out with 144 Swiss Webster male mice, that were 3 months (n = 48), 6 months (n = 48), or 12 months (n =

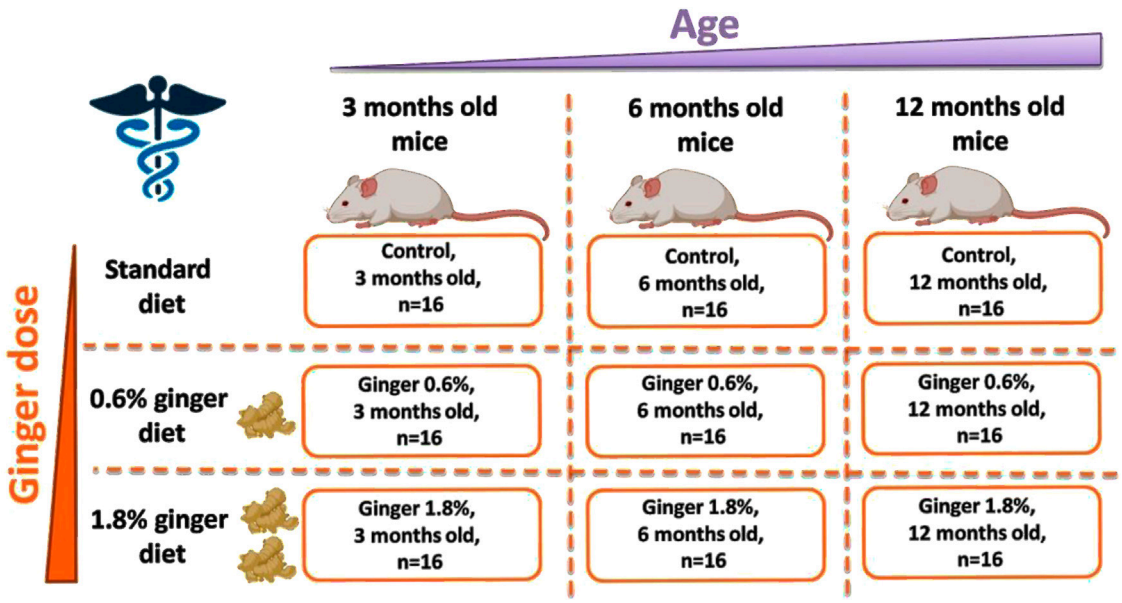

FIGURE 1
Scheme of the conducted ginger supplementation experiment.

48) old at the start of the supplementation (Figure 1). The Swiss Webster mouse strain was selected due to its widespread use in nutritional, toxicological, and pharmacological studies (Meek and Olson, 2007; Abd El-Salam and Hassan, 2017; Glavas et al., 2019). This outbred strain exhibits robust hepatic function and age-dependent redox alterations that make it suitable for modeling liver oxidative stress and assessing dietary antioxidant interventions. Furthermore, Swiss Webster mice are genetically heterogeneous, which allows for broader applicability of the findings and reduces the risk of strain-specific bias in response to dietary supplementation.

Only male Swiss Webster mice were used in this study to reduce variability associated with the estrous cycle, which can influence hormonal regulation of antioxidant enzyme systems and confound interpretation of redox-related endpoints. This approach allows for more consistent assessment of age and dose-related effects of ginger supplementation. However, it is acknowledged that sex-based differences in antioxidant capacity exist, and future studies should aim to include female mice to assess the potential for sex-specific responses to ginger supplementation.

The animals were maintained in standard cages (with four mice per cage), at the temperature of 22°C under standard conditions with 12 h of daylight and 12 h of darkness, with access to food and water. Both food and water were provided *ad libitum* throughout the entire study period, ensuring unrestricted access to standard or ginger-supplemented diets and hydration. For 3 months of supplementation period, the animals were fed with standard mouse feed (Control) or standard diet supplemented with 0.6% (Ginger 0.6%) or 1.8% (Ginger 1.8%) dried ginger (*Z. officinale*) powder (5% gingerols, supplied by Sabinsa Corporation, 20 Lake Drive, East Windsor NJ 08520, United States). Ginger powder was stored at room temperature in a foil-lined, airtight zip bag, protected from light to prevent degradation and oxidation. The ginger-supplemented fodder was prepared freshly by mixing standard feed with the respective amounts of ginger powder (0.6% or 1.8%, corresponding to 0.6 g or 1.8 g per 100 g of fodder) twice a week. After 3 months of supplementation, all animals were anesthetized with diethyl ether and decapitated. Immediately after decapitation, the liver was isolated and further processed for subjection to the described analytical procedures.

The total number of animals (144 mice) was determined based on allocating n = 16 mice per group, with three dietary treatments (control, 0.6% ginger, 1.8% ginger) across three age groups (3, 6, and 12 months). This sample size was chosen to ensure sufficient statistical power to detect medium-to-large effect sizes (Cohen's $f \geq 0.25$) using one-way and two-way ANOVA with multiple comparisons. While a formal *a priori* power analysis was not performed during the initial design phase, assuming an alpha of 0.05 and a power (1-β) of 0.80, the required sample size per group for medium effect detection using ANOVA would typically fall within the range of 12–16 animals per group (Faul et al., 2007). Future studies may refine group sizes using precise *a priori* calculations based on pilot effect sizes.

The selected ginger concentrations (0.6% and 1.8% w/w in the diet) were based on previous rodent studies that demonstrated antioxidant and hepatoprotective effects at similar levels. For instance, Ahmed et al. and Mallikarjuna et al. reported that 1% ginger in rat diets significantly improved oxidative stress biomarkers and liver function (Ahmed et al., 2008; Mallikarjuna et al., 2008). Moreover, dose-dependent antioxidant responses have been observed with 1% and 2% dietary ginger in rats (Shanmugam et al., 2011). In our study, 0.6% served as a moderate dose comparable to those previously shown to be effective, while 1.8% was selected to probe enhanced biological activity without exceeding known safe dietary limits in rodents. These levels also correspond to human-equivalent doses of approximately 3–10 g of ginger daily for a 70-kg adult, which aligns with the range used in multiple clinical supplementation studies (Anh et al., 2020).

The work was performed in the frame of animal handling permission No. 14186201 in accordance with national regulations (Act on the Protection of Animals Used for Scientific or Educational Purposes of 15 January 2015).

2.2 DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay

The potential of liver homogenates to scavenge the free synthetic DPPH radical was determined according to the procedure described by Brand-Williams et al. (1995). A 150 mg portion of liver tissue was cut and mixed with 1.5 mL of ultrapure methanol containing 1% acetic acid. The sample was then homogenized, and incubated in a water bath at 40°C for 2 h. Following incubation, the tubes were removed, allowed to cool briefly, and then centrifuged at 4,000 RPM for 10 min. Next, 0.5 mL of supernatant was mixed with the same volume of ethanolic solution of 0.5 mM DPPH, which was previously diluted to yield an absorbance of 0.9 when measured at a wavelength of λ = 517 nm. Next, the obtained mixture was thoroughly mixed and incubated in a dark and cool place for 30 min for stabilization of the color. Finally, Cary 50 Bio UV-VIS spectrophotometer was used for extinction measurements at a wavelength of λ = 517 nm with the use of Cary WinUV software.

2.3 Antioxidant capacity

The liver samples were handled according to the instructions of the Antioxidant Assay Kit by Cayman Chemical (Ann Arbor, MI, United States), according to the manufacturer's recommendations. The liver tissue (50 mg) was homogenized in 1 mL of Assay Buffer (1X), centrifuged at 10,000 × *g* for 10 min (at 4°C), and the supernatant was promptly used for the test. According to the manufacturer's instructions, the assay was further conducted with the use of 96-well plates in a 210 µL of total volume per well for 5 min, with the inclusion of 10 µL of Trolox standards in each handled plate. After the end of the incubation period, absorbance was read at 750 nm. A Trolox standard curve was plotted, and the antioxidant capacity of the samples was calculated and expressed as millimolar (mM) Trolox equivalents.

2.4 Determination of vitamin C

Vitamin C levels in livers of the experimental animals was determined with a LambdaBio-20 spectrophotometer (Perkin Elmer, Waltham, MA, United States) according to the method described by Omaye et al. (1979) with modification by Jóźwik et al. (2012). For sample preparation, 300 mg portion of liver tissue was weighed and placed into a 5 mL Eppendorf tube. A total of 1.5 mL of phosphate buffer (pH 7.0) was added to the liver tissue. The sample was homogenized thoroughly to ensure uniform consistency. For deproteination and supernatant preparation, 500 µL aliquot of the homogenized sample was transferred into a test tube. An equal volume (500 µL) of tri-chloro-acetic acid (TCA) was added, and the mixture was vortexed thoroughly. The sample was centrifuged at 3,000 RPM for 10 min. A 500 µL aliquot of the resulting supernatant was carefully collected into a clean Eppendorf tube. For colorimetric reaction development, 200 µL of phosphoric acid (H_3PO_4) was added to the supernatant, followed by vortexing. Next, 200 µL of 2,2-dipyridyl was added and the solution vortexed. Lastly, 100 µL of ferric chloride ($FeCl_3$) was added and the solution was vortexed. Two blank samples using 500 µL of TCA were prepared for reference. The reaction mixture was incubated at 37°C for 1 h. Following incubation, the absorbance of the samples was measured at 525 nm using a spectrophotometer.

2.5 Determination of total phenolic content

The total phenolics content of liver homogenates was assessed following the modified method by Škerget et al. (2005) through spectrophotometric measurement of the colorimetric redox reaction upon application of the Folin-Ciocalteu reagent. Preparation of supernatant was the same as the procedure of DPPH described above. 0.5 mL of supernatant were transferred to 6 mL tubes and mixed with 2.5 mL of Folin-Ciocalteu reagent that was diluted 10-fold with demineralized water. Next, the samples were thoroughly mixed and left for 6 min, then 2 mL of saturated sodium carbonate solution was added. The following step was an incubation for 30 min at 40°C (to allow the development of a stable blue color). Again centrifugation was done for 5 min at the speed of 3000 RPM. Finally, the absorbance of the samples was measured at 765 nm wavelength in comparison to a blank sample of double-distilled H_2O (0.5 mL). Evaluation of the results was performed with a calibration curve based on the absorbance of Gallic acid standard solutions in the range 0–0.5 mg/mL. The final results were expressed as milligrams of Gallic acid equivalents (GAE) per Gram of tissue.

2.6 Superoxide dismutase activity assay

The liver tissue samples were perfused with phosphate-buffered saline (PBS; pH 7.4), and homogenized in chilled HEPES buffer (20 mM, pH 7.2, containing 1 mM EDTA, 70 mM sucrose, and 210 mM mannitol). The chilled homogenates were then centrifuged for 5 min at 1,500 x *g* (4°C). The samples were then promptly tested for SOD activity with the Cayman Chemical Superoxide Dismutase Assay Kit (Ann Arbor, MI, United States) according to the manufacturer's manual. The measurement setup included 96-well plates and a total volume of 230 µL of total volume per well, and SOD standard solutions were included on each assayed plate. The absorbance was determined at 450 nm wavelength using Synergy4 microplate reader (Biotek; Winooski, VT, United States). A SOD standard curve was plotted, and the SOD activity of the test samples was calculated and expressed in U/mL.

2.7 Determination of malondialdehyde levels

MDA levels in liver tissue were determined using the Thiobarbituric Acid Reactive Substances (TBARS) Assay Kit (Cayman, Ann Arbor, MI, United States) following the manufacturer's instructions. Briefly, 25 mg of liver tissue was weighed into a 1.5 mL test tube, and 250 µL of RIPA buffer was

added. The mixture was homogenized while being kept on ice. The homogenized samples were centrifuged at 1,600 × g for 10 min at 4°C, and the resulting supernatants were stored on ice. The tissue homogenates did not require dilution before the assay. For the colorimetric assay, 100 µL of each sample or standard was added to appropriately labeled microcentrifuge vials, followed by the addition of 100 µL of 10% trichloroacetic acid (TCA) assay reagent. The mixture was swirled to ensure thorough mixing before the addition of 800 µL of Color Reagent, after which the vials were vortexed. The vials were then incubated in a boiling water bath for 1 h. After incubation, the vials were transferred to an ice bath for 10 min to stop the reaction, followed by centrifugation at 1,600 × g at 4°C. From each vial, 200 µL of the supernatant was transferred (in duplicate) to a clear plate, and the absorbance was measured at 530–540 nm using a Cary Varian 50Bio spectrophotometer (Santa Clara, CA, United States). The MDA concentration was calculated using a calibration curve generated from the MDA standard provided by the manufacturer, with a concentration range of 0–50 µM.

2.8 Determination of reduced glutathione concentration

Reduced glutathione (GSH) concentrations were determined according to the method of Beutler et al. (1963), with minor modifications, using DTNB as the chromogenic reagent and absorbance measured at 412 nm (Beutler et al., 1963). The liver tissue sample was homogenized at a ratio of 50 mg per 250 µL of 0.1 M of phosphoric buffer containing 0.01 M of EDTA (pH = 7.4). The homogenized sample was centrifuged for 10 min at 3000 RPM. For deproteinization of the sample, a mixture was prepared by adding 100 µL of 10% TCA, 100 µL of the supernatant, and 100 µL of 10 mM EDTA and vortexed. The mixture was then left to stand for 10 min. Following this, it was centrifuged again at 3000 RPM for 10 min. For the preparation of the sample and blank solutions, 230 µL of water was added to the plates. In the sample wells, 30 µL of 3.2 M Tris-HCl (pH 8.1), 10 µL of 10 mM EDTA, 25 µL of the obtained supernatant, and 10 µL of 3 mM DTNB were added. For the blank, 30 µL of 3.2 M Tris-HCl (pH 8.1), 20 µL of 10 mM of EDTA, 10 µL of TCA and 10 µL of 3 mM DTNB were added. Plate was placed on the shaker and incubated at room temperature for 5 min. Finally, the absorbance was measured at 412 nm. Bio-Tek Synergy4 microplate reader (Winooski, VT, United States) was used to determine absorbance at 412 nm and data on reaction kinetics. The obtained results were evaluated with the Gen5 program (BioTek), and ultimately the glutathione levels were calculated as µM concentration values.

2.9 Statistical analysis

The data are presented as mean ± standard deviation (SD). Data analysis was performed using the Real Statistics Resource Pack software (Release 8.9.1; copyright 2013–2023; Charles Zaiontz; www.real-statistics.com). Analysis of Variance (ANOVA) at α = 0.05, with Bonferroni correction for multiple comparisons, was used to determine statistical significance. Tukey's Honest Significant Difference (HSD) test was conducted as a follow-up to ANOVA. In the generated graphs, statistically significant differences ($p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$) were indicated by one, two, or three asterisks, respectively.

3 Results

The effects of dietary ginger supplementation on liver antioxidant defense systems were evaluated across different age groups of mice through multiple biochemical and enzymatic tests.

DPPH radical scavenging activity in liver tissue demonstrated both age- and dose-dependent responses to ginger supplementation (Figure 2). In the control group animals, there was a decrease in the DPPH scavenging activity with advancement of age (65.88% ± 5.69%, 55.19% ± 7.87%, and 59.00% ± 6.82%, for the 3-month-old, 6-month-old, and the 12-month-old animals, respectively; with a significance of $p \leq 0.001$ in the 6-month-old control group animals and $p \leq 0.05$ in the 12-month-old control animals, both in comparison to the 3-month-old control group). In the 3-month-old mice, both 0.6% and 1.8% ginger supplementation significantly decreased DPPH radical scavenging activity compared to control (65.88% ± 5.69% for the control, 58.46% ± 6.91% for the 0.6% ginger supplemented animals, and 56.52% ± 3.70% for the 1.8% ginger supplemented animals). This effect was more pronounced in the 1.8% ginger supplemented group ($p \leq 0.001$). The 6-month-old mice and the 12-month-old mice groups showed an inverse pattern, with significant increase ($p \leq 0.001$) in the DPPH radical scavenging in response to ginger supplementation, thus exhibiting ginger supplementation-induced reversal of the age-dependent decrease observed for the control animal groups.

Total antioxidant capacity, measured as Trolox equivalents, declined in the control animals (2.40 ± 0.40, 2.79 ± 0.30, and 1.86 ± 0.66 Trolox equivalents for the 3-month-old, 6-month-old, and the 12-month-old animals, respectively) with significant decrease especially in the 12-month-old control group ($p \leq 0.01$, Figure 3). Ginger supplementation overall displayed a pattern of enhanced total antioxidant capacity across all age groups, with the effect being most pronounced in the 1.8% supplemented 12-month-old group (1.86 ± 0.66 Trolox equivalents in the control group versus 3.31 ± 0.33 Trolox equivalents in the supplemented animals; with $p \leq 0.001$, in comparison to the 12-month-old control group).

Liver vitamin C levels showed an age-dependent decline in control animals (2.91 mg ± 0.36 mg, 2.56 mg ± 0.64 mg, and 1.56 mg ± 0.15 mg, for the 3-month-old, 6-month-old, and the 12-month-old animals, respectively; Figure 4), with the 12-month-old control group exhibiting approximately twice lower vitamin c levels compared to the 3-month-old control group (1.56 ± 0.15 versus 2.91 ± 0.36 mg/100 g; mean ± SD; $p \leq 0.001$). Ginger supplementation overall displayed a pattern of increased vitamin C levels in comparison to the respective controls in all age groups, with a significant effect in the 6-month-old group supplemented with 1.8% ginger (2.56 mg ± 0.64 mg versus 3.46 mg ± 0.60 mg; $p \leq 0.01$ compared to respective age-matched control).

Total phenolic content showed a tendency of age-related decline in control animals (6.47 ± 0.67, 5.77 ± 0.38, and 5.04 ± 1.00 mg GAE/g for the 3-month-old, 6-month-old, and the 12-month-old animals,

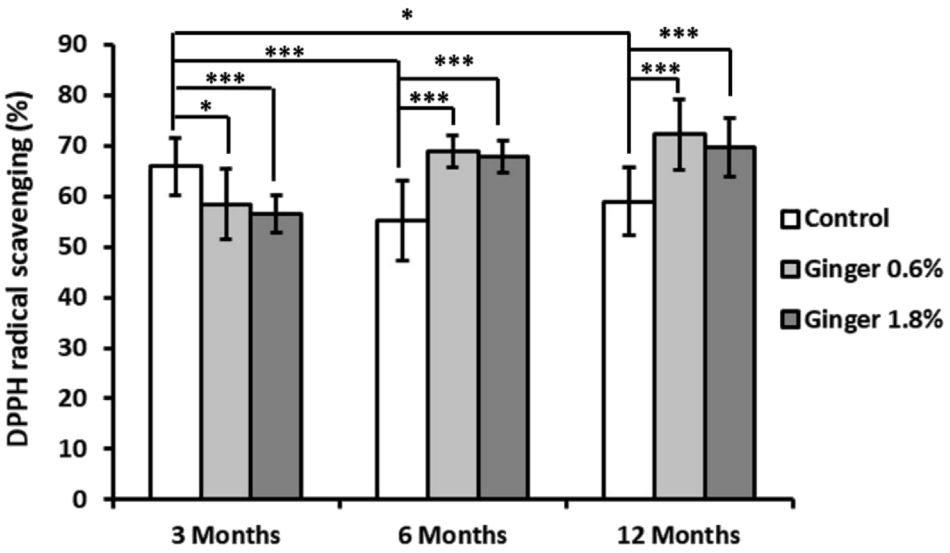

FIGURE 2
Liver DPPH radical scavenging activity. Data are presented as the percentage of DPPH radical scavenging across different age groups (3, 6, and 12 months) and dietary treatments (control, 0.6% ginger, 1.8% ginger). Values are shown as mean ± standard deviation (n = 16). Statistical significance between groups is indicated (ANOVA/Tukey HSD; *p ≤ 0.05; ***p ≤ 0.001).

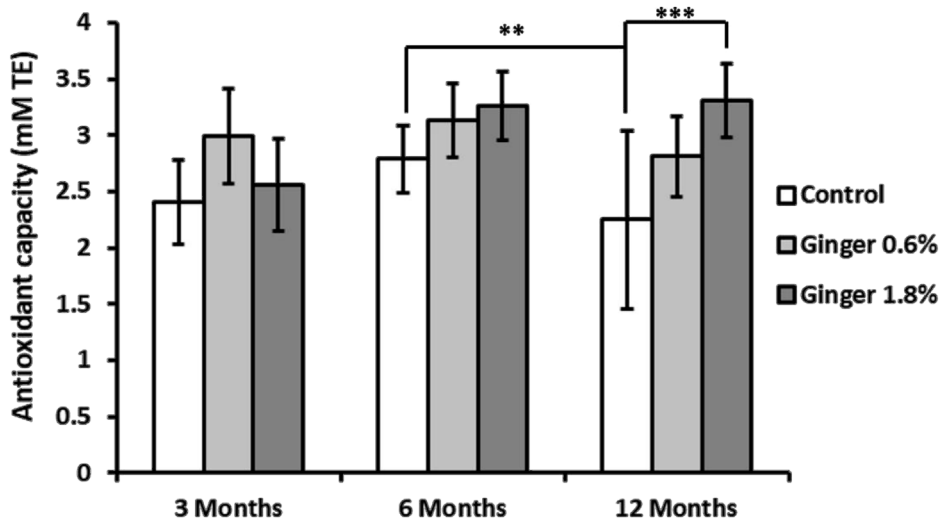

FIGURE 3
Liver total antioxidant capacity. Total antioxidant capacity of liver homogenates was determined as described in detail in the Methods section. The results are expressed in millimolar (mM) Trolox equivalents (TE). Data represent mean ± standard deviation (n = 8), with significance denoted by asterisks. (ANOVA/Tukey HSD; **p ≤ 0.01; ***p ≤ 0.001).

respectively; Figure 5), with the 12-month-old control group showing significantly lower levels compared to the 3-month-old control group (5.04 ± 1.00 versus 6.47 ± 0.67 mg GAE/g tissue; mean ± SD; $p \leq 0.001$). Ginger supplementation led to differential effects in the different age groups, with decrease of phenolic content levels in the 3-month-old animals, increase in the 6-month-old animals (especially the 1.8% supplemented group, 5.77 ± 0.38 versus 8.65 ± 0.89 mg GAE/g tissue; $p \leq 0.001$ in comparison to the age-matched control), and no effect in the 12-months-old animal groups.

Superoxide dismutase (SOD) activity showed significant age-related decline in control animals (10.76 ± 1.15, 6.93 ± 1.05. and 7.04 ± 2.58 U/mL for the 3-month-old, 6-month-old, and the 12-month-old animals, respectively; Figure 6), with both the 6-month-old and the 12-month-old control group exhibiting markedly lower activity compared to the 3-month-old control group (6.93 ± 1.05 and 7.04 ± 2.58 versus 10.76 ± 1.15 U/mL; mean ± SD; $p \leq 0.001$). Ginger supplementation did not produce significantly different effects on SOD activity in comparison to the respective age-matched control groups.

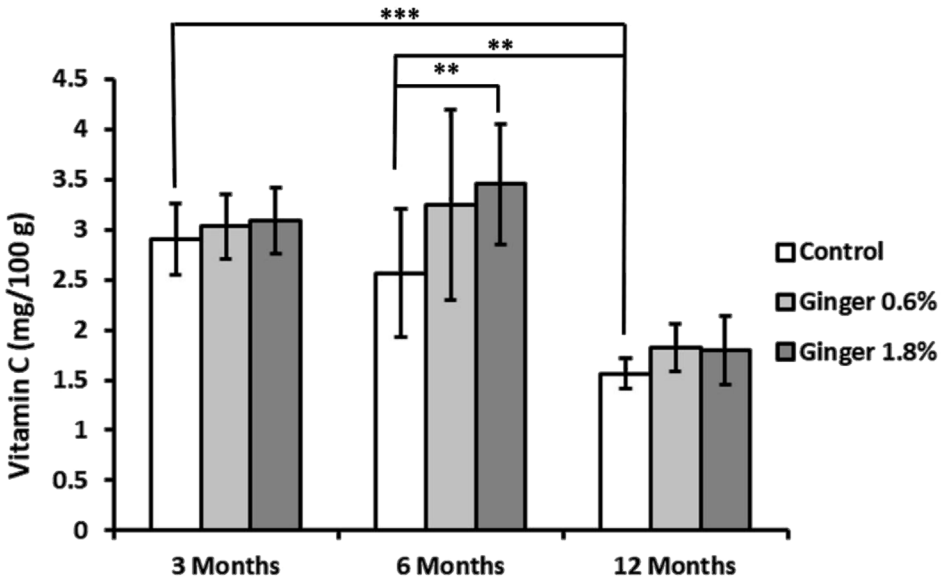

FIGURE 4
Liver vitamin C levels. Vitamin C concentrations in liver tissue were determined spectrophotometrically as described in the Methods section. The results are expressed as milligrams of vitamin C per Gram of tissue. Data represent mean ± standard deviation (n = 8). Statistical significance between groups is indicated (ANOVA/Tukey HSD; **p ≤ 0.01; ***p ≤ 0.001).

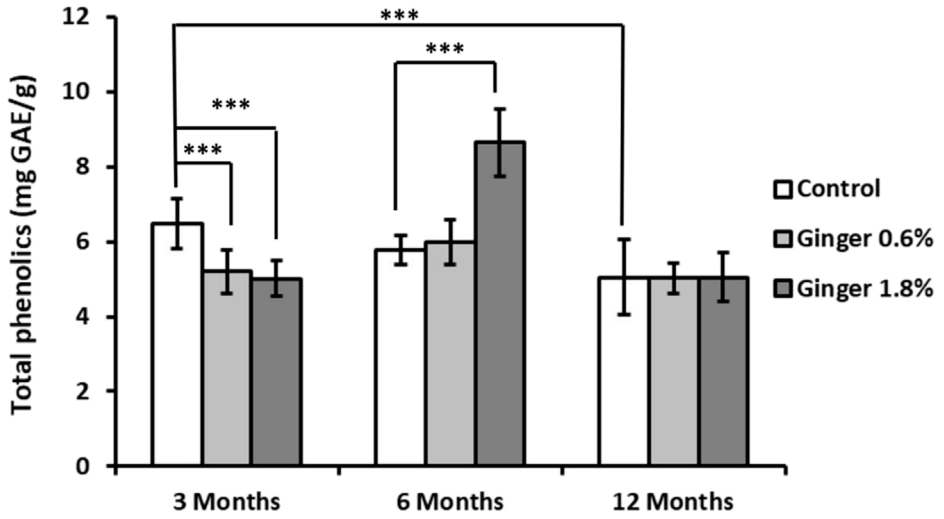

FIGURE 5
Liver total phenolic content. The total phenolic content in liver homogenates was assessed using the Folin-Ciocalteu reagent, as detailed in the Methods section. Results are expressed as milligrams of gallic acid equivalents (GAE) per Gram of tissue. Data are shown as mean ± standard deviation (n = 16). Statistical differences between groups are indicated (ANOVA/Tukey HSD; ***p ≤ 0.001).

Malondialdehyde (MDA) levels demonstrated a significant age-related decrease in control animals (81.08 ± 23.16, 34.86 ± 4.40, and 37.77 ± 5.89 μM for the 3-month-old, 6-month-old, and the 12-month-old animals, respectively; Figure 7), with both the 6-month-old and 12-month-old control groups exhibiting substantially lower levels compared to the 3-month-old control group (34.86 ± 4.40 and 37.77 ± 5.89 versus 81.08 ± 23.16 μM; mean ± SD; $p \leq 0.001$). In 6-month-old and 12-month-old animals, ginger supplementation at both concentrations resulted in moderate decreases in MDA levels compared to age-matched controls, which did not reach statistical significance. The most pronounced effect was observed in 3-month-old animals supplemented with 1.8% ginger, where MDA levels were significantly ($p \leq 0.001$; 81.08 ± 23.16 versus 45.44 ± 5.69 μM) reduced compared to the age-matched control group.

Reduced glutathione (GSH) levels did not display statistically significant age-related variations in control groups animals (Figure 8). Ginger supplementation produced enhancing effects

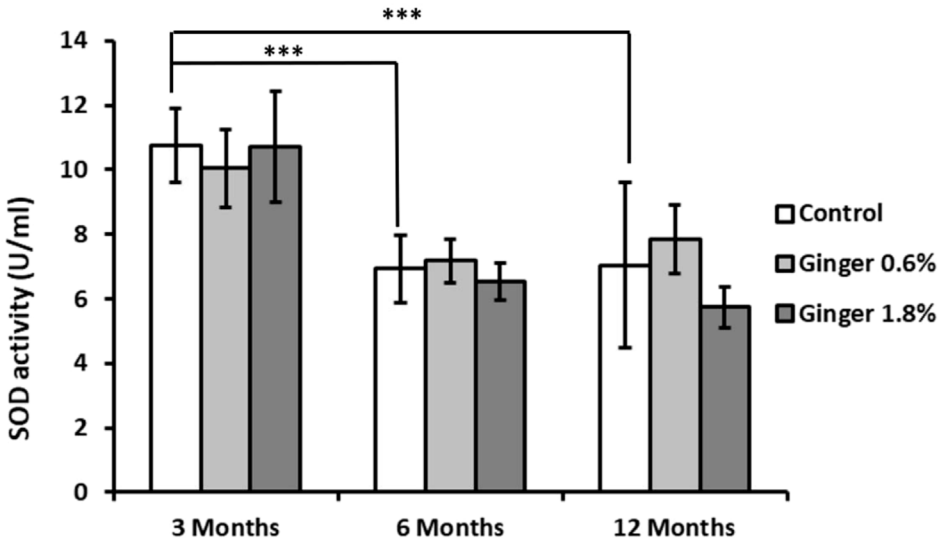

FIGURE 6
Liver superoxide dismutase (SOD) activity. Superoxide dismutase activity in liver homogenates was measured as described in the Methods section and is expressed in U/mL. Data are presented as mean ± standard deviation (n = 8). Statistical significance is indicated (ANOVA/Tukey HSD; ***p ≤ 0.001).

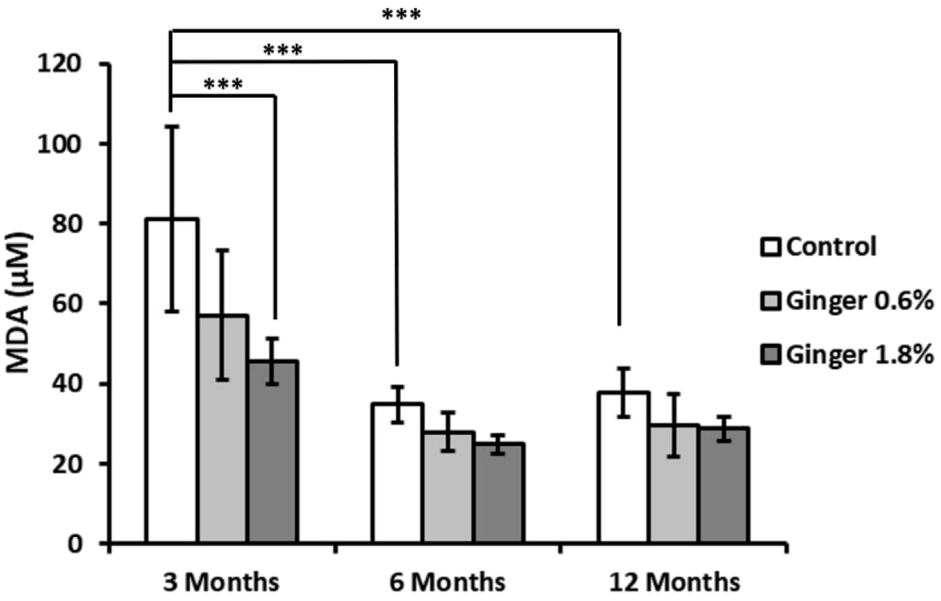

FIGURE 7
Liver malondialdehyde (MDA) levels. Malondialdehyde levels, an indicator of lipid peroxidation, were measured in liver tissue homogenates. Data are expressed as micromolar (μM) concentrations. Results are shown as mean ± standard deviation (n = 8). Statistical significance between groups is indicated (ANOVA/Tukey HSD; ***p ≤ 0.001).

in comparison to the age-matched controls in all age groups, with the 1.8% concentration being more effective (inducing statistically significant effects with $p \leq 0.001$, $p \leq 0.05$, and $p \leq 0.01$ in the 3-month-old, 6-month-old, and 12-month-old animal groups, with 748.07 ± 104.25 versus 962.02 ± 204.59, 623.94 ± 88.30 versus 791.75 ± 132.33, and 634.57 ± 84.85 versus 831.43 ± 83.73 μM, respectively).

4 Discussion

This study provides novel insights into how ginger dietary supplementation affects liver antioxidant defense systems across different age groups in mice. The results demonstrate complex age- and dose-dependent effects of ginger supplementation on various markers of oxidative stress and antioxidant capacity in liver tissue.

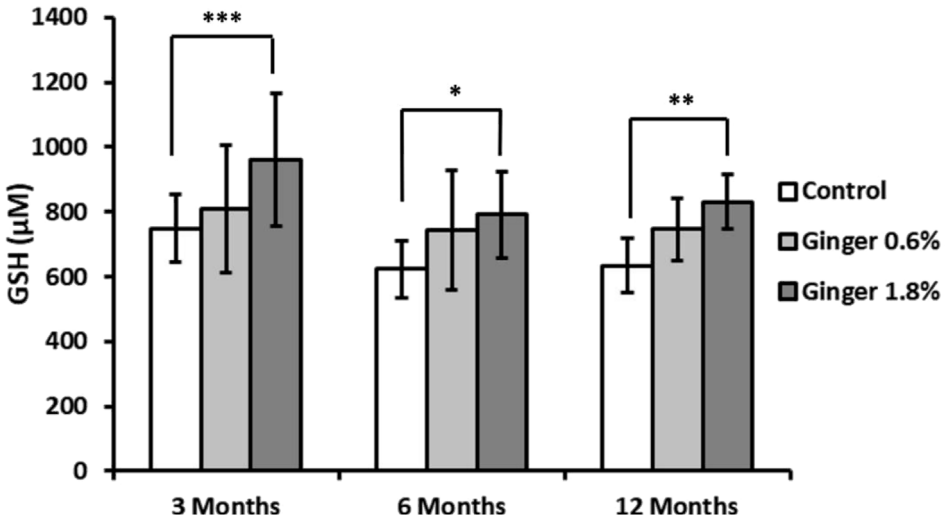

FIGURE 8
Liver reduced glutathione (GSH) levels. Reduced glutathione concentrations in liver homogenates were determined as described in the Methods section and expressed in micromolar (µM) concentrations. Data are shown as mean ± standard deviation (n = 16). Statistical significance is indicated (ANOVA/Tukey HSD; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).

Our results confirm the age-dependent decline in several key antioxidant parameters, including DPPH radical scavenging activity, total antioxidant capacity, vitamin C levels, total phenolic content, and SOD activity. This aligns with previous research suggesting that aging is associated with diminished antioxidant defenses and increased oxidative stress, which contribute to liver dysfunction (Shih and Yen, 2007; Castro Mdel et al., 2012; Lozada-Delgado et al., 2020). Our findings support this paradigm and extend it by demonstrating the differential response of antioxidant defenses to ginger supplementation at various life stages. Moreover, the observed decline in parameters such as SOD activity and vitamin C levels in the control animal groups emphasizes the importance of age-specific interventions to counteract these deficits.

The dose-dependent effects of ginger supplementation were evident across most of the studied biochemical markers. At higher doses (1.8%) and most consistently in older mice, ginger supplementation increased DPPH radical scavenging activity, enhanced total antioxidant capacity, as evidenced by increased Trolox equivalents, and increased reduced GSH levels. These findings align with prior studies suggesting that ginger's antioxidant properties are mediated through effects such as scavenging of reactive oxygen species and enhancement of endogenous antioxidant systems (Stoilova et al., 2007; Lee et al., 2011; Sueishi et al., 2019).

From a mechanistic standpoint, the antioxidant effects of ginger are likely mediated by both direct and indirect pathways. Ginger's bioactive constituents—particularly gingerols, shogaols, paradols, and zingerone—are known to exert free radical scavenging activity, lipid peroxidation inhibition, and upregulation of endogenous antioxidant defenses. These actions are partially attributed to modulation of the Nrf2 (nuclear factor erythroid 2–related factor 2) signaling pathway, which regulates the expression of antioxidant response element (ARE)-dependent genes such as those encoding glutathione S-transferase, heme oxygenase-1 (HO-1), and NAD(P)H:quinone oxidoreductase 1 (NQO1) (Peng et al., 2015). Furthermore, ginger has been shown to inhibit inflammatory signaling cascades like NF-κB, which are often intertwined with oxidative stress responses. In aged organisms, these pathways may become dysregulated or exhibit reduced responsiveness. This could explain the enhanced efficacy of ginger observed in older animals in the present study—where the supplementation may help restore diminished redox regulation by reactivating Nrf2 signaling or replenishing depleted non-enzymatic antioxidants such as GSH and vitamin C. Moreover, pharmacokinetic alterations with age—such as slower metabolism and longer tissue retention of ginger compounds—may enhance their local effectiveness in older livers.

Although the total antioxidant capacity (TAC) appeared numerically higher in the 6-month-old control group than in the 3-month-old control group, and lower in the 1.8% ginger-supplemented group at 3 months compared to the corresponding control, these differences did not reach statistical significance. Therefore, these observations should be interpreted with caution. However, minor fluctuations in TAC between age groups could reflect transitional physiological states in antioxidant regulation during early adulthood. Some studies suggest that compensatory upregulation of endogenous antioxidant systems may transiently occur during midlife before the onset of age-related decline (Benzi et al., 1989; Shih and Yen, 2007), potentially contributing to slightly elevated TAC at 6 months. Regarding the numerically lower TAC in the high-dose ginger group at 3 months, it is possible that high-dose antioxidant supplementation in physiologically young and redox-balanced animals may trigger negative feedback regulation or subtle pro-oxidant shifts, as previously proposed in models of hormesis and antioxidant overcompensation (Calabrese and Baldwin, 2003). Although these effects were not statistically significant in our study, they highlight the importance of considering age and baseline redox status when evaluating the impact of dietary antioxidant interventions. Future studies with larger sample sizes or targeted molecular profiling could help determine whether these trends are biologically meaningful.

A key finding was the age-dependent pattern observed in DPPH radical scavenging activity. While younger (3-month-old) mice showed decreased DPPH radical scavenging with ginger supplementation, both middle-aged (6-month-old) and older (12-month-old) mice exhibited significantly increased activity. This differential response suggests that the impact of ginger on free radical scavenging mechanisms varies with age, potentially due to age-related changes in baseline antioxidant capacity as well as age-related changes of in the rate of metabolizing exogenously supplied bioactive compounds including ginger phytochemicals with antioxidant action (Kim et al., 2002; Lee et al., 2008). Meanwhile, the enhanced DPPH radical scavenging observed in older mice aligns with previous research showing ginger's ability to combat oxidative stress that is increased with aging (Peng et al., 2015; Hosseinzadeh et al., 2017; Al Hroob et al., 2018).

The observed decrease in DPPH radical scavenging activity in 3-month-old mice supplemented with ginger, particularly at the 1.8% dose, may be explained by several interconnected mechanisms. First, young mice generally possess robust endogenous antioxidant systems, with high baseline levels of enzymatic and non-enzymatic antioxidants. Introducing additional exogenous antioxidants like ginger may lead to transient pro-oxidant effects or feedback inhibition of endogenous antioxidant responses—a phenomenon often observed in hormetic responses to phytochemicals. This biphasic behavior is supported by the concept that mild oxidative stress can activate protective mechanisms, whereas excessive antioxidant input in already-balanced systems may dampen radical signaling or paradoxically impair redox balance (Calabrese and Baldwin, 2003). Additionally, certain phenolic compounds in ginger may exhibit dual roles—acting as antioxidants at low or moderate ROS levels but exhibiting pro-oxidant effects under high-reducing or low-ROS cellular states (Maliar et al., 2023). This could explain the reduced DPPH scavenging in young mice where oxidative burden is minimal. Moreover, the higher metabolic activity and detoxification efficiency in younger animals may lead to faster metabolism and clearance of ginger phytochemicals, limiting their local accumulation and effect in liver tissue. Collectively, these age-dependent pharmacodynamic and redox-regulatory differences help explain the attenuated response in younger animals and underscore the importance of physiological context in interpreting antioxidant interventions.

The age-related differences in antioxidant response to ginger supplementation observed in this study highlight a complex interplay between baseline redox status and metabolic responsiveness. Younger mice (3 months old) displayed higher endogenous antioxidant levels in control groups, which may have limited the observable benefits of supplementation or even led to paradoxical effects (e.g., reduced DPPH scavenging and phenolic content). This may be due to a "ceiling effect," where antioxidant systems are already well-regulated and additional stimulation is unnecessary or counterproductive. In contrast, middle-aged and older mice (6 and 12 months) exhibited significant age-associated declines in DPPH scavenging, vitamin C levels, and SOD activity—creating a physiological state of redox vulnerability. Ginger supplementation in these groups likely restored redox homeostasis by bolstering antioxidant reserves, particularly through the replenishment of GSH and enhancement of total antioxidant capacity. This aligns with prior findings that antioxidant interventions have greater benefit in aged or oxidatively stressed organisms compared to healthy young counterparts (Shih and Yen, 2007; Castro Mdel et al., 2012). These observations support the concept of age-personalized antioxidant supplementation, where physiological context determines the efficacy of dietary interventions.

The significant reduction MDA levels, particularly in younger mice receiving high-dose ginger supplementation, reflects the previously reported ability of dietary ginger to counter lipid peroxidation (Ahmed et al., 2000; Ahmed et al., 2008). However, the less pronounced effects in older animals might indicate an age-related resistance to modulation of lipid peroxidation pathways. These findings warrant further investigation into the molecular mechanisms underpinning these differences.

Interestingly, MDA levels in control animals were significantly higher at 3 months compared to 6 and 12 months, which contrasts with the typical expectation of age-related increases in lipid peroxidation. However, several factors may explain this finding. In early adulthood, mice exhibit higher metabolic rates, elevated mitochondrial respiration, and increased membrane lipid turnover, all of which can transiently elevate ROS production and enhance susceptibility to lipid peroxidation (Sohal et al., 1990). Moreover, older tissues may shift toward protein and DNA oxidation markers, with relatively less lipid peroxidation occurring at baseline (Cakatay et al., 2003; Ma et al., 2013). Therefore, MDA should not be viewed in isolation as a direct surrogate of total oxidative burden across ages. These findings highlight that age-related shifts in oxidative stress profiles can vary depending on the molecular target (lipid, protein, DNA), metabolic context, and tissue-specific vulnerability.

Superoxide dismutase activity demonstrated significant age-related decline, confirming previous findings about the deterioration of liver enzymatic antioxidant defenses with age (Reiss and Gershon, 1976; Santa Maria et al., 1996). The lack of significant changes with ginger supplementation suggests that ginger's antioxidant effects might primarily operate through non-enzymatic pathways, rather than by modulating SOD activity directly.

The lack of a measurable effect of ginger supplementation on total SOD activity, despite improvements in other antioxidant parameters, may stem from several factors. First, the assay used in this study measured total SOD activity, without discriminating between isoforms such as SOD1 (cytosolic) and SOD2 (mitochondrial). Prior studies have shown that ginger may preferentially affect mitochondrial oxidative stress pathways and upregulate SOD2 (Homa and Wentz-Hunter, 2018), changes that could remain undetected in whole-tissue homogenates where SOD1 predominates. Second, it is possible that the antioxidant benefits of ginger in this model were mediated more through non-enzymatic antioxidants (e.g., GSH, vitamin C) or via modulation of other enzymatic systems (e.g., catalase, glutathione peroxidase), thereby limiting the need for enhanced SOD activity. Third, redox homeostasis involves complex compensatory networks. Increased glutathione or phenolic content may reduce superoxide burden, alleviating the need for further SOD induction. Finally, assay sensitivity and technical variability may also obscure subtle isoform-specific changes. Further studies examining specific SOD isoforms at the transcriptional and protein level, or

subcellular localization of antioxidant effects, could help clarify these findings.

Although ginger supplementation did not produce statistically significant changes in SOD activity in any group, it is worth noting that SOD activity values in the 1.8% ginger groups at 6 and 12 months were numerically lower than those in the 0.6% ginger groups. This observation, while not statistically significant, may reflect a dose-dependent regulatory ceiling or feedback suppression of enzymatic antioxidants when exogenous antioxidant input is high. At elevated doses, ginger-derived phenolics may strongly increase non-enzymatic antioxidant reserves—such as GSH and vitamin C—which could reduce the cellular demand for SOD activity, leading to compensatory downregulation. This type of adaptive response has been reported in other antioxidant supplementation studies, where excessive exogenous antioxidant availability dampened the expression of genes of endogenous antioxidant defense proteins (Halliwell, 2000; Calabrese and Baldwin, 2003). Another possibility is that higher doses of ginger exert differential effects on antioxidant enzyme regulation depending on cellular redox state and age, which may not follow a linear dose–response pattern. However, due to the lack of statistical significance, these interpretations remain speculative and should be explored in future studies involving isoform-specific enzyme measurements and redox-sensitive gene expression profiling.

Glutathione levels showed a consistent positive response to ginger supplementation across all age groups, with the higher dose (1.8%) being more effective. This enhancement of GSH levels is particularly noteworthy given glutathione's central role in cellular redox regulation and antioxidant defense (Zhang and Forman, 2012). The ability of ginger to boost GSH levels across different age groups suggests it could have broad applications in supporting liver antioxidant defenses.

The findings of the present study collectively support our initial hypothesis regarding ginger's beneficial effects on liver antioxidant systems, particularly at higher doses. However, the age-dependent variations in response highlight the complexity of antioxidant system regulation and suggest that ginger's effects may be modulated by age-related factors. The stronger responses observed in older mice for several parameters (particularly DPPH radical scavenging and total antioxidant capacity) align with our hypothesis about enhanced effects in older animals.

The results also provide insights into potential mechanisms of ginger's antioxidant effects. The simultaneous enhancement of non-enzymatic antioxidants (vitamin C, GSH) and total antioxidant capacity, coupled with reduced lipid peroxidation (MDA), suggests multiple pathways of action. This multi-target effect is consistent with previous studies showing ginger's diverse bioactive properties (Mao et al., 2019; Wu et al., 2023).

These findings have several potential implications for therapeutic applications. The age-dependent effects observed suggest that ginger supplementation might be particularly beneficial in supporting liver antioxidant defenses in older individuals, where natural antioxidant systems may be compromised. This aligns with prior research showing ginger's potential versatile benefits in supporting liver health (Gao et al., 2012; AuthorAnonymous et al., 2017; Hamza et al., 2021; Guo et al., 2022). The study outcomes also hint to potential broader anti-aging benefits of ginger supplementation, in line with its previously characterized versatile bioactivity profile simultaneously contracting different hallmarks of aging (Ozkur et al., 2022; Matin et al., 2024a). Additionally, the dose-dependent effects observed in the present work support the importance of appropriate dosing in ginger supplementation strategies.

Future research could focus on elucidating the molecular mechanisms underlying the age-specific effects observed, particularly the differential responses in young versus older animals. Investigation of changes in antioxidant gene expression and protein levels could provide additional insights into how ginger modulates these systems. Additionally, longer-term studies could help understand the sustainability of these effects and potential adaptations over time.

5 Limitations

This study has several limitations that should be considered when interpreting the findings. While we evaluated total superoxide dismutase (SOD) activity, the assay did not distinguish between SOD isoforms (e.g., SOD1 and SOD2), nor did it assess other antioxidant enzymes such as catalase or glutathione peroxidase. Thus, potential isoform-specific or compensatory effects may have been masked in total activity measurements.

A limitation of this study is the exclusive use of male animals, which, while controlling for hormonal variability, does not account for sex-specific differences in antioxidant defense systems. Estrogen, for instance, has been shown to modulate redox signaling and influence expression of key antioxidant enzymes such as SOD and GPx. Therefore, future research should include both sexes to determine whether the antioxidant effects of ginger exhibit sex-specific patterns, particularly in the context of aging.

Further, although we observed several trends across age and dose groups, some differences—particularly those in DPPH scavenging, total antioxidant capacity, and SOD activity—did not reach statistical significance. These non-significant patterns should be interpreted with caution and may benefit from further validation with larger sample sizes or targeted molecular analyses. In addition, we did not perform transcriptomic or proteomic analyses to directly assess the molecular pathways modulated by ginger, such as Nrf2 or NF-κB signaling, which are known to regulate antioxidant defenses. These mechanistic insights remain speculative and should be explored in future investigations.

Finally, the study was conducted over a 3-month period of supplementation, which may not fully capture the long-term effects of dietary ginger supplementation on liver function and redox balance. Longer-term studies and broader biomarker panels would strengthen understanding of the sustained impact and safety profile of ginger in aging models.

6 Conclusion

This study demonstrates that ginger dietary supplementation exerts significant dose- and age-dependent effects on liver antioxidant defense systems in mice. Our findings confirm the initial hypotheses regarding both the dose-dependency of ginger's effects and their more pronounced effects in older animals for

several key parameters. Ginger supplementation enhanced key markers of antioxidant capacity, including DPPH radical scavenging activity, reduced glutathione (GSH) levels, and total antioxidant capacity, particularly at higher doses and in older animals. These findings highlight ginger's potential to mitigate age-associated oxidative stress and support hepatic antioxidant defenses.

The differential impacts of ginger supplementation observed in this study underscore the importance of tailoring dietary interventions based on age and dosage. The demonstrated dose-dependent effects emphasize the importance of appropriate dosing strategies, while age-specific responses highlight the need for personalized antioxidant supplementation strategies. Future research should focus on elucidating the molecular mechanisms underlying these age-specific effects and investigating the long-term implications of ginger supplementation on liver health across different life stages.

Overall, this study establishes ginger as a promising dietary supplement for supporting liver antioxidant defenses, particularly in aging populations, while also highlighting the complexity of its biological effects across different age groups. These findings contribute to our understanding of natural antioxidant interventions and their potential role in promoting healthy liver aging.

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

The animal study was approved by the animal handling permission No. 14186201 in accordance with national regulations (Act on the Protection of Animals Used for Scientific or Educational Purposes of 15 January 2015). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MM: Writing – original draft, Investigation, Methodology, Conceptualization, Formal Analysis, Project administration, Data curation. KW: Writing – review and editing, Formal Analysis, Investigation. JH: Conceptualization, Writing – review and editing. LR: Writing – review and editing, Conceptualization. AA: Writing – review and editing, Conceptualization.

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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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Dietary supplementation with a designer metabolic modulator improves MASLD and associated anxiety in mice

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Background: Metabolic dysfunction-associated steatotic liver disease (MASLD), formerly known as non-alcoholic fatty liver disease (NAFLD), is a multifaceted condition characterized by excessive liver fat accumulation associated with obesity or other risk factors. Patients with obesity-related MASLD often suffer from comorbid psychiatric conditions, including anxiety. The therapeutic approach for MASLD relies on weight management through dietary and behavioral modifications. Nutritional interventions with essential amino acids (EAAs) have emerged as safe and promising tools in treating metabolic disorders and liver diseases. This study aimed to investigate the effects of dietary supplementation with α5, a designer EAA-based metabolic modulator enriched with tricarboxylic acid cycle intermediates, in a murine model of diet-induced MASLD with associated anxiety.

Methods: Ten-week-old male C57BL/6J mice were fed for 17 weeks either a high-fat, high-sugar diet or a standard purified diet. The α5 compound (1.5 mg/g/day in drinking water) was administered to half of the mice fed each diet (n = 8/group). Mice body weight and energy intake were recorded. Liver and adipose tissue depot weights were calculated as ratios to body weight. Blood analytes were evaluated. Liver samples were analyzed for the enzymatic activity of mitochondrial chain respiratory complexes, gene expression (reverse transcription-qPCR), and histological features (hematoxylin-eosin and Masson's trichrome staining). Liver disease severity was graded using the NAFLD Activity Score. The open field behavioral test was conducted to assess anxiety.

Results: Mice fed the high-fat, high-sugar diet developed obesity, a MASLD phenotype, and anxiety-like behaviors. Dietary supplementation with α5 ameliorated liver pathology, including reduced hepatocellular ballooning, fat lipid droplet diameter, and the expression of genes related to fibrosis, without affecting body weight. Moreover, α5 supplementation significantly reduced the anxiety-like behavior observed in untreated MASLD mice.

Discussion: These results suggest that α5 represents a novel intervention to prevent or mitigate the progression of MASLD and its associated mental health complications.

KEYWORDS

metabolic dysfunction-associated steatotic liver disease, anxiety, mice, western diet, fibrosis, essential amino acids, tricarboxylic acid cycle intermediates, dietary supplement

1 Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD), formerly known as non-alcoholic fatty liver disease (NAFLD), is a progressive condition characterized by triglyceride (TG) liver accumulation in the absence of excessive alcohol intake (Tack et al., 2024). The updated diagnostic criteria emphasize both hepatic fat content exceeding 5%–10% of liver weight and the presence of at least one risk factor among overweight or obesity, hyperglycemia or type 2 diabetes, elevated TGs, reduced HDL-cholesterol, or increased blood pressure (Tack et al., 2024). MASLD affects approximately 30% of the global population, a prevalence that continues to rise alongside the spread of unhealthy lifestyles, including Western-type diets and physical inactivity (Younossi et al., 2025). The disease spectrum ranges from isolated steatosis (termed metabolic dysfunction-associated steatotic liver, MASL) to more advanced stages such as steatohepatitis (MASH), which features hepatic inflammation and hepatocyte ballooning. If untreated, MASH can progress to fibrosis, cirrhosis, and ultimately hepatocellular carcinoma (Huang et al., 2025). A major clinical challenge is the typically silent early phase of MASLD, during which symptoms are either absent or nonspecific, leading to delayed diagnosis (Eskridge et al., 2023).

Despite growing research efforts, the pathophysiological mechanisms of MASLD remain incompletely understood. Known contributors include genetic predisposition and excessive intake of dietary fat and sugar, which promote adipocyte dysfunction, leading to the release of free fatty acids and ectopic accumulation of lipids in the liver (Huang et al., 2025). In parallel, increased hepatic *de novo* lipogenesis and mitochondrial dysfunction contribute to oxidative stress and impaired ATP production, ultimately leading to hepatocellular injury, chronic inflammation, and fibrosis (Huang et al., 2025; Radosavljevic et al., 2024).

Emerging evidence also links MASLD to neuropsychiatric comorbidities. Patients with MASLD/NAFLD exhibit higher rates of anxiety and depression (Hadjihambi et al., 2023; Labenz et al., 2020; Shea et al., 2024; Wang et al., 2024), even in the early, pre-diagnosis stages of the disease (Eskridge et al., 2023). These associations appear to be multifactorial and bidirectional, involving insulin resistance, low-grade chronic inflammation, and alterations in the gut microbiome (Shea et al., 2024). Possibly perpetuating a detrimental cycle between metabolic dysfunction and mental health (Shea et al., 2024).

Currently, resmetirom (a thyroid hormone β-receptor agonist) is the only approved pharmacological treatment for MASH with advanced fibrosis. Still, other therapeutic agents have demonstrated promising results in randomized controlled trials (Huang et al., 2025; Lin et al., 2024). Due to the complex nature of MASLD/MASH, combination therapies are also under investigation (Dufour et al., 2020). For now, weight-loss programs, including the adoption of healthy dietary patterns and lifestyle modification, represent the cornerstone of MASLD treatment (Segala et al., 2024). Unfortunately, adherence to such interventions is generally low among patients with MASLD (Frith et al., 2010), and the presence of comorbid anxiety may further reduce compliance.

Our research group has long-standing experience in supplementing diets with essential amino acids (EAAs). We extensively investigated an EAA balanced formula, termed branched-chain amino acid-enriched mixture (BCAAem), which enhances mitochondrial metabolism and the endogenous antioxidant response, exerting beneficial effects in various organs and pathophysiological conditions (see Ruocco et al., 2021, for a review). Of note, we previously demonstrated that the BCAAem prevents liver steatosis in a rodent model of alcoholic liver disease (Tedesco et al., 2018). Building on our previous work, we recently designed novel EAA-based metabolic modulators incorporating tricarboxylic acid (TCA) cycle intermediates, which showed superior efficacy in promoting mitochondrial function compared to the original BCAAem formulation (Brunetti et al., 2020; Ruocco et al., 2021). One such compound, referred to as α5, consists of eleven EAAs, including balanced stoichiometric ratios of the BCAAs (leucine:isoleucine:valine ratio, 3:1:1), enriched with three TCA cycle intermediates (citric, malic, and succinic acids) and cofactors (Ruocco et al., 2021; Tedesco et al., 2020). The α5 compound has been shown to support neuronal energy metabolism *in vitro* and *in vivo* (Bifari et al., 2020; Dolci et al., 2022), suggesting its safety and therapeutic potential not only in metabolic disease, but also in neuropsychiatric disorders.

In this study, we fed adult mice a high-fat, high-sugar diet (HFHSD) that was previously shown to induce a MASLD/MASH phenotype closely resembling the human disease (Verbeek et al., 2015). Fare clic o toccare qui per immettere il testo. Our findings demonstrate that dietary supplementation with the designer metabolic modulator α5 improves liver pathology and alleviates anxiety-like behavior in mice with a Western-type diet-induced MASLD.

2 Materials and methods

2.1 Animals, diets, and treatment

All experiments were performed in accordance with the European Directive 2010/63/EU and current Italian law (D. Lgs. n. 26/2014). The protocol was approved by the General Direction of Animal Health and Veterinary Drugs of the Italian Ministry of Health with the authorization n. 498/2018-PR. Eight-week-old male C57BL/6J mice (n = 32) (Charles River, Calco, Italy) were housed under controlled temperature and humidity conditions, in a 12-h light-dark cycle, with *ad libitum* access to food and water. After

2 weeks of acclimatization, mice were randomized into four groups (n = 8/group, 4/cage) and fed for 17 weeks with either Standard Purified Diet (SPD; AIN-93M formula, TD.94048, Envigo, Italy) or HFHSD (TD.08811, Envigo, Italy) (Supplementary Table S1). The length of the dietary intervention was based on previous reports using the same HFHSD (Gariani et al., 2016; Verbeek et al., 2015). Half of the mice fed each of the two diets were supplemented with the α5 (Professional Dietetics S. p.A, Milan, Italy) for the entire experiment duration (Figure 1A). Body weight and food intake were recorded weekly, and drinking volume was measured three times a week. The composition of the α5 supplement is detailed in Supplementary Table S2. It was administered at a dose of 1.5 mg/g body weight/day in drinking water, as previously described (Tedesco et al., 2020), and the solution was replaced three times a week. The amount of α5 to be dissolved in water was calculated for each experimental group based on the average body weight from the most recent measurement and the average daily water consumption over the previous 2 weeks. This calculation was adjusted regularly according to these parameters. Blood was collected from the tail vein at week 16 or via submandibular venipuncture immediately before sacrifice. All blood drawings were performed after a 7-h fast. At the end of the study, mice were euthanized by cervical dislocation. Tissue, serum, and plasma samples were collected and snap-frozen in liquid nitrogen, then stored at −80 °C. One mouse in the SPD group died soon after randomization. One mouse in the SPD+α5 group showed a suspected liver neoplasm at sacrifice, and its data were excluded from analyses.

2.2 Behavioral tests

The open field test was used to assess anxiety as described (Keleher et al., 2018) with minor modifications. The arena consisted of a plastic box [40 (length) × 40 (width) × 40 (height) cm] with a grid on the floor to identify a 25 × 25 cm area defined as the central zone. The test was conducted in a quiet, dimly lit room, during the light phase of the light-dark cycle between 9 a.m. and 4 p.m. Briefly, mice were individually placed in the center of the arena and allowed to explore freely for 5 min. Mice's behavior was recorded and analyzed by a video tracking system (ANY-Maze, Stoelting Co., IL, United States). The following measurements were collected: total distance traveled, average speed, time spent moving, time spent in the peripheral zone, number of entries into the central zones, and number of fecal boli produced. Preference to stay in the peripheral zones, reduced visits to the central zone, and increased fecal boli production were interpreted as indicators of anxiety.

2.3 Circulating analytes and glucose homeostasis evaluation

Blood samples for serological analyses and assessment of glucose homeostasis were collected from the tail vein after a 7-h fast at week 16 of treatment. Serum levels of insulin, C-reactive protein (CRP), and tumor necrosis factor-alpha (TNF-α) were measured by ELISA immunoassay. We used Mouse Insulin ELISA Kit (ab277390, Abcam, Cambridge, United Kingdom), Mouse C Reactive Protein ELISA Kit (PTX1) (ab157712, Abcam, Cambridge, United Kingdom), Mouse TNF-alpha Quantikine HS ELISA (MHSTA50, R&D Systems, Bio-techne, Minneapolis, MN, United States), respectively, according to the manufacturers' instructions. For the glucose tolerance test (GTT), mice received an intraperitoneal (i.p.) injection of glucose (0.75 mg/g body weight; Sigma-Aldrich, Milan, Italy) (n = 4 mice/group). Blood glucose concentrations were measured from the tail vein at baseline (0 min) and at 15, 30, 60, and 120 min post-injection using the OneTouch Verio Reflect glucometer (LifeScan, Sesto San Giovanni, Italy) (Ruocco et al., 2020). The area under the curve (AUC) was calculated using the trapezoidal rule (Ruocco et al., 2020). Blood samples for biochemical analysis were obtained via submandibular venipuncture before sacrifice, collected in EDTA-containing tubes, and then centrifuged to separate the plasma. The following analytes were measured: total cholesterol, TGs, alanine aminotransferase (ALT), and aspartate aminotransferase (AST). The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated by multiplying fasting insulin (ng/mL) by fasting glucose (mg/dL) and dividing the result by 405 (Verbeek et al., 2015).

2.4 Mitochondrial respiratory complex activities

Homogenates of frozen mouse liver samples were prepared as described by Spinazzi and colleagues (Spinazzi et al., 2012), with minor modifications (Brunetti et al., 2020). Enzymatic activities of individual MRC complexes were measured spectrophotometrically. Assays of complex I (CI, NADH:ubiquinone reductase), II (CII, succinate dehydrogenase), III (CIII, decylubiquinol cytochrome c oxidoreductase), and IV (cytochrome *c* oxidase), as well as the citrate synthase assay, were performed as described in detail (Spinazzi et al., 2012). The complex V (CV, F_1-ATPase) assay was performed according to Frazier and Thornburn (Frazier and Thorburn, 2012). All enzymatic activities were normalized to the citrate synthase activity.

2.5 Reverse transcription and quantitative PCR

For the analysis of mRNA expression, total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). RNA concentration and purity were assessed with the NanoDrop™ OneC Microvolume UV-Vis Spectrophotometer (Thermo Scientific, Milan, Italy). Total RNA (2 μg) was reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Segrate, Italy). cDNA was diluted 1:5 in DNAse-free water, and 2 μL were amplified by real-time quantitative PCR with iTaq Universal SYBR Green SuperMix (Bio-Rad Laboratories) on a ViiA7 Real-Time PCR system (Applied Biosystems). Each sample reaction was conducted in triplicate. Primer sequences (Supplementary Table S3) were designed using Primer3 software (version 0.4.0). Relative gene expression was calculated by a comparative method ($2^{-\Delta\Delta CT}$). Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as a housekeeping gene after evaluating its stable expression.

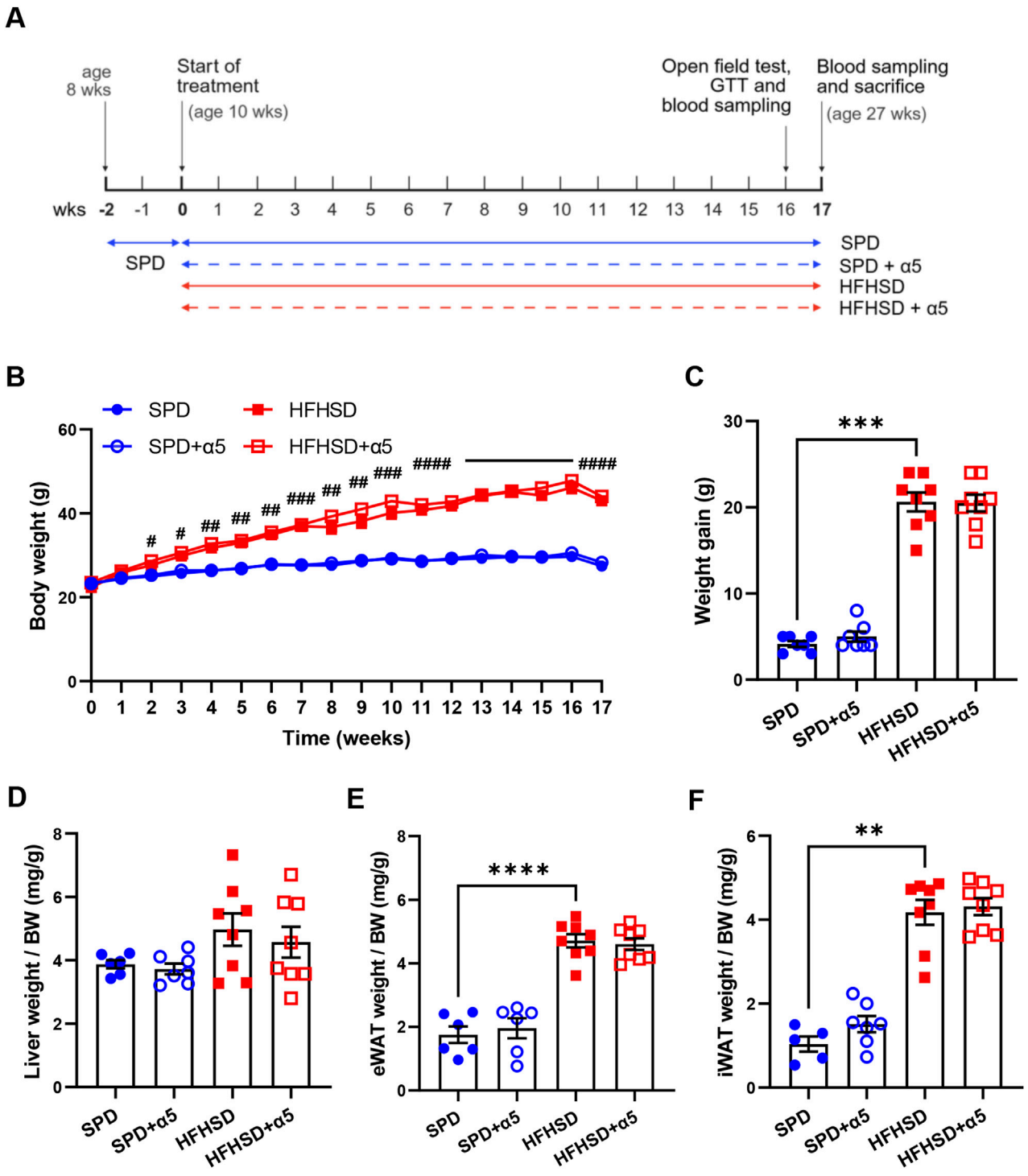

FIGURE 1
Effects of the nutritional interventions on body and organ weight. **(A)** Schematic representation of the experimental design timeline. Ten-week-old C57BL/6J mice were fed *ad libitum* with standard purified diet (SPD) or high-fat, high-sugar diet HFHSD with or without α5 supplementation for 17 weeks. Image was created with BioRender.com. **(B)** Weekly measurement of body weight; **(C)** Body weight gain, measured as the difference between the final and initial (at the start of the treatment) body weight. **(D)** Liver weight normalized to body weight (BW); **(E,F)** weight of visceral (eWAT, epididymal white adipose tissue) and subcutaneous (iWAT, inguinal WAT) fat pads normalized to BW. Data represent mean ± SEM (n = 5-8 mice/group; missing points are due to technical issues). Statistical analysis was performed by the two-way ANOVA, followed by Tukey's multiple comparisons test **(B)**; the ordinary one-way ANOVA, followed by Šídák's multiple comparisons test **(D,E)**, or the Kruskal-Wallis test, followed by Dunn's multiple comparison test **(C-F)**. **p < 0.01, ***p < 0.001 and ****p < 0.0001; #p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001 in HFHSD vs SPD.

To measure mtDNA content, total DNA was extracted using the QIAamp DNA Mini Kit (Qiagen). Then, qPCR was performed using primers specific for the mitochondrially encoded gene Cytochrome c oxidase subunit I (*Co1*) and the nuclear gene Ribonuclease P (*Rnase P*) (Supplementary Table S3). The mtDNA copy number (mtDNAcn) was calculated as previously described (Segala et al., 2024).

2.6 Liver morphometrical analyses and triglyceride content assay

Liver samples were fixed in a 4% paraformaldehyde solution for 48 h and embedded in paraffin wax. Serial sections (5 µm thick) were cut with a semiautomatic microtome. Alternate sections were deparaffinized, rehydrated, and stained with hematoxylin-eosin (H&E) to assess overall liver parenchymal morphology, as well as liver and hepatocyte abnormalities (including ballooning, steatosis, and inflammatory infiltration), and the diameter of lipid droplets (Raffaele et al., 2019). Masson's trichrome staining was performed to evaluate liver fibrosis, with fibrotic connective tissue stained blue, and the hepatocyte cytoplasm red (Raffaele et al., 2019). Sections were observed with an optical light microscope (Olympus BX50, Hamburg, Germany) at a final magnification of ×200. Image analysis was performed using the Image Pro Premier 9.1 software program (Media Cybernetics, Rockville, MD, United States). Lipid droplet diameter was measured by evaluating a minimum of 10 droplets per randomly chosen field on five non-consecutive sections per mouse. In addition, a frequency analysis of lipid droplets was performed based on arbitrary diameter clustering into the following five classes: <3.5 µm, between 3.5 and 7 µm, between 7 and 15 µm, between 15 and 25 µm, and >25 µm. The presence and percentage of fibrosis were measured in five randomly chosen fields per mouse. All analyses were evaluated in a single-blind manner by an experienced examiner. Liver disease severity was histologically assessed according to the NAFLD activity score (NAS) system as follows: degree of steatosis (grade 0 ≤ 5%; grade 1 = 5–33%; grade 2 = 34%–66%; grade 3 ≥ 66%), inflammation (0: no foci, 1 < 2 foci per 200x field, 2: 2 to 4 foci per 200x field, and 3: >4 foci per 200x field) and ballooning (0: none; 1: rare or few; 2: many). The NAS is the sum of these indices (Verbeek et al., 2015). The hepatic TG content was measured with the Triglyceride Colorimetric Assay Kit (10010303, Cayman Chemical, Ann Arbor, MI, United States) using a standard curve according to the manufacturer's instructions.

2.7 Statistical analysis

The normality of the data was assessed with the Shapiro-Wilk test. Data without a Gaussian distribution were analyzed using the non-parametric Kruskal-Wallis test, followed by Dunn's multiple comparison test; data with a Gaussian distribution were analyzed with an Ordinary One-way ANOVA test, followed by Šídák's multiple comparisons test. The following comparisons were performed: SPD vs. SPD + α5, SPD vs. HFHSD, and HFHSD vs. HFHSD + α5. Behavioral test parameters were analyzed using the Mann-Whitney test if they were non-normal, or the unpaired t-test if they were normally distributed. Data were expressed as mean ± SEM. A p-value <0.05 was considered statistically significant. Outliers were identified by the ROUT or Grubbs' methods. Statistical analyses and graphs were performed using Prism 9.0.0 (GraphPad, La Jolla, California, United States).

3 Results

3.1 Dietary α5 supplementation does not affect mice body weight and fat depots

We first characterized weight-related changes in male C57BL/6J mice during 17 weeks of nutritional interventions. Starting at week 2, C57BL/6J mice fed HFHSD showed a progressive, significant increase in body weight compared to SPD-fed mice (Figure 1B). Consistently, we observed a significantly higher weight gain (Figure 1C), supported by a higher daily average calorie intake, in the HFHSD-compared to the SPD-fed group (Supplementary Figure S1B). The weight increase in HSHSD-fed mice was associated with augmented depots of visceral and subcutaneous white adipose tissues (WAT), as measured by body weight-normalized epididymal and inguinal WAT weight (Figures 1E,F). These changes occurred with a slight, non-significant increase in liver weight relative to body weight in HFHSD-fed mice compared to the SPD group (Figure 1D). Administration of the α5 compound reduced food intake in SPD- but not in HFHSD-fed mice (Supplementary Figure S1A) with no changes in energy intake (Supplementary Figure S1B), and increased water intake both in SPD- and HFHSD-fed mice (Supplementary Figure S1C). Supplementation with α5 did not significantly affect body weight, body weight gain, or body weight-normalized organ weight in SPD- nor HFHSD-fed mice (Figures 1B–F).

3.2 Dietary α5 supplementation does not affect biomarkers of glycemic or lipid homeostasis

As expected (Verbeek et al., 2015), feeding mice with HFHSD induced insulin resistance, characterized by significantly increased glycemia and insulinemia, as well as a higher HOMA-IR index, compared to the SPD (Figures 2C–E). This finding was not accompanied by a worse response of HFHSD-fed mice to the GTT, measured at week 16 after a 7-h fast (Figures 2A,B). The 17-week HFHSD also altered lipid homeostasis, with significantly increased total cholesterol levels (Figure 2I), but no difference in TG levels (Figure 2H), as previously shown (Verbeek et al., 2015). Compared to mice fed SPD, mice fed HFHSD for 17 weeks exhibited hepatic damage, as indicated by strongly increased circulating ALT levels (Figure 2G) and an inflammatory status, characterized by elevated serum CRP and TNFα levels (Figures 2L,M).

Dietary supplementation with α5 elicited a non-significant improvement in glucose tolerance in SPD-fed mice (Figures 2A,B). The α5 compound did not alter the levels of the dosed biochemical analytes in either SPD-fed or HFHSD-fed mice (Figures 2C–I). A visible but non-significant trend towards reduced ALT and CRP levels was found in mice fed HFHSD when supplemented with α5 (Figures 2G-L).

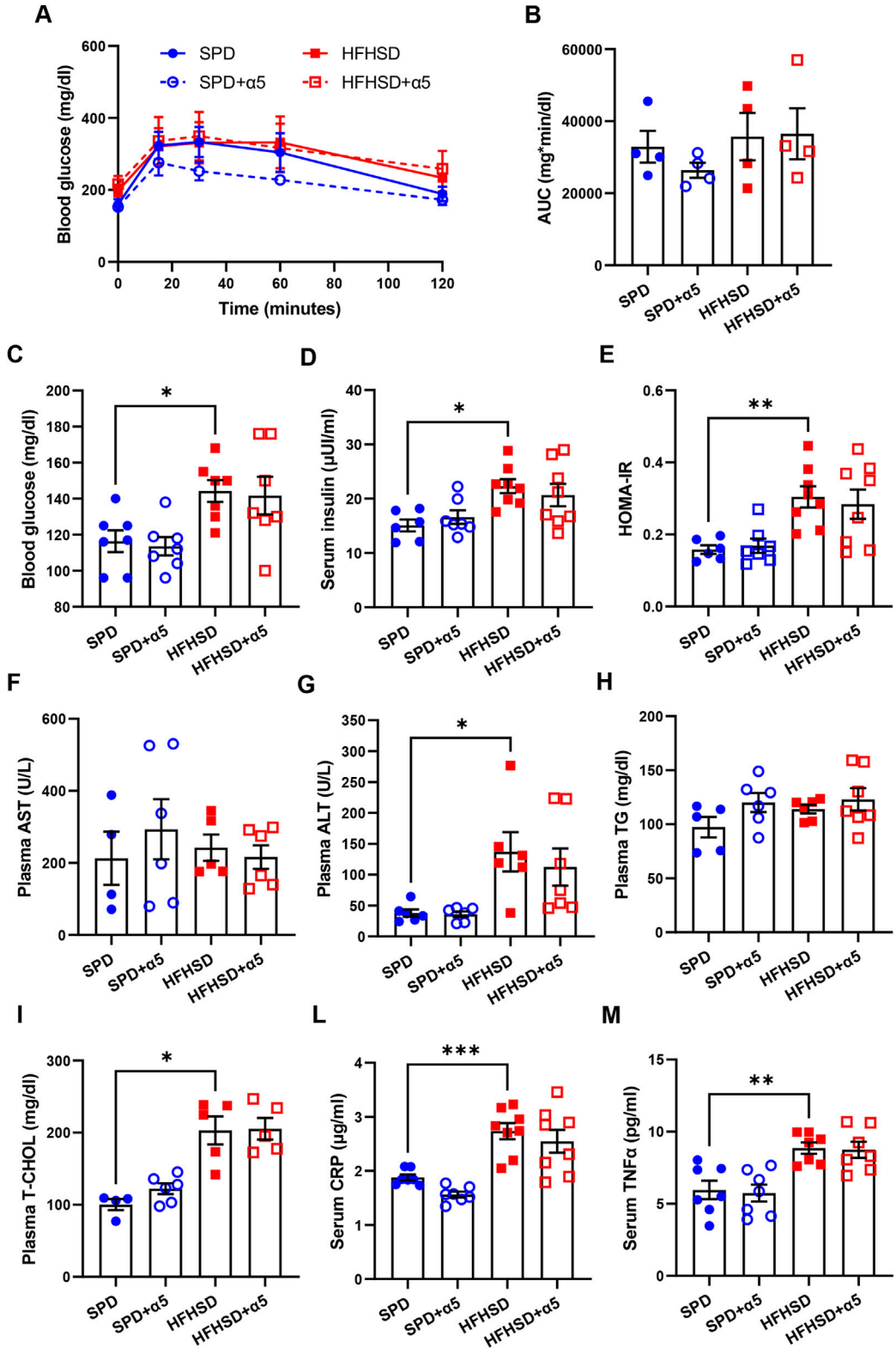

FIGURE 2
Assessment of blood biochemical analytes and inflammatory biomarkers. **(A,B)** Blood glucose after a glucose tolerance test; the panel on the right **(B)** shows the area under the curve (AUC). **(C,D)** Blood glucose and serum insulin levels; **(E)** homeostasis model assessment of insulin resistance (HOMA-IR) index; **(F–I)** plasma aspartate aminotransferase (AST), alanine transaminase (ALT), triglycerides (TG), total cholesterol (T-CHOL) levels; **(L,M)** serum C-reactive protein (CRP) and tumor necrosis factor-α (TNFα) levels. All analytes were measured after a 7-h fast. Data represent mean ± SEM (n = 4-8 mice/group; missing points are due to technical issues). Outliers were excluded by the ROUT method. Statistical analyses were performed using one-way ANOVA, followed by Šídák's multiple comparisons test **(B–H,L,M)** or the Kruskal-Wallis test, followed by Dunn's multiple comparison test **(G-I)** and. *p < 0.05, **p < 0.01 and ***p < 0.001.

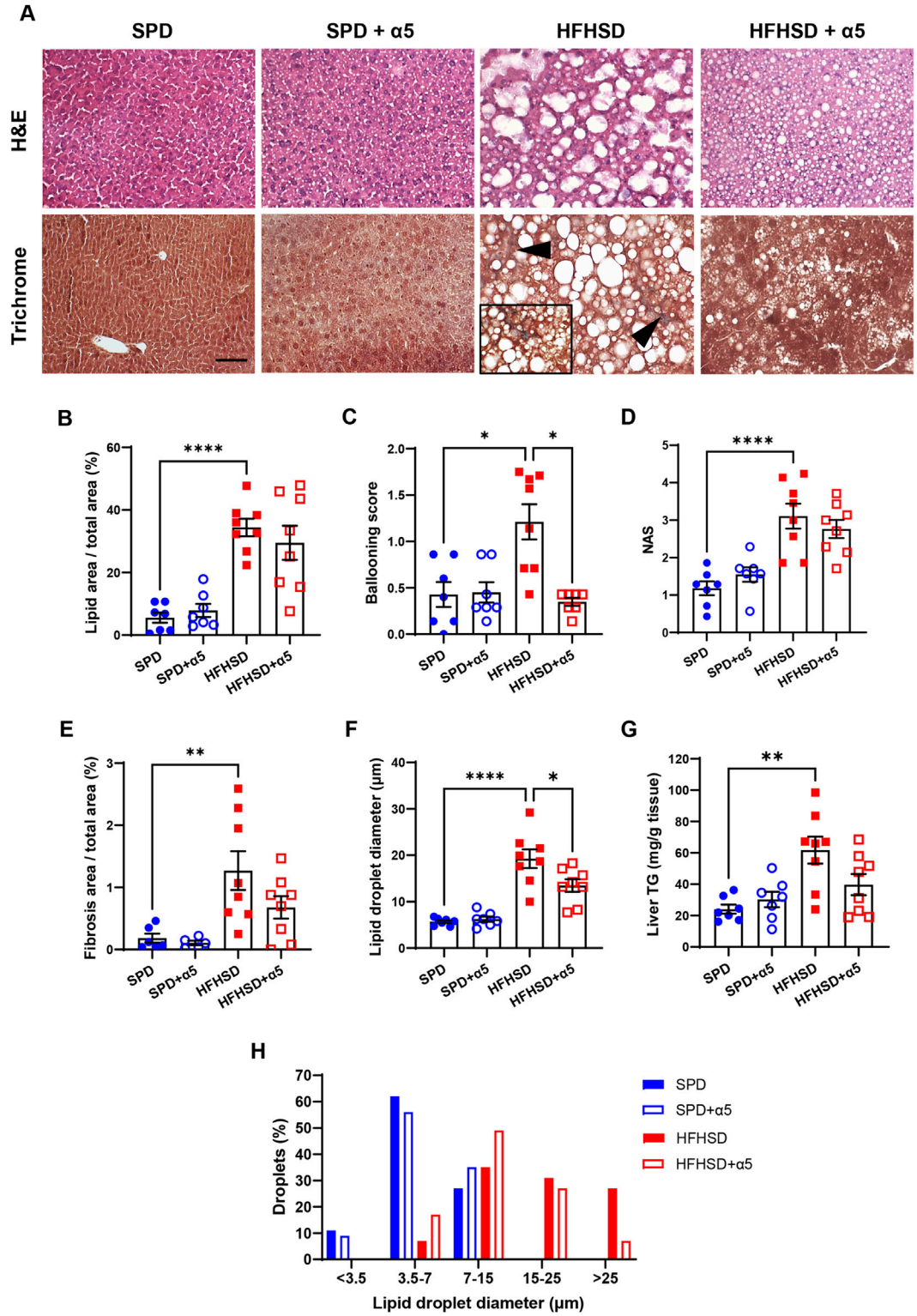

FIGURE 3
Liver histological analyses. **(A)** Representative photomicrographs of hematoxylin and eosin (H&E) or Masson's trichrome-stained specimens of mouse liver. Arrowheads and 40× frame denote areas of blue-colored collagen, indicating fibrosis. Bar, 50 μm. **(B–F)** Morphometric analysis parameters: percent steatosis measured as lipid area/total area; ballooning score; NAFLD activity score (NAS); percent fibrosis measured as collagen area/total area; lipid droplet diameter. **(G)** Frequency distribution of lipid droplet diameter classes. Data represent mean ± SEM (n = 5-8 mice/group). Outliers were excluded by the ROUT method. The Statistical analysis was performed using one-way ANOVA, followed by Šídák's multiple comparisons test **(B,D–F)** or the Kruskal-Wallis test, followed by Dunn's multiple comparison test **(C)**.*$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$.

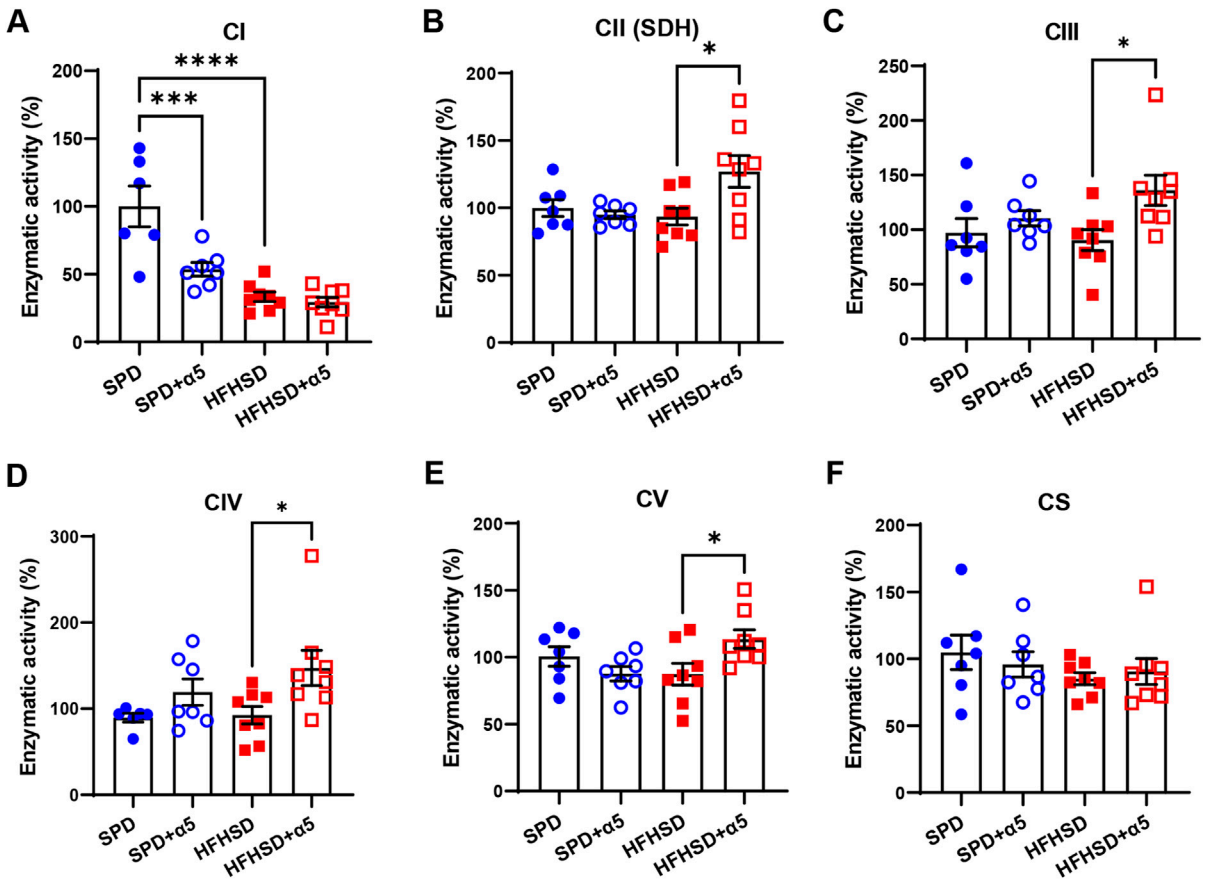

FIGURE 4
Assessment of liver mitochondrial function. **(A–F)** Enzymatic activities of the mitochondrial respiratory chain complex I (CI), II (CII), III (CIII), IV (CIV), and citrate synthase (CS). Data represent mean ± SEM (n = 6-8 mice/group). One outlier was excluded by the ROUT method. The statistical analysis was performed using one-way ANOVA, followed by Šídák's multiple comparisons test **(A–C,E)** or the Kruskal-Wallis test, followed by Dunn's multiple comparison test **(D-F)**. *p < 0.05, ***p < 0.001 and ****p < 0.0001.

3.3 Dietary α5 supplementation attenuates HFHSD-induced fatty liver disease

Liver histology was performed to assess parenchymal architecture and associated pathological changes. Representative images are presented in Figure 3A. Mice fed SPD displayed occasional hepatocyte ballooning but no signs of steatosis, inflammatory infiltration, or fibrosis. In contrast, after 17 weeks on HFHSD, mice developed marked hepatic steatosis and pronounced hepatocyte ballooning (swollen hepatocytes containing large lipid droplets and peripherally displaced nuclei) along with sporadic lobular inflammation and mild fibrosis. While α5 supplementation did not alter hepatic histology in SPD-fed mice, it improved liver morphology in HFHSD-fed mice, which exhibited fewer and smaller lipid droplets, as well as decreased steatosis and ballooning. Morphometric analysis confirmed significant increases in lipid area, hepatocyte ballooning, fibrosis area, and histological score of fatty liver disease severity (NAS) in HFHSD-fed mice compared to the SPD-fed ones (Figures 3B–E). The diameter of hepatic lipid droplets was also significantly greater in the HFHSD group (Figure 3F), with 26% of droplets displaying a diameter >25 µm (Figure 3H). These results were accompanied by a significant increase in the hepatic amount of TG in HFHSD-fed mice (Figure 3G). The α5 supplementation showed only a trend towards improvement of some parameters (Figures 3B,D,E), including a non-significant but consistent amelioration of liver TG content ($p = 0.0581$ vs HFHSD) (Figure 3G). Notably, α5 supplementation was significantly effective in reducing both hepatocyte ballooning (Figure 3C) and lipid droplet diameter (Figure 3F). The treatment increased the percentage of droplets with a diameter between 3.5 and 15 µm and reduced that of droplets with a diameter ≥25 µm (Figure 3H). Collectively, these findings indicate that α5 acts as a metabolic modulator capable of ameliorating diet-induced MASLD in mice.

3.4 Treatment with α5 supports hepatic mitochondrial activity in HFHSD-fed mice

Analysis of the enzyme activity of mitochondrial respiratory chain complexes in liver showed a strongly reduced CI activity in HFHSD-fed compared to SPD-fed mice (Figure 4A), as previously described (Verbeek et al., 2015). The activity of the other liver respiratory chain complexes remained unchanged after a 17-week

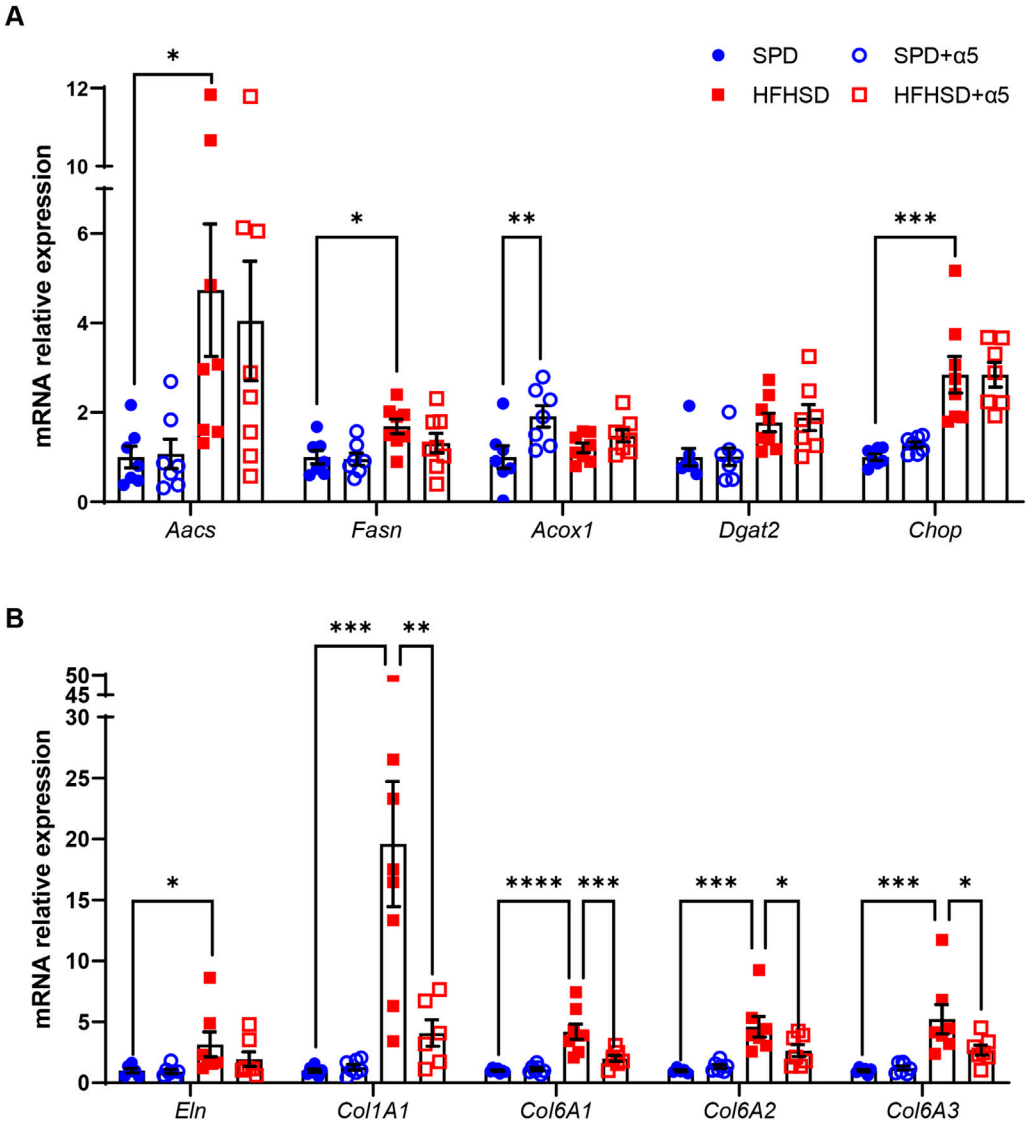

FIGURE 5
Relative mRNA expression in mouse liver. Reverse transcription-qPCR data of **(A)** Acetoacetyl-CoA Synthetase (*Aacs*), Fatty acid synthase (*Fasn*), Acyl-CoA oxidase 1 (*Acox1*), Diacylglycerol O-acyltransferase 2 (*Dgat2),* and C/EBP homologous protein (*Chop*); **(B)** Elastin (*Eln*), Collagen type I alpha 1 (*Col1A1*), Collagen type VI alpha 1 (*Col6A1*), Collagen type VI alpha 2 (*Col6A2*), and Collagen type VI alpha 3 (*Col6A3*). Data were normalized to *Hprt* expression. Values are reported as relative expression compared to the SPD group, taken as 1. Data represent mean ± SEM (n = 5-8 mice/group). Outliers were excluded by the Grubbs' method. The statistical analysis was performed by the Kruskal-Wallis test, followed by Dunn's multiple comparison test *(Aacs, Dgat2, Chop, Eln)*) or one-way ANOVA, followed by Šídák's multiple comparisons test (all other mRNAs). *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

HFHSD under our conditions. Unexpectedly, we observed reduced hepatic CI activity in SPD-α5-treated mice when compared to SPD mice, without changes in the HFHSD-α5 vs the HFHSD group (Figure 4A). Treatment with α5 did not affect other MRC complexes in SPD-fed mice (Figures 4B–E). Of interest, the α5 supplement significantly increased the CII, CIII, CIV, and CV activities in livers from HFHSD-fed mice (Figures 4B-E). The enzymatic activity of citrate synthase was not modified by any treatment (Figure 4F), suggesting unchanged mitochondrial mass. Accordingly, neither HFHSD nor α5 supplementation altered the mRNA levels of peroxisome proliferator-activated receptor γ coactivator 1α (*PGC-1α*) or mtDNA content in the liver (Supplementary Figure S2).

3.5 Dietary α5 supplementation reverses the HFHSD-mediated increase in fibrosis markers

We investigated the changes in gene expression that may be altered in diet-induced MASLD. The mRNA levels of diacylglycerol O-acyltransferase 2 (*Dgat2*), a key player in liver TG synthesis, appeared slightly but non-significantly augmented by HFHSD. Still, those of two key enzymes involved in *de novo* lipogenesis, the acetoacetyl-CoA synthetase (*Aacs*) and the fatty acid synthase (*Fasn*), were significantly increased in the liver from HFHSD-fed compared to the SPD-fed group (Figure 5A). In addition, increased mRNA levels of the endoplasmic reticulum (ER) stress marker

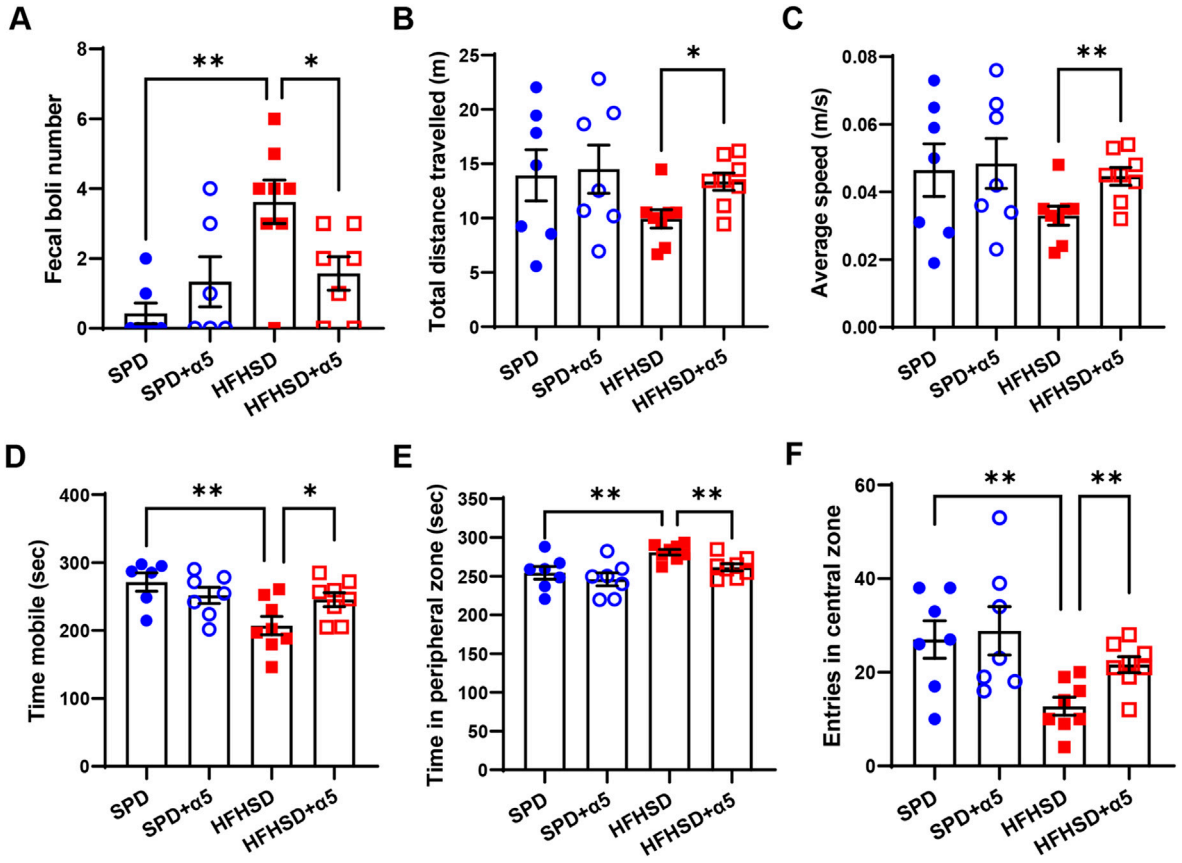

FIGURE 6
Characterization of the anxiety behavior in HFHSD-fed mice with and without α5 supplementation. **(A)** Fecal boli number count in the arena at the end of the test; **(B–F)** Open field test parameters. Data represent mean ± SEM (n = 6-8 mice/group). One outlier was excluded by the ROUT method. The unpaired t-test (B–F) and the Mann-Whitney test **(A)** were used for statistical analysis. *p < 0.05 and **p < 0.01.

C/EBP homologous protein (*Chop*) were observed in the HFHSD mice compared to the SPD group (Figure 5A). Supplementation with α5 did not modify these lipogenesis or ER stress markers in HFHSD-fed mice (Figure 5A). While the expression of the acyl-CoA oxidase 1 (*Acox1*), which promotes peroxisomal β-oxidation and catabolism of very long-chain fatty acids (VLCFAs), was not affected by HFHSD, α5 administration *per se* induced a significant increase in *Acox1* mRNA levels in mice fed with SPD (Figure 5A). Livers from HFHSD-fed mice displayed augmented expression of genes involved in fibrogenic pathways. Elastin (*Eln*) mRNA levels were increased, with a trend toward reduction in mice treated with α5. Further, collagen type I alpha 1 (*Col1A1*) mRNA was induced 15-fold, and *Col6A1*, *Col6A2*, and *Col6A3* mRNAs were significantly increased in the HFHSD group (Figure 5B). Notably, dietary supplementation with α5 almost completely reversed the increase of all the collagen types investigated (Figure 5B).

3.6 The anxious behavior of MASLD mice is prevented by α5 supplementation

We assessed the behavioral performance of mice at the 16th experimental week. The open field test was adopted to evaluate anxiety status as thigmotaxis (i.e., the tendency to remain in a protected area close to the arena walls) and defecation level (Bourin et al., 2007). Representative track plots of mice movements in the arena are shown in Supplementary Figure S3. We observed an increase in the number of fecal boli (Figure 6A) and a corresponding increase in time spent in the peripheral zone of the arena, accompanied by a significant reduction in time spent mobile and the number of entries in the central zone in HFHSD-fed mice compared to SPD-fed mice (Figures 6D–F). Although not statistically significant, a downward trend in total distance traveled and average speed of movement was also observed (Figures 6B,C). Together, these data demonstrate that HFHSD-fed mice experience anxiety symptoms. When compared to unsupplemented HFHSD mice, α5-treated HFHSD mice exhibited significantly increased total distance travelled, average speed, mobility time, and number of entries in the central zone, as well as reduced number of fecal boli and time spent in the peripheral zone. Thus, our results support the efficacy of α5 in rescuing anxious symptoms in a fatty liver mouse model obtained by HFHSD feeding.

4 Discussion

Despite various promising preclinical and clinical studies, MASLD remains an unmet medical need. Weight loss achieved

through diet and lifestyle modifications is the primary approach for patients in the early stage of the disease, i.e., those presenting signs of liver steatosis (MASL) without evidence of fibrosis. However, early treatment is of paramount importance in reducing liver fat deposition and preventing disease progression to advanced stages with severe fibrosis (MASH). Hence, novel therapeutic strategies are needed. In the present work, we investigated the efficacy of a nutritional intervention based on the α5 metabolic modulator in a mouse model of diet-induced MASLD.

After 17 weeks on the HFHSD, mice developed obesity, with increased weight of subcutaneous and visceral fat depots, and insulin resistance (as indicated by an increased HOMA-IR index). Their unchanged glucose tolerance could be related to the smaller group size in the GTT experiment. Mice under HFHSD also exhibited hypercholesterolemia, without differences in triglyceridemia. Though we used a different reference diet (i.e., SPD instead of chow diet) and introduced minor modifications in the experimental plan, these results are consistent with those reported with the same Western-type diet by Verbeek and collaborators (Verbeek et al., 2015). The systemic metabolic effects induced by HFHSD were accompanied by increased plasma ALT (an indicator of liver damage), augmented hepatic TG content, liver histological abnormalities (steatosis, hepatocyte ballooning, and fibrosis), and a higher histological score for the severity of fatty liver disease (NAS), which recapitulated the advanced stages of MASLD. Reduced CI enzymatic activity completed the picture. Notably, mice on the HFHSD also exhibited behavioral alterations, characterized by decreased exploratory activity in the open field test. Clear signs of an anxiety state (Bourin et al., 2007) included augmented defecation episodes during the test, spending more time moving in the periphery of the arena, and entering the center area fewer times. Thus, this Western diet model is well-suited to investigating the effects of novel therapeutic approaches to liver pathology and coexisting anxiety symptoms.

Our data demonstrate that dietary supplementation with α5 is efficacious against HFHSD-related MASLD, with no effect on energy intake or body weight. The intervention did not alter glycemic or lipidic homeostasis, nor modify any biochemical analyte in either SPD or HFHSD conditions, confirming its safety on chronic administration in mice. In HFHSD-fed mice supplemented with α5, we observed only a trend toward a reduction in plasma ALT levels, liver TG content, and some liver morphometric parameters (lipid area, fibrosis area, NAS). The failure to achieve significance could likely be attributed to the limited sample size or the high variability of the unsupplemented or α5-supplemented HFHSD groups for these parameters.

The unexpected reduction in liver CI activity observed in α5-treated mice under SPD conditions (but not under HFHSD) currently lacks a biological explanation. Importantly, the α5 supplement did not affect the activity of other MRC complexes in SPD-fed animals. Previous studies reported reduced CI and CIV activity in HFHSD-fed mice compared with chow-fed controls, which is partly consistent with our observations (Verbeek et al., 2015). As cellular respiration relies on coordinated MRC activity, selective changes in individual complexes should be viewed in this integrated context. Despite some conflicting results, evidence from both rodent and human studies suggests that mitochondrial alterations evolve during MASLD progression, with early adaptive adjustments in hepatic energy metabolism giving way to later-stage loss of metabolic flexibility (Fromenty and Roden, 2023). This dynamic complexity, compounded by variability in experimental models and conditions, makes the interpretation of liver MRC activity in diet-induced MASLD particularly challenging. In our study, no changes in markers of mitochondrial biogenesis (PGC-1α expression) or mass (citrate synthase activity, mtDNA content) were detected across the four experimental groups at the 17-week time point. Nevertheless, α5-supplementation was associated with significant increases in CII, CIII, CIV, and CV activity under HFHSD conditions, suggesting a diet-dependent effect. Further mechanistic studies will be required to elucidate the pathways underlying this α5-mediated modulation and its role in the progression of MASLD.

Except for increased Acox1 expression under SPD conditions, which may favor β-oxidation and catabolism of VLCFA, the compound did not affect the mRNA levels of genes involved in lipogenesis under HFHSD. Remarkably, dietary α5 supplementation significantly counteracted hepatocyte ballooning, an indicator of cellular damage and a prognostic factor for a greater risk of fibrosis progression (Singla et al., 2023). Further, α5 significantly reduced the number and diameter of lipid droplets in the liver from HFHSD-fed mice. Formerly considered inert markers of liver disease, lipid droplets indeed play active roles in MASLD pathophysiology, and their size correlates with the degree of fibrosis (Reid et al., 2024). Consistently, α5 treatment almost completely reversed the HFHSD-mediated increase in collagen transcripts, thereby strengthening the possibility of its favorable effect against hepatic fibrosis. Assays of fibrosis markers at the protein level may confirm these findings. Since the increased number and size of lipid droplets are associated with disease progression to steatohepatitis, strategies to modulate their biogenesis and growth are being proposed for their promising therapeutic potential (Bilson and Scorletti, 2024). Thus, α5 seems to act at one of the core mechanisms through which MASLD occurs and progresses (Figure 7). While further investigation should clarify α5-mediated mechanisms regulating lipid droplet size (including DGAT1, autophagy, or ER stress), it is worth noting that mitochondrial dysfunction is a key driver of disrupted lipid metabolism and fibrogenesis, which influence each other's progression in MASLD.

Supplementation with BCAAs in liver disease has raised considerable interest, as well as controversy, due to the heterogeneity of the studies (Zhang et al., 2024). Previous work from our research group demonstrated that a specific EAA mixture enriched in BCAAs (BCAAem), unlike one based on the amino acid profile of casein, prevented liver steatosis, mitochondrial impairment, and oxidative stress in a rodent model of alcoholic liver disease (Tedesco et al., 2018). Liver protection was likely attributable to the BCAAem-mediated preservation of the mammalian/mechanistic inhibitor of rapamycin 1 (mTORC1) pathway, as found in ethanol-exposed HepG2 cells (Tedesco et al., 2018). Recently, other authors reported the multifaceted beneficial effects of metabolic modulators containing a specific combination of five amino acids in human cell model systems that mimic the NASH phenotype (Daou et al., 2021). The latter formula was well

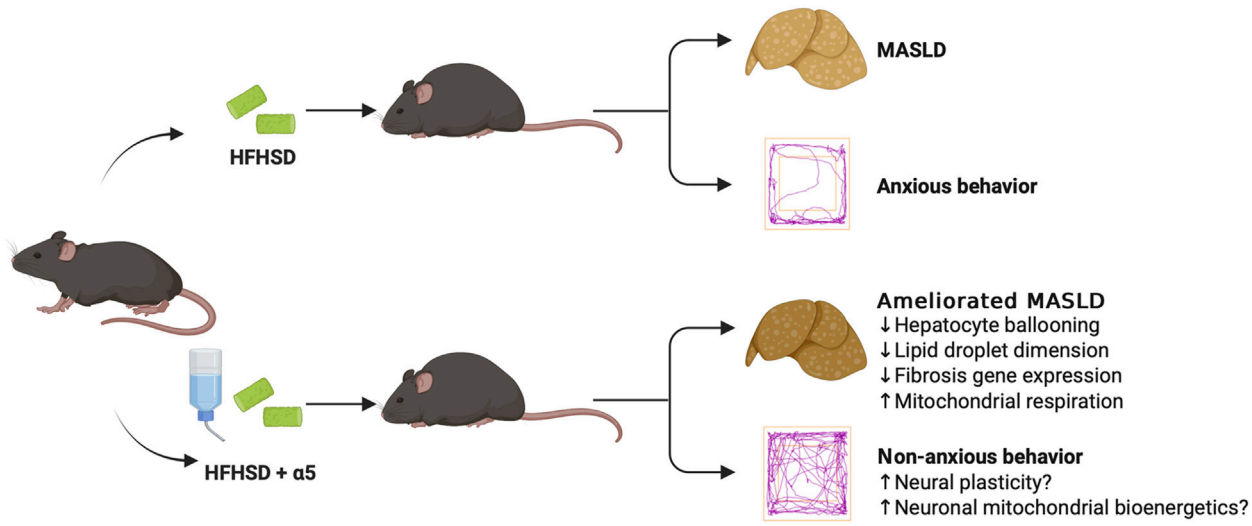

FIGURE 7
Conceptual schematic of the proposed effects of α5 supplementation. The diagram illustrates multiple mechanisms through which α5 may improve liver pathology in HFHSD-fed mice, independent of body weight. In parallel, α5 may modulate systemic and brain pathways, contributing to reduced anxiety-like behavior. Together, these actions support an integrated model in which α5 exerts protective effects across metabolic and neurobehavioral domains. Created with BioRender.com.

tolerated and decreased liver fat content in MASLD patients with or without type 2 diabetes (Harrison et al., 2021), encouraging further research in this field.

An additional relevant finding of the present study is the efficacy of dietary α5 supplementation in reducing MASLD-related anxiety, as revealed by the complete recovery of specific exploratory behaviors altered by HFHSD. We can only speculate about the potential mechanism(s) underlying the α5 psychotropic effect (Figure 7). We previously found that this compound promoted the full differentiation of murine and human neural stem cells into neuronal phenotypes, characterized by increased dendritic arborization and maturation of dendritic spines (Bifari et al., 2020). Enhanced mitochondrial energy metabolism, mediated by the activation of the mTORC1 and its downstream target p70 S6 kinase 1 (S6K1), appeared to be involved in these α5-mediated phenomena in newborn neurons (Bifari et al., 2020). Altered dendritic branching and spine density are observed in chronic stress and anxiety (McEwen et al., 2012), while neural plasticity is the target of conventional and emerging interventions to treat depression and anxiety disorders (Scangos et al., 2023). Interestingly, mice with genetic deletion of S6K1 displayed a robust anxiety-like behavior associated with reduced adult hippocampal neurogenesis (Koehl et al., 2021). The antioxidant capacity of α5 (Bifari et al., 2020; Dolci et al., 2022) could also contribute to its antianxiety effect. Finally, we cannot exclude the involvement of the gut-brain axis. Gut dysbiosis has been considered among the processes shared by MASLD and mental health disturbances (Shea et al., 2024). Therefore, modulation of gut microbiota, as observed with other EAA-based interventions (Ruocco et al., 2020) could have a role in both conditions.

This study has certain limitations. First, the small number of animals may have contributed to the lack of statistically significant changes in some parameters. Second, the α5-mediated mechanisms in MASLD improvement are not substantiated enough. Third, we cannot exclude the possibility that the observed mood alterations resulted from diet-induced obesity rather than being directly attributable to MASLD. Fourth, additional studies will be necessary to establish more precise correlations between HFHSD-induced liver disease and anxiety.

Our primary objective was to evaluate the efficacy of dietary α5 supplementation in ameliorating MASLD. While we also aimed to assess whether mice fed a Western diet would develop anxiety-like behaviors and whether EAA supplementation could mitigate these outcomes, the pronounced effect of the metabolic modulator on the anxiety phenotype was unexpected. This finding warrants further investigation to elucidate the underlying psychotropic mechanisms and to explore the potential efficacy of α5 supplementation in other models of mood disorders.

In conclusion, our data demonstrate that dietary supplementation with the EAA-based α5 designer formula, enriched with TCA cycle intermediates and cofactors, effectively counteracts the development of Western diet-induced MASLD in mice. Concurrently, α5 supplementation substantially reduced the anxiety behavior in this model. Notably, these beneficial effects occurred independently of changes in body weight or adiposity, suggesting that they are mediated by direct mechanisms activated by the specific combination of EAAs and metabolic enhancers. Further studies will be essential to fully characterize these mechanisms and to assess the translational potential of α5 supplementation in the management of metabolic and mood disorders.

Data availability statement

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

Ethics statement

The animal study was approved by General Direction of Animal Health and Veterinary Drugs of the Italian Ministry of Health (authorization n. 498/2018-PR). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AS: Data curation, Formal Analysis, Investigation, Writing – original draft. GF: Investigation, Methodology, Writing – original draft. EB: Investigation, Methodology, Writing – review and editing. AiV: Investigation, Visualization, Writing – review and editing. EG: Investigation, Writing – review and editing. EP: Formal Analysis, Writing – review and editing. CR: Investigation, Writing – review and editing. MR: Investigation, Writing – review and editing. RR: Funding acquisition, Supervision, Writing – review and editing. EN: Conceptualization, Funding acquisition, Writing – review and editing. AeV: Conceptualization, Funding acquisition, Supervision, Writing – original draft.

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Conflict of interest

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Targeting emerging amino acid dependencies and transporters in cancer therapy

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Amino acid metabolism is an important vulnerability in cancer. Established strategies such as arginine depletion, glutaminase inhibition, tryptophan-kynurenine modulation, and methionine restriction have shown that these pathways can be targeted in patients. At the same time, clinical trials reveal two consistent challenges: tumors can adapt by redirecting their metabolism, and reliable biomarkers are needed to identify patients who are most likely to benefit. Recent studies point to additional amino acids with translational potential. In pancreatic cancer, histidine and isoleucine supplementation has been shown in preclinical models to be selectively cytotoxic to tumor cells while sparing normal counterparts. In glioblastoma, threonine codon-biased protein synthesis programs that support growth; in other contexts, lysine breakdown suppresses interferon signaling through changes in chromatin structure; and alanine released from stromal cells sustains mitochondrial metabolism and therapy resistance. These dependencies are closely tied to amino acid transporters, which act as both nutrient entry points and measurable biomarkers. In this review, we summarize current evidence on histidine, isoleucine, threonine, lysine, and alanine as emerging metabolic targets, and discuss opportunities and challenges for clinical translation, with emphasis on transporter biology, biomarker development, and therapeutic combinations.

KEYWORDS

amino acid metabolism, cancer therapy, nutritional intervention, dietary modulation, metabolic targeting, amino acid transporters, glutaminase inhibition, SLC transporters

1 Introduction

Amino acids play a central role in cancer metabolism, serving as building blocks for protein synthesis and regulators of signaling, redox balance, and immune function (Liu et al., 2024). They are often classified as essential, non-essential, or conditionally essential, and further grouped by structural or metabolic features such as branched-chain amino acids (BCAAs: leucine, isoleucine, valine), aromatic amino acids (tryptophan, phenylalanine, tyrosine), and sulfur-containing amino acids (methionine, cysteine). These categories are associated with distinct biological roles in cancer research. For example, BCAAs activate mechanistic target of rapamycin complex 1 (mTORC1), a growth pathway that senses nutrient availability (Saxton and Sabatini, 2017). Serine, glycine, and methionine feed one-carbon (1C) metabolism, which provides nucleotides for DNA synthesis and supports epigenetic regulation (Ducker and Rabinowitz, 2017), while arginine and tryptophan

regulate immune responses via T-cell activity and kynurenine–aryl hydrocarbon receptor (AhR) signaling (Carpentier et al., 2024; Ghorani et al., 2023).

Disruptions in amino acid pathways are a hallmark of cancer. Cancer cells reprogram amino acid uptake and utilization to sustain biomass accumulation, maintain antioxidant defenses, and promote growth signaling (Saxton and Sabatini, 2017). These adaptations create differences in tumor and normal tissues that can be therapeutically exploited. Clinical studies have already established proof-of-concept. For example, arginine depletion has shown activity in tumors deficient in argininosuccinate synthase 1 (ASS1) (Chan et al., 2021; Chan et al., 2022; Szlosarek et al., 2021), the enzyme that enables cells to synthesize arginine *de novo*. Glutaminase inhibitors, which block the enzyme responsible for converting glutamine into glutamate and feeding the tricarboxylic acid (TCA) cycle, have been evaluated in glutamine-dependent cancers, both as single drug therapy and in rational drug combinations (Patel et al., 2024; Wicker et al., 2021; DiNardo et al., 2024). Similarly, inhibition of tryptophan catabolism through indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO), enzymes that degrade tryptophan to kynurenine, has been explored to restore antitumor immunity (Wu et al., 2023; Zakharia et al., 2021). These studies establish the feasibility of targeting amino acid metabolism in patients and demonstrate two recurring challenges: tumors can rewire their metabolism in ways that allow them to escape treatment, and the lack of reliable biomarkers makes it difficult to identify which patients are most likely to benefit from treatment (Akinlalu et al., 2025; Balamurugan et al., 2025; Rasuleva et al., 2023; Rasuleva et al., 2021). Recent advances have begun to address these limitations, with ASS1 loss used as a biomarker for arginine deprivation (Carpentier et al., 2024), kynurenine-to-tryptophan ratios to monitor IDO/TDO inhibition (Wu et al., 2023; Huang et al., 2022), and circulating metabolites or amino acid-based positron emission tomography (PET) tracers to confirm drug activity (Galldiks et al., 2023). These developments represent important steps toward more precise, biomarker-guided use of amino acid therapies.

In parallel, new amino acid targets have come into focus. In pancreatic cancer, histidine and isoleucine supplementation are selectively cytotoxic to cancer cells while sparing non-malignant counterparts (Akinlalu et al., 2024). In glioblastoma (GBM), threonine drives tumor growth by fueling codon-biased protein synthesis through transfer RNA (tRNA) modifications mediated by the enzyme YRDC (Wu et al., 2024). Lysine catabolism promotes immune evasion by altering histone modifications (crotonylation) that suppress interferon signaling (Yuan et al., 2023). Alanine released by stromal fibroblasts fuels mitochondrial metabolism in cancer cells, enabling survival and resistance to therapy (Zhu et al., 2023; Gauthier-Coles et al., 2022).

Importantly, these emerging amino acid targets are linked to their associated transporters that act as nutrient gateways and functional biomarkers. The L-type amino acid transporter 1 (LAT1; gene name *SLC7A5*) mediates uptake of histidine, isoleucine, and threonine (Bo et al., 2021). The cationic amino acid transporter 1 (CAT1; gene name *SLC7A1*) transports lysine and contributes to immune regulation (You et al., 2022). The sodium-coupled neutral amino acid transporter 2 (SNAT2; gene name *SLC38A2*) facilitates the uptake of alanine (Gauthier-Coles et al., 2022). Advances in transporter inhibitors (Bo et al., 2021; Lyu et al., 2023), metabolic imaging using amino acid PET tracers (Galldiks et al., 2023; Jakobsen et al., 2023), and studies linking transporter activity to immune regulation (Bo et al., 2021; Guo et al., 2023) underscore their central role in clinical translation.

Here, we focus on histidine, isoleucine, threonine, lysine, and alanine as emerging metabolic vulnerabilities in cancer. We emphasize their mechanism, associated transporters, and translational relevance. Drawing on lessons from established amino acid interventions, we outline the opportunities and challenges that will shape the clinical development of these emerging strategies (Figure 1; Table 1).

2 Emerging amino acids in cancer therapy

2.1 Histidine

Histidine is an essential amino acid with unique chemical properties. Its imidazole side chain can buffer protons and bind metals, giving histidine a central role in pH balance and metal-dependent enzymatic reactions (Kessler and Raja, 2023). Historically, histidine has not been considered a major driver of cancer metabolism. However, recent work in pancreatic ductal adenocarcinoma (PDAC) has highlighted histidine as a potential therapeutic target.

Our recent study (Akinlalu et al., 2024) demonstrated that histidine combined with isoleucine induces selective cytotoxicity in PDAC. Across *in vitro* cell culture models and *in vivo* nude mice xenografts, supplementation with these two amino acids reduced PDAC cell viability while sparing non-malignant counterparts. This selectivity suggests an intrinsic sensitivity to histidine-isoleucine overload, likely linked to altered amino acid handling by tumor cells. The study also pointed to the mRNA exonuclease XRN1 as a possible mechanism: when histidine and isoleucine accumulated, RNA processing was perturbed, adding to cellular stress. Rather than starving tumors of nutrients, this approach overloads them with amino acids they cannot manage, leading to metabolic crisis and cell death.

A complementary study (Kumar et al., 2023) independently showed that histidine supplementation disrupts tumor metabolic balance through a different mechanism. In PDAC models, histidine overload depleted amino acids required for glutathione synthesis, leading to loss of redox homeostasis, accumulation of hydrogen peroxide, and oxidative stress. This sensitized tumors to gemcitabine; exogenous glutathione rescued the effect, confirming the oxidant-antioxidant mechanism. These data suggest two converging mechanisms in PDAC: interference with RNA processing when histidine is paired with isoleucine, and induction of oxidative stress when histidine is used alone. Both selectively stress PDAC cells over normal tissues. While these preclinical findings are compelling, their current limitation lies in model specificity, as most evidence arises from xenograft and cell-line systems. Independent confirmation in patient-derived models and pharmacokinetic studies will be necessary to determine whether histidine modulation is feasible and safe in clinical settings.

Translationally, histidine could be explored as an adjuvant to chemotherapy, as a co-supplement with isoleucine to induce direct cytotoxicity, or in combination with therapies that generate

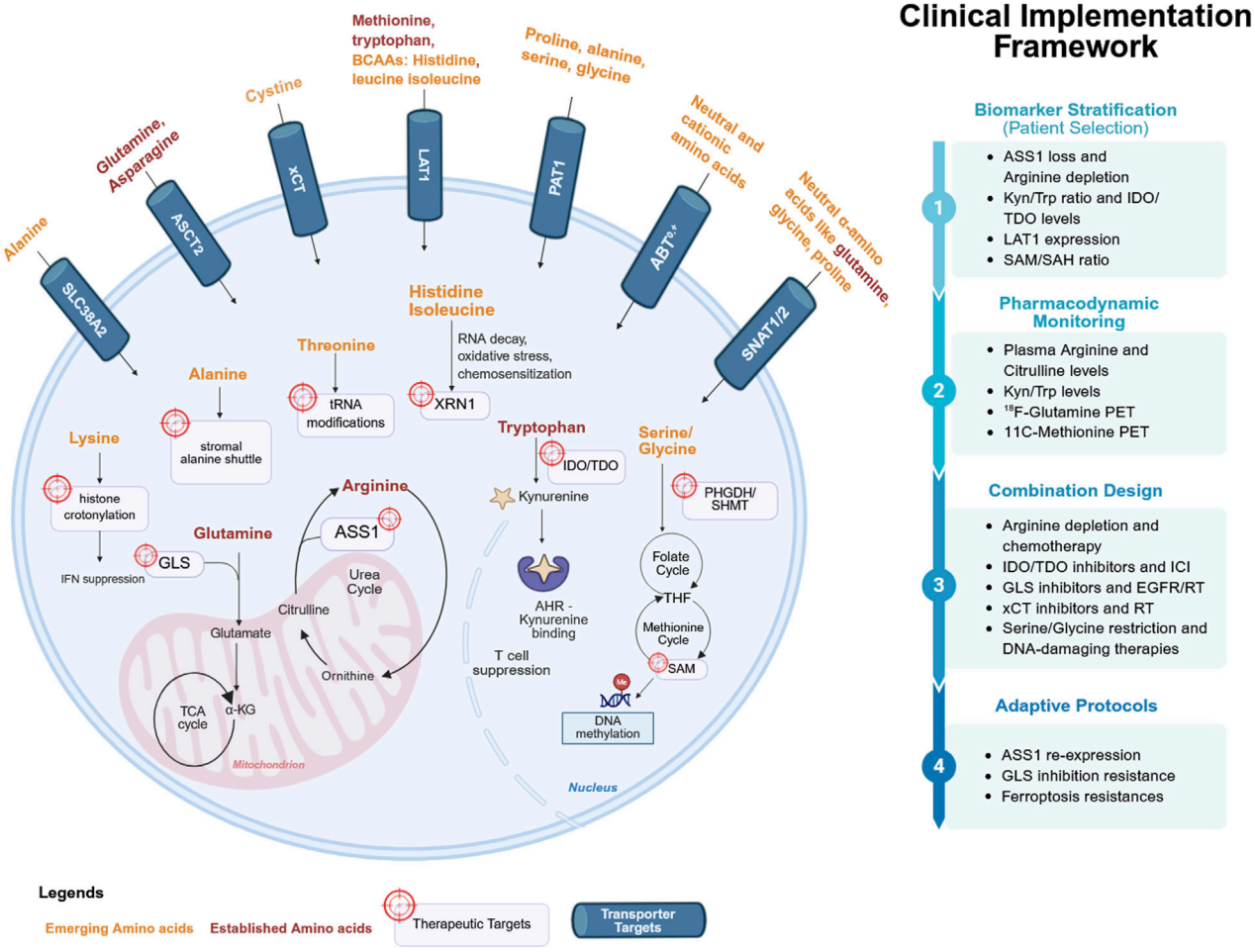

FIGURE 1
Amino acid metabolism and transporters as therapeutic targets in cancer. The cellular pathway illustrates the tumor cell with established dependencies (arginine, glutamine, tryptophan, methionine, asparagine; red) and emerging amino acids (histidine, proline, serine–glycine, aspartate, branched-chain amino acids, threonine, lysine, alanine; orange). Transporters (LAT1, ASCT2, xCT, SLC6A14, SNAT1/2, PAT1; blue) are localized at the plasma membrane. Arrows represent nutrient influx and metabolic pathways, with icons denoting therapeutic interventions (enzyme depletion, pharmacologic inhibitors, dietary restriction, transporter blockade). The clinical implementation framework emphasizes biomarker stratification, pharmacodynamic monitoring, combination design, and adaptive protocols, reflecting how amino acid biology is translated into precision oncology. Figure was created in Biorender. Akinlalu, A. (2025).

oxidative stress. Recent studies show that PDAC tumors exhibit abnormal histidine uptake and catabolism mediated by histidine ammonia-lyase (HAL), which may contribute to reduced circulating histidine levels and increased oxidative stress (Kumar et al., 2023; Wu et al., 2025; McDonnell et al., 2025). Although stromal barriers in PDAC may limit direct correspondence between plasma and intratumoral metabolites, these observations suggest that HAL expression or activity within the tumor, and possibly systemic histidine availability, may serve as exploratory biomarkers for identifying patients most likely to benefit from histidine-based interventions.

2.2 Isoleucine

Isoleucine is one of the BCAAs, along with leucine and valine. BCAAs support protein synthesis, activate mTORC1, and influence immune function (Dimou et al., 2022). In PDAC cells, proteomic analysis of extracellular vesicles (EVs) suggests that tumor cells actively dispose of isoleucine and histidine, consistent with intracellular accumulation being stressful (Akinlalu et al., 2024). Re-supplementation of isoleucine, alone or with histidine, overwhelmed PDAC cells and triggered necrotic death, while non-malignant cells tolerated supplementation (Akinlalu et al., 2024).

Clinically, isoleucine supplementation may be feasible because BCAA-enriched nutrition is used in perioperative and supportive care for cancer patients. Meta-analyses and randomized trials in gastrointestinal cancers report improved nitrogen balance, fewer infections, and better postoperative recovery with BCAAs (Matsui et al., 2024; Ma et al., 2024). This safety profile suggests that controlled isoleucine supplementation could be integrated into therapeutic regimens. However, dosing will require careful monitoring to avoid systemic imbalance. While short pulses of

TABLE 1 Established and emerging amino acid dependencies and transporter targets in cancer. The table summarizes mechanisms, transporters, biomarkers, strategies, and evidence tiers for the four established pathways (arginine, glutamine, tryptophan, methionine) and five emerging amino acid cancer therapy, histidine, isoleucine, threonine, lysine and alanine. Transporters central to these targets are shown as both therapeutic entry points and functional biomarkers.

Target/ Transporter	Therapeutic strategy	Cancer type(s)	Mechanism of action	Biomarker(s)	Clinical stage	References
Arginine/ASS1	Pegargiminase (ADI-PEG 20), rhArg, combinations with chemotherapy and ICI	Mesothelioma, HCC, NSCLC, Sarcoma	Arginine depletion; mTORC1 and polyamine suppression	ASS1 loss (IHC, methylation), plasma arginine/citrulline	Phase II/III	Carpentier et al. (2024), Chan et al. (2021), Chan et al. (2022), Szlosarek et al. (2021), Szlosarek et al. (2024), Rogers et al. (2023), Strømland et al. (2025)
Tryptophan/IDO1-TDO2-AHR	IDO1/TDO2 inhibitors, AHR antagonists, and PD-1 blockade	Melanoma, NSCLC, Colon	Kynurenine accumulation, AHR-mediated immunosuppresion	Kyn/Trp ratio, IDO/TDO expression, AHR activity	Phase I/II	(Wu et al., 2023; Tokito et al., 2024; Yap et al., 2025; Schlichtner et al., 2023; Campesato et al., 2020)
Glutamine/ Glutaminase	GLS inhibitors (CB-839), diet and targeted therapy	Renal, Colon, TNBC	Blockade of glutamine anaplerosis; impaired nucleotide synthesis, redox stress	GLS expression, LAT1/ASCT 2 profile,^{18}F-Gln PET	Phase I/II	(Patel et al., 2024; Wicker et al., 2021; DiNardo et al., 2024; Ciombor et al.,2025; Tsujimoto et al., 2023; Chen et al., 2025; Gouda et al., 2025; Kuroki et al., 2023; Lee et al., 2022)
Methionine/One-carbon	Dietary restriction, methioninase, MAT2A/ PHGDH/SHMT inhibitors	Prostate, Colon, Pancreas, glioma	SAM depletion; epigenetic deregulation, impaired 1C flux	SAM/SAH ratios, PHGDH/ SHMT expression	Phase I/ Preclinical	(Mattes et al., 2024; Han and Hoffman, 2021; Bernasocchi and Mostoslavsky, 2024; Tong et al., 2024; Gounder et al., 2025)
Histidine	Dietary supplementation and chemo/radiotherapy	Pancreas	Redox stress via GSH depletion/ROS; RNA turnover stress	Low plasma histidine; ROS/GSH	Preclinical	(Akinlalu et al., 2024; Kumar et al., 2023)
Isoleucine and BCAAs	Acute/pulsed supplementation	PDAC	mTORC1 activation, EV-mediated amino acid overload	BCAA plasma levels; stress markers	Preclinical	(Akinlalu et al., 2024; Matsui et al., 2024; Ma et al., 2024)
Threonine	Dietary restriction; YRDC/tRNA modification pathway inhibition	Glioblastoma	tRNA modification codon-biased translation and proliferation	YRDC, tRNA-modification enzymes accumulation	Preclinical	(Wu et al., 2024; Harris et al., 2011)
Lysine	Dietary restriction; inhibit lysine catabolism	Glioblastoma; HCC	Catabolism, crotonyl-CoA, histone crotonylation, IFN suppression	Crotonylation markers; GCDH elevation, IFN signatures	Preclinical	(Yuan et al., 2023; Jing et al., 2025; Johal et al., 2024)
Alanine/SNAT2	Block SNAT2 uptake or alanine overload	PDAC, ovarian, SMARCA4/2-deficient tumors	Stromal alanine shuttle fueling anaplerosis; genotype-selective dependence	SNAT2 and GPT2 elevation; stromal-tumor alanine flux	Preclinical	(Zhu et al., 2023; Nie et al., 2025)
LAT1 (*SLC7A5*)	JPH203 transporter inhibition, imaging-guided targeting	Glioma, NSCLC, breast, GI	Leu/Ile/Met uptake, mTORC1 activation	LAT1 expression,^{11}C-Met PET	Early Clinical	(Galldiks et al., 2023; Bo et al., 2021; Sato et al., 2021)
ASCT2 (*SLC1A5*)	C118P, siRNA, small-molecule inhibitors	Breast, PDAC	Glutamine uptake, supports glutamine addiction	ASCT2 expression	Preclinical	(Lyu et al., 2023; Shi et al., 2024)

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TABLE 1 (*Continued*) Established and emerging amino acid dependencies and transporter targets in cancer. The table summarizes mechanisms, transporters, biomarkers, strategies, and evidence tiers for the four established pathways (arginine, glutamine, tryptophan, methionine) and five emerging amino acid cancer therapy, histidine, isoleucine, threonine, lysine and alanine. Transporters central to these targets are shown as both therapeutic entry points and functional biomarkers.

Target/ Transporter	Therapeutic strategy	Cancer type(s)	Mechanism of action	Biomarker(s)	Clinical stage	References
xCT (*SLC7A11*)	Sulfasalazine, sorafenib, ferroptosis sensitizers	Colon, liver, lung, pancreas	Inhibition depletes glutathione and increases ferroptotic susceptibility	SLC7A11 expression, GSH/ GSSG ratios	Early clinical/ Preclinical	(Hu et al., 2020; He et al., 2025; Yan et al., 2023; Messier et al., 2024)
SNAT1/2 (*SLC38A1/2*)	Transporter inhibition	Breast, ovarian	Alanine uptake; stress-induced upregulation	SLC38A1/2 expression	Preclinical	(Jakobsen et al., 2023; Gauthier-Coles et al., 2022)

high isoleucine may selectively stress tumors, prolonged elevation of circulating BCAAs could increase the risk of side effects, such as insulin resistance or neurological complications (Shah et al., 2024; De Simone et al., 2013).

2.3 Threonine

Threonine is essential for protein synthesis and one-carbon metabolism (Tang et al., 2021). In GBM, one of the most aggressive brain tumors, threonine plays a distinct role in translational control, growth and survival (Wu et al., 2024). Wu et al. (2024) demonstrated that GBM cells accumulate unusually high levels of threonine, which fuels a specific tRNA modification (Wu et al., 2024). The enzyme YRDC (YrdC domain–containing protein) uses threonine to generate N^6-threonylcarbamoyl adenosine (t^6A), a modification found on tRNAs that decode ANN codons (Perrochia et al., 2013; Harris et al., 2011). This modification skews protein translation toward mitosis-related and proliferative proteins, giving GBM cells a growth advantage. When threonine availability was reduced or YRDC was inhibited, GBM cells showed impaired t^6A modification, reduced protein synthesis, and suppressed proliferation. In mouse models, dietary threonine restriction significantly slowed tumor growth, validating threonine as a nutrient that sustains GBM progression (Wu et al., 2024).

For clinical translation, two approaches may be explored. First, dietary modulation, where controlled threonine restriction could selectively stress tumors with high translational demand while sparing normal tissues. Second, pharmacological inhibition of YRDC or related enzymes in the tRNA modification pathway. Such drugs could directly impair the codon-biased translation mechanism that GBM depends on. Although this modification has been observed in a subset of GBM models, potential biomarkers may include YRDC enzymatic activity, high t^6A modification levels in tumor RNA, or metabolic features of threonine accumulation. Because these findings are still limited, larger patient studies will be needed to validate these biomarkers and to determine which tumors are most likely to respond to threonine-targeted therapy. Moreover, while threonine availability has been mechanistically linked to YRDC-dependent translation and tumor growth, this evidence comes from GBM models and tumor subtypes. Whether similar vulnerabilities occur across other tumor types or in patients remains uncertain, demonstrating the need for broader validation and biomarker-guided trial designs.

2.4 Lysine

Lysine is an essential amino acid that serves as both a protein building block and a site for multiple post-translational modifications, including acetylation, ubiquitination, and methylation (Wang and Cole, 2020). In GBM, excess lysine breakdown produces crotonyl-CoA, which drives histone crotonylation and suppresses type I interferon signaling (Yuan et al., 2023). This epigenetic silencing weakens immune surveillance by reducing cytotoxic T cell infiltration into tumors. Limiting lysine availability, through dietary restriction or inhibition of catabolic enzymes, lowers crotonyl-CoA levels, restores interferon pathways, and enhances the response to immunotherapy. These findings place lysine metabolism at the center of a metabolic–epigenetic control that tumors exploit to hide from the immune system.

Also, lysine modulation can act in multiple directions depending on tumor type and context. In another GBM study, restricting lysine or using lysine mimics that compete with its metabolic functions enhanced the efficacy of standard chemotherapy, demonstrating the value of nutrient limitation approaches (Jing et al., 2025). Conversely, in hepatocellular carcinoma (HCC), lysine transporter-mediated uptake improved the effects of targeted therapy combined with immune checkpoint inhibition (ICI), indicating that in some tumors, additional lysine may tip the balance toward improved immune activity (Chang et al., 2025). Meanwhile, systemic studies in mice have shown that lysine restriction is physiologically tolerable and can reduce the buildup of harmful lysine catabolites, further supporting the safety of metabolic modulation (Johal et al., 2024).

These results illustrate that lysine-based therapies cannot be viewed through a single lens of "restriction" or "supplementation". Instead, lysine represents a flexible metabolite whose impact depends on the interplay between tumor metabolism, the immune microenvironment, and therapy context. Other lysine-derived modifications, including succinylation and lactylation, are gaining attention as regulators of gene expression and immune signaling, pointing to additional therapeutic targets in lysine metabolism.

For clinical translation, lysine restriction may be advantageous in tumors that exploit catabolism to evade immunity, while supplementation could enhance therapy in settings where immune cells compete with tumors for lysine. Future clinical translation will require careful patient selection, biomarker development, and context-specific strategies that account for this duality.

2.5 Alanine

Alanine, a non-essential amino acid, is increasingly recognized as a fuel in the metabolic cooperation between tumor cells and their microenvironment. Beyond its traditional role in the glucose-alanine cycle between muscle and liver, alanine has been shown to act as a stromal-derived nutrient that sustains tumor mitochondrial metabolism (Parker et al., 2020). In PDAC, pancreatic stellate cells release alanine into the tumor microenvironment, which is subsequently imported by cancer cells and converted to pyruvate (Be et al., 2019). Pyruvate replenishes the TCA cycle through anaplerosis, the process of refilling depleted metabolic intermediates. This exchange allows PDAC cells to preserve oxidative phosphorylation and biosynthetic activity under nutrient stress, demonstrating alanine as a central metabolite in stromal-tumor metabolic crosstalk.

Alanine dependence has also been observed in other cancers with defined genetic backgrounds. In ARID1A-mutant ovarian cancers, where loss of the ARID1A chromatin-remodeling gene rewires metabolic programs, tumor cells show heightened reliance on alanine uptake (Nie et al., 2025). Blocking this uptake impaired tumor growth in preclinical models, revealing a genotype-selective vulnerability. Similarly, in SMARCA4/2-deficient tumors, which lack components of the SWI/SNF chromatin-remodeling complex, exogenous alanine overload demonstrated cytotoxicity (Zhu et al., 2023). Supplementation with alanine disrupted metabolic balance and induced cell death in cell cultures and xenograft models (Zhu et al., 2023). These findings suggest that the role of alanine in cancer is not uniform but shaped by tumor genotype, with certain mutations conferring heightened susceptibility. A notable strength of these studies is the integration of metabolic tracing and co-culture systems, which reveal real-time nutrient exchange between stromal and cancer cells. However, translating these observations remains challenging, as stromal heterogeneity and tissue architecture may alter alanine dynamics *in vivo*.

Clinically, targeting alanine metabolism presents dual opportunities. One approach is to inhibit alanine uptake by blocking sodium-coupled neutral amino acid transporter 2 (SNAT2; *SLC38A2*), which mediates alanine import. Early studies indicate that pharmacological inhibition of SNAT2 suppresses tumor growth and can synergize with inhibitors of glucose metabolism (Gauthier-Coles et al., 2022). Alternatively, in specific genetic contexts such as SMARCA4/2 deficiency, alanine supplementation itself may be leveraged to overwhelm tumor metabolic capacity, a strategy that contrasts with nutrient-depletion approaches.

3 Amino acid transporters as targets and functional biomarkers

3.1 LAT1 (*SLC7A5*)

L-type amino acid transporter 1 (LAT1) mediates the uptake of large neutral amino acids such as, leucine, and threonine (Scalise et al., 2018a). LAT1 is frequently overexpressed in solid tumors, including pancreatic, breast, and brain cancers (Sato et al., 2021; Okano et al., 2020). High LAT1 expression correlates with poor prognosis and resistance to standard therapies, reflecting its role in fueling mTORC1 signaling (Shibasaki et al., 2023). Pharmacological inhibitors of LAT1, such as JPH203, have entered early-phase clinical testing and demonstrated manageable safety profiles with preliminary signs of antitumor activity (Bo et al., 2021; Okano et al., 2020). More recent studies suggest that LAT1 also modulates the tumor immune microenvironment by controlling the amino acid supply to both tumor cells and infiltrating lymphocytes, making it a potential dual-purpose target (Zhao et al., 2025).

3.2 ASCT2 (*SLC1A5*)

The alanine/serine/cysteine transporter 2 (ASCT2; gene name *SLC1A5*) primarily mediates glutamine uptake but also transports neutral amino acids. While glutamine metabolism is well established in cancer, ASCT2 is a vital target in nutrient uptake. Inhibitors such as C118P block ASCT2-mediated transport and demonstrate antitumor efficacy in preclinical breast cancer models (Lyu et al., 2023). Recent structural studies have improved the understanding of ASCT2's substrate-binding dynamics, allowing for new inhibitors with greater selectivity. Because ASCT2 expression can be detected via immunohistochemistry or transcriptomic profiling, it is also being evaluated as a biomarker for glutamine dependence in patient tumors (Lyu et al., 2023; Garibsingh et al., 2021).

3.3 xCT (*SLC7A11*)

The cystine/glutamate antiporter (xCT; gene name *SLC7A11*) imports cystine in exchange for glutamate, coupling amino acid transport to redox homeostasis through glutathione synthesis (Hu et al., 2020). Overexpression of xCT protects tumors from oxidative stress but also creates therapeutic opportunities: targeting xCT sensitizes cancers to radiotherapy and ferroptosis-inducing agents (He et al., 2025; Yan et al., 2023). Recent work has shown that xCT levels predict response to radiotherapy in colorectal cancer liver metastases, highlighting its value as both a target and a clinical biomarker (He et al., 2025). Additionally, inhibitors of xCT are being explored in combination with immune checkpoint inhibitors, where depletion of cystine may amplify T cell–mediated oxidative killing (He et al., 2025; Messier et al., 2024; Xu et al., 2021).

3.4 SNAT2 (*SLC38A2*)

The sodium-coupled neutral amino acid transporter 2 (SNAT2; gene name *SLC38A2*) regulates transport of alanine and other small

neutral amino acids. SNAT2 expression is upregulated in nutrient-stressed tumors and correlates with therapy resistance (Gauthier-Coles et al., 2022). In ovarian cancers with ARID1A mutations, reliance on SNAT2-mediated alanine uptake has been identified as a selective vulnerability (Gauthier-Coles et al., 2022). Pharmacological inhibitors of SNAT2 are in preclinical development and demonstrate synergy with glucose metabolism inhibitors, demonstrating their role as a metabolic checkpoint (Jakobsen et al., 2023; Gauthier-Coles et al., 2022).

3.5 CAT1 (*SLC7A1*)

The cationic amino acid transporter 1 (CAT1; gene name *SLC7A1*) imports positively charged amino acids such as lysine and arginine. Recent studies link CAT1 to immune regulation, showing that lysine transport influences epigenetic programs that modulate interferon responses (You et al., 2022). In GBM, upregulation of lysine transport enhances histone crotonylation, leading to suppression of type I interferon signaling and immune evasion (Yuan et al., 2023). Although CAT1 has not yet been the focus of clinical trials, its role in metabolic–epigenetic reprogramming highlights its translational potential.

3.6 Transporters as imaging and circulating biomarkers

Amino acid transporters also provide opportunities for non-invasive imaging and biomarker development. Radiolabeled amino acid analogs such as ^{18}F-fluoroethyltyrosine (FET) and ^{11}C-methionine are used in PET to visualize transporter activity *in vivo* (Galldiks et al., 2023). Recent studies explore the use of LAT1-specific tracers to monitor tumor metabolism and treatment response dynamically (Achmad et al., 2025). In parallel, circulating metabolite profiles, such as plasma amino acid ratios, are being tested as functional markers of transporter activity, offering accessible biomarkers for patient selection in clinical trials (Chan et al., 2021; Zakharia et al., 2021; Chang et al., 2025).

3.7 Clinical and safety considerations for amino acid transporter-targeted therapies

As amino acid transporters continue to emerge as therapeutic targets, it is important to consider their functions in normal physiology to ensure treatment safety. Most of these transporters are not tumor-specific; they are also expressed in healthy tissues that rely on continuous amino acid exchange. LAT1, for example, is highly active at the blood–brain barrier and placenta, mediating the transfer of large neutral amino acids essential for brain and fetal development (Ohgaki et al., 2017). ASCT2 and SNAT1/2 regulate the movement of neutral amino acids across liver, muscle, and kidney cells, contributing to energy metabolism and osmotic balance (Scalise et al., 2018b; Ge et al., 2018), while xCT maintains antioxidant defense by exchanging cystine and glutamate in many epithelial tissues (Martis et al., 2020). Because of these physiological roles, broad or prolonged inhibition of these transporters can potentially cause nutrient imbalance, neurotoxicity, or organ dysfunction if monitoring is inadequate or drug exposure is not properly controlled. Early clinical and preclinical studies indicate that transporter inhibitors can be administered safely when dosing is well managed, but they also highlight the need for continuous safety assessment given the essential metabolic functions of these proteins. Emerging strategies such as tumor-targeted delivery systems, intermittent or adaptive dosing, and combination therapies are being developed to minimize systemic exposure while maintaining anti-tumor activity. Although transporter-targeting strategies such as LAT1 and ASCT2 inhibition show reproducible metabolic effects, clinical translation is constrained by transporter redundancy and expression in normal organs. These factors complicate dose optimization and emphasize the need for transporter-selective or tumor-targeted approaches. Integrating transporter biology with pharmacokinetic monitoring in future trials will be crucial for translating these approaches safely into clinical use.

4 Oncogenic regulation, opportunities and challenges for clinical translation

4.1 Oncogenic regulation of amino acid metabolism and transporters

Amino acid metabolism and transporter activity are tightly linked to oncogenic signaling. Many of the enzymes and transporters discussed in this review are directly regulated by transcription factors that coordinate nutrient use with cell growth and stress response. MYC (myelocytomatosis oncogene), one of the most studied oncogenes, increases the expression of several amino acid transporters, including ASCT2 and LAT1, to enhance amino acid uptake and sustain mTORC1 activity (Hansen et al., 2015). YAP (Yes-associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif), the downstream effectors of the Hippo pathway, also stimulate amino acid transport by upregulating LAT1, which promotes leucine uptake and mTORC1 signaling, especially under nutrient-limited conditions (Hansen et al., 2015; Bertolio et al., 2023). In addition, mutant p53 (tumor protein p53) reprograms amino acid metabolism by promoting serine–glycine synthesis and LAT1/CD98 expression, while repressing xCT through interaction with NRF2 (nuclear related factor 2), thereby altering redox balance and nutrient handling (Tombari et al., 2023). These oncogenic influences show that metabolic rewiring is not a passive adaptation to nutrient stress but a genetically driven feature of tumor progression. Recognizing how oncogenic drivers shape amino acid dependencies can guide both biomarker development and inform the design of pathway-specific therapeutic combinations.

4.2 Opportunities for translational targeting

4.2.1 Biomarker-driven patient selection

Clinical experience with arginine and tryptophan has shown that amino acid metabolism is most effectively targeted when linked to biomarkers, such as ASS1 loss for arginine or the kynurenine/tryptophan ratio for tryptophan catabolism. For emerging amino acids, parallel opportunities exist: plasma histidine depletion or high expression of histidine ammonia-lyase (HAL) may predict response to

histidine–isoleucine supplementation; YRDC catalytic activity or elevated tRNA modification signatures could identify threonine-dependent glioblastomas; lysine-crotonylation signatures or GCDH overexpression may stratify patients for lysine restriction; and transporter expression, such as SNAT2 for alanine or LAT1 for histidine/isoleucine/threonine, could provide functional markers of dependency.

4.2.2 Exploiting nutrient surplus as a therapeutic target

Most metabolic therapies have focused on nutrient deprivation, such as starving tumors of arginine or methionine. By contrast, histidine and isoleucine supplementation demonstrate that amino acid supplementation can selectively induce tumor stress. This conceptual shift broadens the therapeutic approach, allowing tumors' own metabolic vulnerabilities (such as amino acid export or catabolic upregulation) to be used against them.

4.2.3 Combination therapies and synergy

Just as glutaminase inhibition has shown greatest promise in combination with radiation or chemotherapy, emerging amino acid strategies may synergize with existing treatments. Histidine supplementation enhances gemcitabine activity by disrupting redox balance, lysine restriction boosts immune checkpoint blockade by reactivating interferon pathways, and alanine modulation sensitizes tumors to mitochondrial stress and chemotherapy. Rationally designed combinations could overcome the limited durability seen with single therapies.

4.2.4 Transporters as tractable drug targets and imaging tools

LAT1, SNAT2, and CAT1 represent pharmacologically accessible points that couple extracellular nutrient supply with tumor growth. Notably, transporter activity can also be visualized non-invasively through PET tracers, enabling real-time monitoring. This dual role, both as targets and biomarkers, strengthens the translational case for amino acid–based therapies.

4.3 Challenges

4.3.1 Tumor microenvironment metabolism and therapy resistance

Amino acid metabolism in tumors represents a network shared between cancer cells, stromal fibroblasts, and immune populations within the tumor microenvironment (TME). This metabolic cooperation shapes both nutrient access and treatment response. Cancer-associated fibroblasts (CAFs) can secrete alanine, sustaining oxidative metabolism in pancreatic tumors during nutrient stress (Sousa et al., 2016), while aspartate-glutamate exchange between stromal and tumor cells buffers redox status and supports biosynthesis (Be et al., 2019). Within the immune compartment, depletion of tryptophan by IDO1/TDO2 and arginine by myeloid cells suppresses T-cell proliferation and effector function (Günther et al., 2019). Beyond these established pathways, emerging amino acid dependencies, such as those involving histidine, isoleucine, threonine, lysine, or alanine, may further influence the TME by modifying oxidative balance, nutrient signaling, and stress adaptation. Integrating these newer observations with known mechanisms suggests that tumors exploit a flexible amino acid economy in which both stromal support and immune suppression converge to sustain growth. This metabolic reciprocity explains why amino acid-targeted interventions often show context-dependent efficacy and demonstrates the need for therapeutic strategies that account for tumor microenvironmental nutrient exchange, immune cell competition, and tumor metabolic plasticity. These tumor-stromal-immune exchanges illustrate the multifactorial nature of amino acid metabolism in the TME, setting the stage for broader challenges in clinical translation. Collectively, the studies reviewed provide valuable mechanistic insight but vary in translational depth. Many rely on metabolic or xenograft models that simplify the TME, whereas patient-derived organoids and isotope tracing in clinical samples remain limited. These methodological differences partly explain why results are sometimes inconsistent across tumor types. Recognizing these constraints allows a more accurate assessment of which amino-acid pathways are most actionable in patients.

4.3.2 Metabolic plasticity and adaptive resistance

Experience with arginine and glutamine has shown how tumors adapt when a single nutrient pathway is blocked, rerouting flux through compensatory pathways. Emerging strategies will face similar resistance: tumors may adjust transporter expression, shift metabolic routing, or draw on stromal support to bypass amino acid targeting.

4.3.3 Balancing systemic safety with tumor selectivity

Unlike small-molecule inhibitors with defined targets, dietary or metabolic interventions risk perturbing systemic amino acid pools. Although preclinical studies suggest that histidine, isoleucine, and threonine interventions are tolerated, long-term effects on protein synthesis, immune function, and normal tissue homeostasis must be carefully evaluated.

4.3.4 Heterogeneity of tumor microenvironment

Alanine exemplifies how stromal fibroblasts supply tumors with metabolic support. This highlights a challenge: vulnerabilities may not arise from tumor cells alone but from the interaction between tumor and microenvironment. Therapies will need to consider this complexity, as targeting stromal-tumor nutrient exchange is inherently more difficult than inhibiting tumor-intrinsic enzymes.

4.3.5 Limited clinical data and trial design hurdles

While arginine and glutamine have advanced into randomized trials, most emerging amino acid strategies remain in early preclinical stages. Translating them will require innovative trial designs, such as adaptive protocols that pivot therapy upon biomarker-detected resistance, as well as careful patient selection to ensure only biomarker-positive patients are enrolled.

4.4 Future direction

The successful translation of emerging amino acid therapies will depend on a precision-guided approach. This means linking basic mechanistic insights with reliable biomarkers, designing smart treatment combinations, and selecting patients based on their genetic or metabolic profile. For example, methionine restriction

is being tested with radiation therapy, and arginine depletion is used in patients whose tumors lack ASS1. In the same way, new amino acid strategies must align the biology of each target with matching biomarkers and treatment designs. Equally important, future progress will require integrating these mechanistic findings with carefully designed clinical studies that address current limitations in model relevance, patient selection, and biomarker validation. Combining metabolic profiling with functional assays in patient-derived systems will help distinguish true causal dependencies from correlative observations, ensuring that only the most actionable pathways advance into clinical testing. Incorporating transporter biology, monitoring plasma metabolite levels, and building rational combinations with existing therapies will be key to moving these approaches from preclinical promise to real clinical benefit.

5 Conclusion

Amino acid metabolism is a promising frontier in cancer research. Established interventions such as arginine depletion, glutaminase inhibition, tryptophan catabolism blockade, and methionine restriction have demonstrated both the feasibility and the complexity of targeting metabolic pathways in patients. Building on these lessons, recent discoveries highlight histidine, isoleucine, threonine, lysine, and alanine as new amino acid dependencies with translational potential. Each of these amino acids introduces distinct mechanisms, from selective cytotoxicity and redox imbalance to translational control, epigenetic reprogramming, and stromal–tumor nutrient exchange. Notably, these new targets are closely tied to amino acid transporters such as LAT1, CAT1, and SNAT2, which function as metabolic gateways and potential therapeutic targets or imaging biomarkers. This dual role strengthens the rationale for incorporating transporters into biomarker-driven clinical strategies. Opportunities include exploiting nutrient surplus, pairing amino-acid modulation with chemotherapy, radiotherapy, and immunotherapy, and deploying functional biomarkers for patient selection. Challenges include metabolic plasticity, microenvironmental heterogeneity, and the need for careful trial design to balance systemic safety with tumor selectivity. Looking forward, progress will depend on precision-oriented implementation that combines mechanistic insight with biomarker development and rational therapeutic combinations. By aligning amino acid mechanisms with transporter targeting, metabolic imaging, and patient selection, the field can move closer to translating emerging amino acid dependencies into durable clinical benefit. Ultimately, integrating these strategies into precision oncology frameworks has the potential to expand treatment options and improve outcomes for patients with some of the most difficult-to-treat cancers.

Author contributions

AA: Data curation, Formal Analysis, Investigation, Validation, Visualization, Writing – original draft, Writing – review and editing. EO: Validation, Writing – review and editing. TG: Validation, Writing – review and editing. DS: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review and editing.

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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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Glossary

1C	One carbon
AA	Amino acid
ADI-PEG 20	Pegylated arginine deiminase
AHR	Aryl Hydrocarbon Receptor
ALT2/GPT2	Alanine Aminotransferase 2 (Glutamic-Pyruvic Transaminase 2)
ASCT2 (*SLC1A5*)	Alanine-Serine-Cysteine Transporter 2
ASS1	Argininosuccinate Synthase 1
BCAAs	Branched-Chain Amino Acids
CAT1 (*SLC7A1*)	Cationic amino acid transporter 1
EVs	Extracellular vesicles
GBM	Glioblastoma (glioblastoma multiforme)
GCDH	Glutaryl-CoA dehydrogenase
GLS	Glutaminase
GSH	Reduced glutathione (GSSG = oxidized glutathione)
HCC	Hepatocellular carcinoma
HAL	Histidine ammonia-lyase
ICI	Immune checkpoint inhibitor
IDO1/TDO2	Indoleamine 2,3-dioxygenase 1/Tryptophan 2,3-dioxygenase 2
IHC	Immunohistochemistry
Kyn/Trp	Kynurenine-to-tryptophan ratio
LAT1 (*SLC7A5*)	L-type amino acid transporter 1
mTORC1	Mechanistic target of rapamycin complex 1
NSCLC	Non-small cell lung cancer
PD-1	Programmed cell death protein 1
PDAC	Pancreatic ductal adenocarcinoma
PET	Positron emission tomography
ROS	Reactive oxygen species
SLC	Solute carrier (transporter family prefix)
SNAT2 (*SLC38A2*)	Sodium-coupled neutral amino acid transporter 2
TCA cycle	Tricarboxylic acid cycle
t^6A	N^6-threonylcarbamoyl adenosine (tRNA modification)
Trp	Tryptophan
xCT (*SLC7A11*)	Cystine/Glutamate Antiporter
XRN1	5′→3′exoribonuclease 1
YRDC	YrdC-domain–containing protein (enzyme for t^6A formation)

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Essential amino acids preserve intestinal barrier integrity *via* mitochondrial protection in obesity and gut inflammation

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Objective: Obesity disrupts intestinal homeostasis, leading to increased permeability ("leaky gut"), mucosal inflammation, and systemic metabolic dysfunction. Mitochondrial impairment in intestinal epithelial cells (IECs) is a central driver of this process. Essential amino acids (EAAs) improve mitochondrial function in metabolic tissues, but their impact on intestinal health remains underexplored. Here, we investigated whether dietary EAAs preserve gut barrier integrity through mitochondrial protection in obesity and inflammation.

Methods: Male C57BL/6N mice were fed a high-fat diet (HFD) or an isocaloric, isonitrogenous EAA-substituted HFD (HFD-EAA) for 33 weeks to assess metabolic outcomes, intestinal barrier function, inflammation, and mitochondrial biogenesis. Parallel, *in vitro* studies in differentiated Caco-2 cells tested an EAA formula enriched with Krebs cycle intermediates (E7), under basal and pro-inflammatory conditions (IL-1β, TNF-α, LPS).

Results: HFD-EAA supplementation prevented and reversed obesity, improved glucose tolerance, reduced mesenteric fat expansion, and preserved intestinal barrier integrity while attenuating inflammation. EAAs restored intestinal length and weight, lowered plasma calprotectin, and normalized citrulline, a biomarker of enterocyte mass. Tight and adherens junction proteins (zonulin-1, occludin, E-cadherin, claudins) were maintained, while pore-forming claudin-2 was reduced. EAAs also upregulated PGC-1α and mitochondrial electron transport chain genes in intestinal epithelial cells (IECs). Their direct effects were confirmed *in vitro* in Caco-2 cells, where E7 increased transepithelial electrical resistance (TEER), enhanced mitochondrial respiration, suppressed inflammation-induced glycolytic reprogramming, activated antioxidant defenses, and reduced IL8 secretion. Mechanistically, E7 promoted eNOS phosphorylation and inhibited mTORC1 signaling.

Conclusion: EAAs protect gut barrier integrity by sustaining mitochondrial biogenesis and function in IECs, thereby reducing obesity- and stress-induced

inflammation. These findings highlight EAAs as a promising nutritional strategy to counteract mitochondrial dysfunction and prevent or reverse gut barrier disruption in obesity-related and inflammatory disorders.

KEYWORDS

essential amino acids, intestinal barrier function, mitochondrial function, gut inflammation, mTOR signaling, leaky gut, intestinal epithelial cells, obesity

1 Introduction

In 2021, an estimated 1.00 billion adult males and 1.11 billion adult females were classified as living with overweight or obesity. If historical trends continue, by 2050, the number of adults living with overweight and obesity is projected to reach 3.80 billion, representing over half of the global adult population (Ng et al., 2025). Clinical obesity is a chronic illness characterized by systemic and organ-specific dysfunctions directly induced by excess adiposity (Rubino et al., 2025). In contrast, preclinical obesity refers to a state of excess adiposity with preserved tissue and organ function but an increased risk of progressing to clinical obesity and other non-communicable diseases (Rubino et al., 2025). These dysfunctions involve multiple organ systems, including the liver, skeletal muscle, cardiovascular and central nervous systems, leading to severe, potentially life-threatening complications such as type 2 diabetes mellitus, metabolic dysfunction–associated steatotic liver disease and steatohepatitis, asthma, cardiovascular diseases, cancer, and neurodegenerative disorders (Jin et al., 2023). These pathological consequences are driven by mechanisms such as chronic inflammation, mitochondrial dysfunction, fibrosis, ectopic fat deposition, and mechanical or hemodynamic stress (Reilly and Saltiel, 2017; Xu et al., 2025). In particular, evidences indicate that low-grade, chronic inflammation plays a central role in linking adipose tissue dysfunction to multi-organ impairment, thereby contributing to the wide spectrum of metabolic, cardiovascular, and neurobehavioral complications associated with obesity (Hotamisligil, 2006).

Alteration in gut homeostasis has been implicated as a key contributor to the progression of obesity-related metabolic and inflammatory complications (Cani and Jordan, 2018). Increased intestinal permeability ("leaky gut") and low-grade mucosal inflammation are key features of obesity, promoting endotoxemia and insulin resistance. Emerging evidence suggests that obesity is also a significant factor in the pathogenesis of inflammatory bowel diseases (IBDs), with pro-inflammatory cytokines derived from adipose tissue exacerbating disease progression (Harper and Zisman, 2016). Experimental studies in mice have shown that obesity worsens colitis pathology (Cheng et al., 2016; Wunderlich et al., 2018). Both diet-induced obesity (DIO) models in mice (Stenman, 2012) and humans with obesity (Genser et al., 2018) show increased intestinal permeability to bacterial products such as lipopolysaccharide (LPS), a potent inflammatory agent. Mouries et al. demonstrated that just 1 week of an obesogenic diet in mice induces dysbiosis, disrupts the gut vascular barrier, and promotes bacterial translocation to the liver, culminating in non-alcoholic steatohepatitis (Mouries et al., 2019).

Mitochondrial dysfunction in intestinal epithelial cells (IECs) plays a key role in driving barrier breakdown, epithelial cell apoptosis, and inflammation in both obesity and IBDs. Guerbette et al. highlighted the differential impact of saturated fatty acids on mitochondrial function, and further described how high-fat diet (HFD) intake leads to metabolic adaptations in IECs (Guerbette et al., 2024). Specifically, excessive lipid consumption reduces mitochondrial number in these cells, impairs their differentiation, and contributes to increased epithelial permeability (Guerbette et al., 2025). Also, DIO exacerbates experimental colitis by elevating oxidative stress and disrupting mitochondrial function, which in turn activates pro-apoptotic pathways in the colon (Li and Li, 2020).

Tight junction (TJ) remodeling and inflammatory cytokine exposure further exacerbate epithelial barrier dysfunction (Ahmad et al., 2017; Bhat et al., 2019; AlMarzooqi et al., 2024). Three main types of cell–cell junctions are known: TJs [occludins, claudins, and zonulin (ZO)], adherens junctions (AJs, such as E-cadherin), and gap junctions. These structures regulate intercellular communication and play distinct roles in tissue homeostasis (AlMarzooqi et al., 2024). Furthermore, serum ZO is a biomarker of impaired intestinal permeability and is associated with diarrhea, dysbiosis, and poor metabolic health. Its levels tend to normalize following sustained weight loss through lifestyle modification or bariatric surgery (Aasbrenn et al., 2020).

Although lifestyle modification and high-protein diets are effective in improving metabolic outcomes (Te Morenga and Mann, 2012; Sanders et al., 2019; Zhu et al., 2020), their impact on intestinal integrity remains poorly defined. In particular, the role of dietary amino acids in modulating epithelial mitochondrial function and gut barrier integrity is incompletely understood. Specific amino acids, including glutamine, arginine, glycine, glutamic acid, and tryptophan, exert local anti-inflammatory effects and support mucosal repair in IBDs (He et al., 2018; Suzuki, 2020; Duanmu et al., 2022; Ji et al., 2023), but studies investigating essential amino acids (EAAs)—the subset required for protein synthesis and metabolic regulation—on intestinal health are scarce. Notably, EAAs stimulate mitochondrial biogenesis and function in metabolic tissues, to improved insulin sensitivity and reduced inflammation (Valerio et al., 2011; Ruocco et al., 2020; 2021; Ragni et al., 2023). These metabolic benefits are largely mediated by increased nitric oxide (NO) production—via endothelial nitric oxide synthase (eNOS) activation—, reduced oxidative stress (Nisoli et al., 2008), and modulation of mechanistic target of rapamycin complex 1 (mTORC1) (D'Antona et al., 2010; Valerio et al., 2011). Yet, whether EAAs can directly preserve intestinal epithelial barrier function, especially in the context of obesity and inflammation, remains unknown.

Here, we tested the hypothesis that dietary EAAs protect gut barrier integrity by preserving mitochondrial function in IECs. We combined an *in vivo* model of DIO with *in vitro* studies using differentiated Caco-2 monolayers under inflammatory stress to (i) assess the impact of EAAs on barrier function, TJ proteins, and epithelial inflammation, and (ii) dissect the mitochondrial and

signaling pathways involved. This integrated approach addresses a critical gap linking nutrition, mitochondrial health, and gut barrier integrity in obesity-related gut dysfunction.

2 Materials and methods

2.1 Animal model of diet-induced obesity and EAA treatment

Male C57BL/6N mice (8 weeks old; Charles River, Calco, Italy) were housed under controlled temperature and humidity with a 12 h light/dark cycle and *ad libitum* access to food and water. After randomization by body weight, mice were fed for 33 weeks with: (i) a HFD (60% fat, 5.2 kcal/g; D12492, Research Diets Inc., New Brunswick, United States; n = 10 mice); (ii) an isocaloric, isonitrogenous, HFD in which casein protein was replaced by an EAA mixture (HFD-EAA; D17073104, Research Diets; n = 10 mice); or (iii) standard chow (3.2 kcal/g; V1534-300, ssniff-Spezialdiäten, Soest, Germany; n = 6 mice) (Supplementary Figure S1). After 25 weeks, a subgroup of obese HFD-fed mice (n = 5) was switched to HFD-EAA for 8 weeks to assess therapeutic effects (HFD > HFD-EAA). The number of mice used for the *in vivo* experiments was calculated taking into account inter-individual variability in HFD responses in C57BL/6N mice (~18% obesity-resistant) (Boulangé et al., 2013). The dietary EAA content was based on previous studies demonstrating its specificity compared to a control free-amino acid mix matched to casein composition (D'Antona et al., 2010; Ruocco et al., 2020). All diets were irradiated, stored in cool, dry conditions, and used within 6 months. Body weight (BW) and food intake (FI) were recorded weekly. At sacrifice (week 33), mice were euthanized by cervical dislocation. Visceral (epididymal and mesenteric), subcutaneous (inguinal), and brown (interscapular) adipose depots, liver, and intestine, were collected, weighed, and snap-frozen. Intestinal length and wet weight were measured as indirect markers of barrier dysfunction and inflammation. All animal procedures complied with the European Directive 2010/63/EU and Community guidelines and ARRIVE guidelines (Percie du Sert et al., 2020) and were approved by the Italian Ministry of Health (Protocol No. 15/2024-PR).

2.2 Glucose homeostasis and insulin sensitivity

Glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed to assess glucose homeostasis and insulin sensitivity. GTT was conducted after 14 and 31 weeks of treatment (*i.e.*, 6 weeks after diet shift in the HFD > HFD-EAA group) following an overnight fast (16–18 h). Mice received an intraperitoneal (i.p.) glucose bolus (1.5 g/kg BW; #G6152, Sigma Aldrich, Milan, Italy). ITT was performed after 4 h daytime fast, with i.p. injection of insulin (0.75 U/kg BW; Apidra, Sanofi, Milan, Italy). Blood glucose was measured from tail-vein at 0, 15, 30, 60, and 120 min using a glucometer (OneTouch Verio Reflect, LifeScan, Sesto San Giovanni, Italy) with corresponding strips. Glucose tolerance and insulin sensitivity were quantified by calculating the area under the curve (AUC) using the trapezoid method (Ruocco et al., 2022).

2.3 Faecal output, energy absorption and digestive efficiency

At week 27, mice were individually housed for 48 h with *ad libitum* access to their assigned diets to assess faecal output. FI (g) and faecal mass (g) were measured, and the percentage of food excreted was calculated as faecal weight/FI x 100. At week 33, faecal energy content (kJ/kg) and elemental composition (carbon, nitrogen, hydrogen; % dry mass) were determined by bomb calorimetry (LabAnalysis Group, Casanova Lonati, Pavia, Italy). Energy intake (kJ) was calculated as FI (g) x diet caloric density (kcal/g) x 4.184 (kcal to kJ). Faecal energy excretion (kJ) was determined as faecal weight (g) x faecal energy content (kJ/kg)/1,000. Digestive efficiency (%) was calculated as digestive efficiency (index of intestinal energy absorption) = [1 – (energy excreted/energy ingested)] × 100 (Meyer et al., 2009).

2.4 Plasma profiling of amino acids and calprotectin

At the end of treatment, blood was collected *via* submandibular venipuncture into EDTA-treated tubes (3 mM; Sigma Aldrich, Merck Life Science, Milan, Italy) and centrifuged (8,000 × g, 10 min, 4 °C) to obtain plasma. *Amino acid profiling*. Circulating amino acids were quantified by cation-exchange chromatography with post-column ninhydrin derivatization using a Biochrom 30+ Amino Acid Analyzer (Biochrom Ltd, ERRECI S.r.l, Pieve Emanuele, Milan, Italy). Briefly, plasma was mixed 1:1 with 5% sulphosalicylic acid containing L-norleucine (250 mM) as an internal standard, incubated for 30 min at 4 °C, and centrifuged (10,000 × g, 5 min, 4 °C). Supernatants were filtered (0.22 µm) and analyzed against calibration standards (250 mM for each amino acid). Amino acids were detected at 440 and 570 nm after ninhydrin derivatization (135 °C) and quantified spectrophotometrically (Ragni et al., 2022). *Calprotectin*. Plasma calprotectin levels were measured using Mouse S100A8/S100A9 ELISA Kit (#EM67RB; Invitrogen, Thermo Fisher, Monza, MI, Italy), following the manufacturer's instructions.

2.5 Caco-2 cells and *in vitro* model of gut inflammation

2.5.1 Cell culture and differentiation

Human colon adenocarcinoma Caco-2 cells (ATCC-HTB-37, LGC, Milan, Italy) were cultured in Eagle's Minimum Essential Medium (EMEM; ATCC 30-2003) supplemented with 10% fetal bovine serum (FBS; ATCC 30-2020), 100 U/mL penicillin, and 100 µg/mL streptomycin (Euroclone, Milan, Italy) at 37 °C in 5% CO_2. Cells were seeded at passages 10–20 and allowed to differentiate for 14–21 days post-confluence, forming polarized monolayers with TJs and AJs and brush borders (Wang et al., 2011).

2.5.2 Amino acid mixture treatments

Differentiated Caco-2 cells were treated with an EAA mixture enriched with Krebs cycle intermediates—citric, malic, and succinic acids—referred to as E7 (0.1% or 1.0% w/v) for 24 or 48 h to assess dose- and time-dependent effects (Supplementary Figure S2). A standard EAA mixture (without Krebs cycle intermediates) was used for comparison (1.0% w/v; Supplementary Figure S2). Concentrations were selected based on previous studies (D'Antona et al., 2010; Ruocco et al., 2020; Tedesco et al., 2020a).

2.5.3 Inflammatory model

To model gut inflammation, differentiated Caco-2 cells (day 14) were pre-treated with E7 (1.0%, 1 h) (Hollebeeck et al., 2012), followed by a 24–72 h exposure to a mixture of inflammatory stimuli (IS), including IL-1β (25 ng/mL), TNF-α (50 ng/mL), and LPS (10 μg/mL) in EMEM with 1% heat-inactivated FBS (Van De Walle et al., 2008; 2010) (Supplementary Figures S2A–C).

2.5.4 mTOR pathway inhibition

To assess pathway involvement, cells were pre-treated with rapamycin (50 nM, Sigma Aldrich) for 1 h, with or without E7, followed by IS exposure for 48 h (Tedesco et al., 2022).

2.5.5 Mitochondrial function inhibition

Differentiated Caco-2 cells (14 days) were pre-treated with E7 (1.0%) for 24 h and subsequently exposed to antimycin A (0.1–1.0 μM; Sigma-Aldrich), a selective complex III inhibitor, for 24 h. Cell viability was assessed by MTT (Crakes et al., 2019).

2.6 Cell vitality assay

Caco-2 cell viability was assessed using two complementary methods, the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma Aldrich) and the Sulforhodamine B (SRB)-based *In Vitro* Toxicology Assay Kit (TOX6, Sigma Aldrich). For the MTT assay, differentiated Caco-2 cells were seeded 75,000 cells/well in 96-well plates (100 μL/wells) and treated with E7 (0.1% or 1.0%) with or without IS for 24, 48, or 72 h. At the end of the treatment, MTT (5 mg/mL in PBS; 20 μL) was added and incubated for 4 h. Formazan crystals were dissolved overnight in 5% SDS/0.1 M HCl (100 μL/well) at 37 °C, and absorbance was measured at 570 nm/655 nm using a microplate reader (Ragni et al., 2022). Alternatively, the SRB-based TOX6 assay was performed according to the manufacturer's instructions to confirm cell viability.

2.7 Gene expression analysis

Total RNA was isolated from jejunal samples or differentiated Caco-2 cells (seeded 5×10^5 cells/well; 24 well plates) using the RNeasy Mini Kit (Qiagen, Milan, Italy) and treated with DNase according to the manufacturer's protocol (Bio-Rad Laboratories, Milan, Italy). cDNA was synthesized from 1 μg of RNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Quantitative real-time PCR (qRT-PCR) was performed with iTaq Universal SYBR Green SuperMix (BioRad Laboratories) on a CFX Connect Real-Time PCR System (Bio-Rad Laboratories). Primer sequences are listed in Supplementary Table S1, designed using Primer3 software (version 4.1.0). Gene expression was normalized to GAPDH using the ΔΔCt method. Relative expression was calculated as $2^{-\Delta\Delta CT}$, where ΔΔCt represents the difference between ΔCt of each sample and ΔCt of the control group (Corsetti et al., 2014).

2.8 Immunoblot analysis

Protein extracts were prepared from differentiated Caco-2 cells (seeded 1.5×10^6 cells/well; 6-well plates) using Mammalian Protein Extraction Reagent (M-PER, #78501, Pierce, Thermo Fisher Scientific, Merck, Milan, Italy), supplemented with protease and phosphatase inhibitors (#PPC1010, Sigma-Aldrich). Protein concentration was determined using the bicinchoninic acid (BCA) assay (#EMP014500, Euroclone). Equal amounts of protein were resolved by SDS-PAGE under reducing conditions and transferred to nitrocellulose or polyvinylidene difluoride (PVDF) membrane (#1704158 and #1704156, Bio-Rad Laboratories). Membranes were incubated with primary antibodies against: ZO-1 (#40-2300; Invitrogen, Thermo Fisher Scientific), Occludin (ab216327, Abcam, Prodotti Gianni, Milan, Italy), peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α, ab191838, Abcam), cytochrome c oxidase subunit IV (COX-IV, #4844, Cell Signaling Technology, Euroclone), phospho-eNOS (Ser1177, #9571), total eNOS (#9572), phospho-p70S6K (Thr389, #9205), total p70S6K (#9202), phospho-S6 (Ser235/236, # 4858), total S6 (# 2217) (all Cell Signaling Technology, Euroclone). Antibody dilutions ranged from 1:500 to 1:1,000; vinculin (1:3,000; V9131, Sigma-Aldrich) served as loading control. After visualization of phosphorylated proteins, membranes were stripped (Restore™ Stripping Buffer, #EMP100500, Euroclone) and reprobed for total proteins. Detection was performed with HRP-conjugated secondary antibodies (Cell Signaling Technology) and SuperSignal Substrate (#EMP011005, Euroclone) (Corsetti et al., 2014). Bands were imaged with Chemidoc XRS+ (Bio-Rad Laboratories) and quantified by ImageLab software 6.1 (BioRad Laboratories).

2.9 Evaluation of intestinal barrier integrity *in vitro*

Caco-2 cells were seeded on Transwell® polycarbonate membrane inserts (0.4 μm pore size; #CLS3470-48EA, Sigma-Aldrich) at a density of 1×10^5 cells/well (24 well plates). After 14 days of differentiation to form polarized monolayers, barrier integrity was evaluated under both basal and inflammatory conditions. *Basal conditions*. Cells were treated apically with E7 (0.1% or 1.0%) for 24 or 48 h. *Inflammatory conditions*. Cells were pre-treated apically with E7 (1.0%) for 24 h, then exposed to IS consisting of IL-1β (25 ng/mL) and TNF-α (100 ng/mL) added to the basolateral side, and LPS (5 μg/mL) added to both apical and basolateral compartments, for 24, 48 or 72 h (Van De Walle et al., 2010; Varasteh et al., 2018). *Mitochondrial function inhibition*. Cells were apically pre-treated with E7 (1.0%) for

24 h, followed by antimycin A (0.1–1.0 μM) for 24 h. *Barrier assessment*. Transepithelial electrical resistance (TEER) was measured using an EVOM3 epithelial volt/Ω m (World Precision Instruments, MatTek *In Vitro* Life Science Laboratories, Slovak Republic) at indicated time points. TEER values were expressed as % relative to untreated control (Hiebl et al., 2020).

2.10 Mitochondrial respiration analysis

2.10.1 Clark electrode

Basal oxygen consumption rate (OCR) was measured in differentiated Caco-2 cells using a Clark-type oxygen electrode (Rank Brothers Ltd., Newbury, UK). Cells were seeded at 2×10^6 in a 100 mm^2 dish and, upon reaching differentiation (14 days), were treated with E7 (1.0%) for 48 h. Cells were then harvested, resuspended in EMEM (1% heat-inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin), and transferred to the respiration chamber at 37 °C. Sequential additions included oligomycin (0.01 mg/mL; #O4876 Sigma-Aldrich) to inhibit ATP synthase, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, 500 nM; #C2920 Sigma-Aldrich) to assess maximal uncoupled respiration, and rotenone/antimycin (500 nM each; #R8875/#A8674 Sigma-Aldrich). OCR was normalized to total protein content (BCA assay).

2.10.2 Seahorse XF

Mitochondrial respiration and glycolysis were assessed in the inflammatory model using a Seahorse XFe24 Extracellular Flux Analyzer (Agilent, Santa Clara, CA, United States). Caco-2 cells (25,000 cells/well) were seeded in Seahorse XFe24 V7 PS Cell Culture Microplates (#100777-004, Agilent) and cultured for 48 h. Cells were then pre-treated with E7 (1.0%) for 1 h, followed by exposure to IS for an additional 24 h (Crakes et al., 2019). On the day of assay, cells were incubated in Seahorse XF DMEM Medium (pH 7.4; #103575-100, Agilent) containing 5.5 mM glucose (#G8270, Sigma-Aldrich, Milan, Italy), 2 mM L-glutamine (#G7513, Sigma-Aldrich), and 1 mM sodium pyruvate (#S8636, Sigma-Aldrich) for 1 h. OCR and extracellular acidification rate (ECAR) were measured using the Seahorse XF T Cell Metabolic Profiling Kit (#103772-100, Agilent), following sequential injections: oligomycin A (1.5 μM; for proton leak), N5,N6-bis(2-Fluorophenyl) [1,2,5]oxadiazolo[3,4-b]pyrazine-5,6-diamine] (BAM15, 2.5 μM; uncoupler for maximal respiration), and rotenone/antimycin A (0.5 μM each; mitochondrial respiration inhibitors) (Ruocco et al., 2020). Data were normalized to DNA content (CyQUANT™ Cell Proliferation Assay, #C7026, Thermo Fisher Scientific) and analyzed using Wave software (Agilent).

2.11 Immunofluorescence staining and image analysis

Caco-2 cells (300,000 cells/well) were seeded on poly-L-lysine-precoated glass coverslips in a 24-well plates. After 14 days of differentiation, cells were pre-treated with E7 (1.0%) and exposed to IS for 48 h. Cells were then fixed with cold methanol (100%, −20 °C, 10 min), blocked with 3% BSA (#A2153, Sigma-Aldrich) and 1% normal goat serum (NGS, #5425, Cell Signaling Technology) in PBS for 1 h at room temperature, and incubated overnight at 4 °C with anti-occludin antibody (#ab216327, 1:250) in PBS 0.2% BSA and 1.0% NGS. After washing, cells were incubated with Alexa Fluor® 488-conjugated goat anti-rabbit IgG (1:1,000; #111-485-144, Jackson ImmunoResearch, Euroclone) in PBS with 0.1% Triton X-100 (#T-8787, Sigma-Aldrich) for 1 h at room temperature, and nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 1:1,500, D1306, Molecular Probes-Invitrogen) in PBS. Images were acquired using a Zeiss Axio Observer Apotome three microscope (×63 oil immersion objective; 2752 × 2208 pixels). At least 25 random fields per condition were analyzed. Uniform thresholding was applied (Zen Lite v3.11 software, Zeiss) to remove background. Occludin-positive fluorescence areas and nuclei counts were quantified with ImageJ (Fiji).

2.12 IL8 secretion in cell culture supernatants

Differentiated Caco-2 cells (14 days; 24-well plate) were pre-treated with E7 (1.0%) and subsequently exposed to IS for 24, 48, or 72 h. At each time point, culture supernatants (500 μL/well) were collected and centrifuged (2,000 × g, 10 min) to remove debris. IL8 concentrations were measured using the Human IL8 ELISA Kit (ab214030, Abcam) following the manufacturer's instructions and expressed as pg/mL.

2.13 Statistical analysis

Data are expressed as mean ± SEM, with n indicating independent biological replicates (see figure legends). Outlier analysis was performed using the ROUT method (Q = 1%). Normality of data distribution was assessed using multiple tests (D'Agostino–Pearson, Anderson–Darling, Shapiro–Wilk, and Kolmogorov–Smirnov). The homogeneity of variances was verified using Brown–Forsythe and Bartlett's tests. For data that met the assumption of normality, parametric tests were used to assess statistical significance. Comparisons between two groups were conducted using an unpaired Student's t-test, while one-way or two-way ANOVA followed by Tukey's *post hoc* test was applied for multiple comparisons. When data did not meet the assumption of normality, non-parametric tests were employed. Comparisons between two groups were performed using the Mann–Whitney test, and the Kruskal–Wallis test followed by Dunn's *post hoc* test was applied for multiple comparisons. Significance was set at $p < 0.05$. Statistical analyses were performed using the Prism 6.0 software (GraphPad Software, Inc.).

3 Results

3.1 EAA diet prevents and reverts obesity and type 2 diabetes in DIO mice

To assess the preventive and therapeutic effects of EAAs in DIO, male C57BL/6N mice were fed HFD or HFD-EAA for 33 weeks, with a subgroup of obese HFD-fed mice switched to HFD-EAA after

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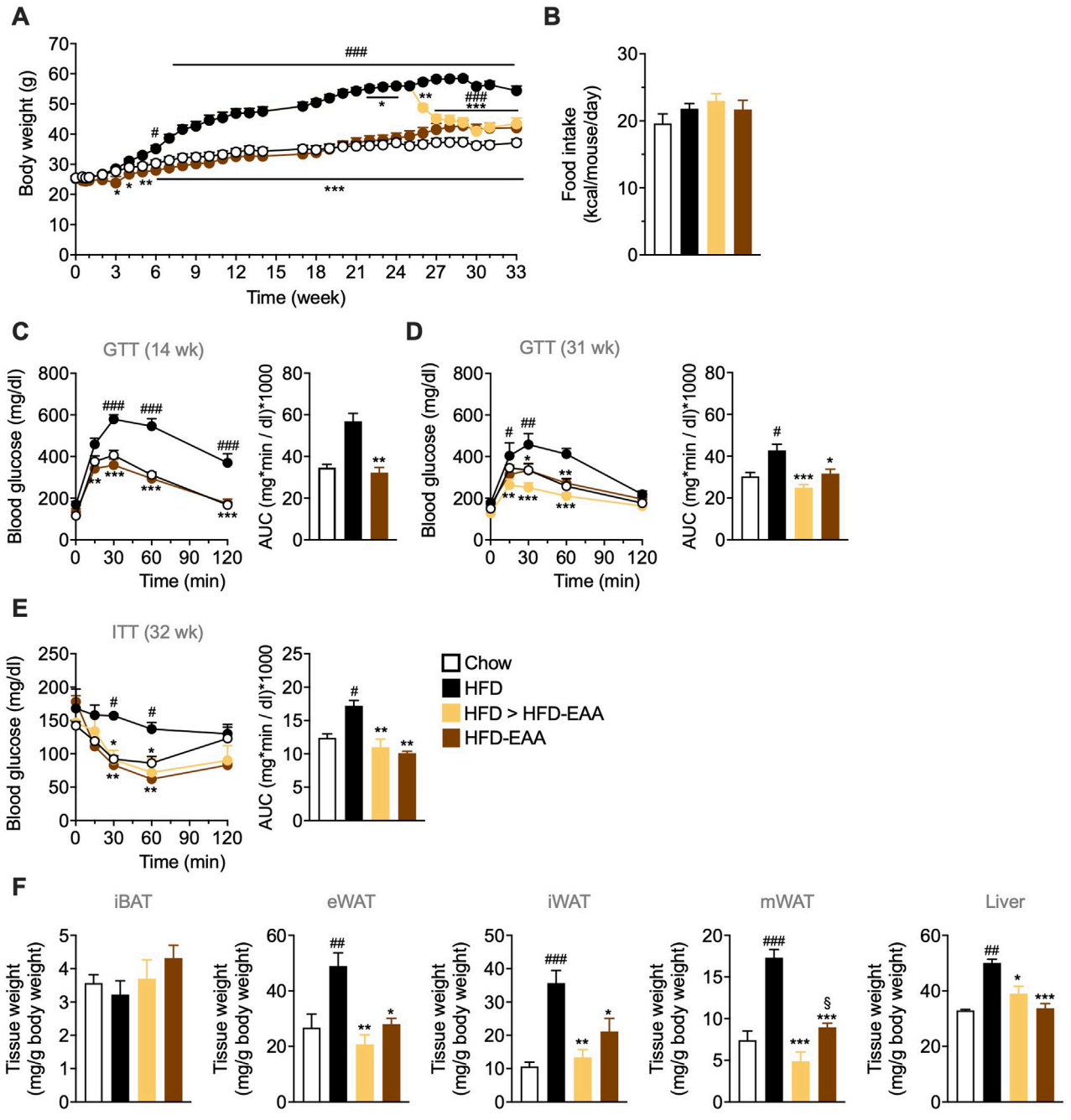

FIGURE 1
EAA-based diet consumption prevents and reverts obesity and glucose intolerance in diet-induced obese mice. **(A)** Body weight (BW, g). **(B)** Cumulative food intake (kcal/mouse/day). **(C, D)** Glucose tolerance test (GTT): glucose (1.5 g/kg BW; i.p.) administered after overnight fasting at the indicated time points. **(E)** Insulin tolerance test (ITT): insulin (0.75 U/kg BW; i.p.) administered after 4 h of fasting. Blood glucose was measured from tail-vein blood. Results are reported as time-course curves (*left*) and area under the curve (AUC) (*right*). **(F)** Weight of interscapular brown adipose tissue (iBAT), epididymal white adipose tissue (eWAT), inguinal white adipose tissue (iWAT), mesenteric white adipose tissue (mWAT), and liver, expressed as mg per g BW. Data are mean ± SEM (**A, B, F** n = 6–10/group; **C** n = 6–7/group; **D, E** n = 5/group). Two-way ANOVA (**A, C–E** left) or one-way ANOVA (**B, D and E** right, **(F)** Kruskal–Wallis test (**C** right). #p < 0.05, ##p < 0.01, ###p < 0.001 vs. Chow; *p < 0.05, **p < 0.01, ***p < 0.001 vs. HFD; §p < 0.05 vs. HFD > HFD-EAA.

25 weeks (HFD > HFD-EAA). HFD feeding induced progressive weight gain (+47% vs. chow), with no substantial differences in FI (Figures 1A,B). In contrast, HFD-EAA diet significantly reduced BW both in the preventive (−22% vs. HFD) and therapeutic (−23% vs. HFD) settings, despite similar caloric intake (Figures 1A,B). Furthermore, HFD feeding impaired glucose tolerance and insulin sensitivity and promoted the accumulation of visceral (epididymal and mesenteric) and subcutaneous (iWAT) fat, accompanied by increased liver weight (Figures 1C–F). Strikingly, HFD-EAA both prevented and reversed these metabolic disturbances, concomitant with reduced adipose depots and decreased liver weight (Figures 1C–F). Notably, mesenteric fat reduction is particularly relevant

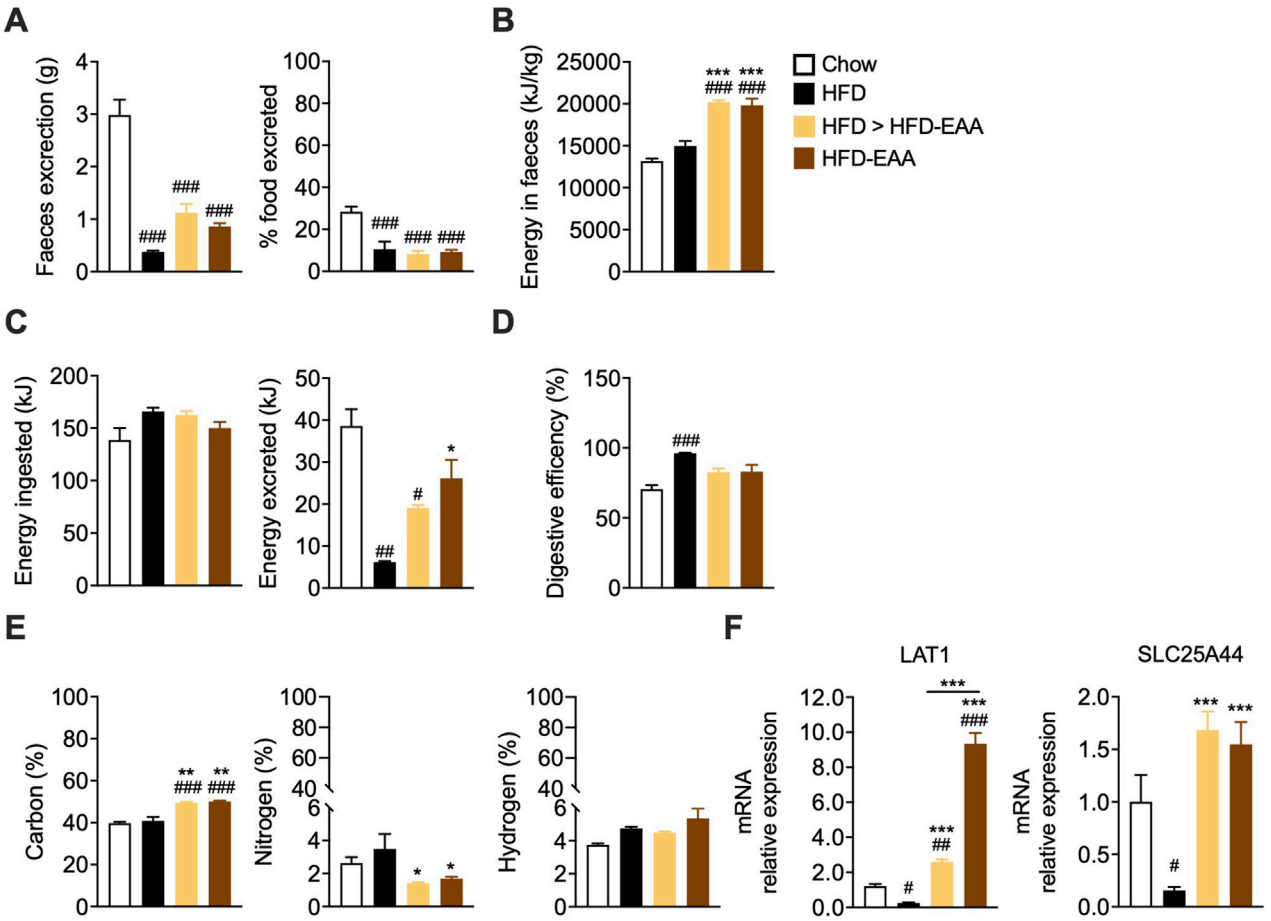

FIGURE 2
EAA-based diet modulates nutrient absorption and enhances amino acid transport. **(A)** Faecal weight (g; *left*) and percentage of food excreted (%; *right*), calculated as faecal weight (g)/food intake (g) × 100, measured in mice fed *ad libitum* for 48 h, at week 27 of treatment. **(B)** Faecal energy content (kJ/kg), assessed by bomb calorimetry at week 33. **(C)** Energy intake (kJ; *left*), calculated as food intake (g) × dietary energy density (kcal/g) × 4.184 (kcal to kJ conversion), and faecal energy excreted (kJ; *right*), calculated as faecal weight (g) × faecal energy content (kJ/kg) ÷ 1,000 at week 33. **(D)** Digestive efficiency (%), calculated as [1 − (energy excreted/energy ingested)] × 100 (week 33). **(E)** Carbon, nitrogen, and hydrogen content in faeces (% dry mass), determined by bomb calorimetry (week 33). **(F)** mRNA expression of amino acid transporters in the jejunum. Transcript levels were normalized to GAPDH and expressed relative to chow-fed mice (set as 1.0). Data are mean ± SEM (**A**: n = 6–10/group; **B–E**: n = 3/group; **F** n = 3–4/group). One-way ANOVA **(A–F)**. Kruskal–Wallis test (**E** right). #p < 0.05, ##p < 0.01, ###p < 0.001 vs. Chow; *p < 0.05, **p < 0.01, ***p < 0.001 vs. HFD.

because visceral adiposity contributes to systemic low-grade inflammation and intestinal barrier dysfunction (Kredel and Siegmund, 2014). Mesenteric fat expansion ("creeping fat") is a hallmark of Crohn's disease and aggravates intestinal inflammation (Bryant et al., 2019; Ha et al., 2020). Consistent with this, EAA-mediated reduction of visceral fat mass has been linked to improved metabolic and intestinal outcomes (Luck et al., 2015). In summary, HFD-EAA both prevented and reversed obesity-associated metabolic disturbances, reduced visceral adiposity—including mesenteric fat—and improved glucose homeostasis, establishing a favorable systemic context for intestinal barrier protection.

3.2 EAA diet modulates nutrient gut absorption favoring amino acid transport

We next examined how EAA supplementation influenced nutrient handling in DIO. Consistent with previous findings in low-fat contexts (Ruocco et al., 2020), HFD and HFD-EAA reduced faecal output and the percentage of excreted food compared to chow (Figure 2A), indicating slower intestinal transit, a feature often associated with obesity-related gut dysfunction. However, when evaluating caloric absorption using bomb calorimetry—a measure of digestive function and caloric bioavailability—a different pattern emerged. EAA treatment increased the faecal energy content, which remained unchanged in the HFD group compared to the chow group (Figure 2B). This suggests that, despite the reduced faecal output, HFD-fed mice do not exhibit greater energy loss, indicating a potentially non-selective and passive nutrient absorption likely due to increased intestinal permeability (*i.e.*, leaky gut). Accordingly, despite similar FI, the amount of energy excreted in faeces was lower, and the absorption efficiency (*i.e.*, retained energy relative to ingested food) was higher in HFD-fed mice (Figures 2C,D). This effect may reflect a compensatory metabolic adaptation or result from increased paracellular transport due to gut barrier dysfunction (Murphy et al., 2015). In contrast, EAA-treated mice exhibited

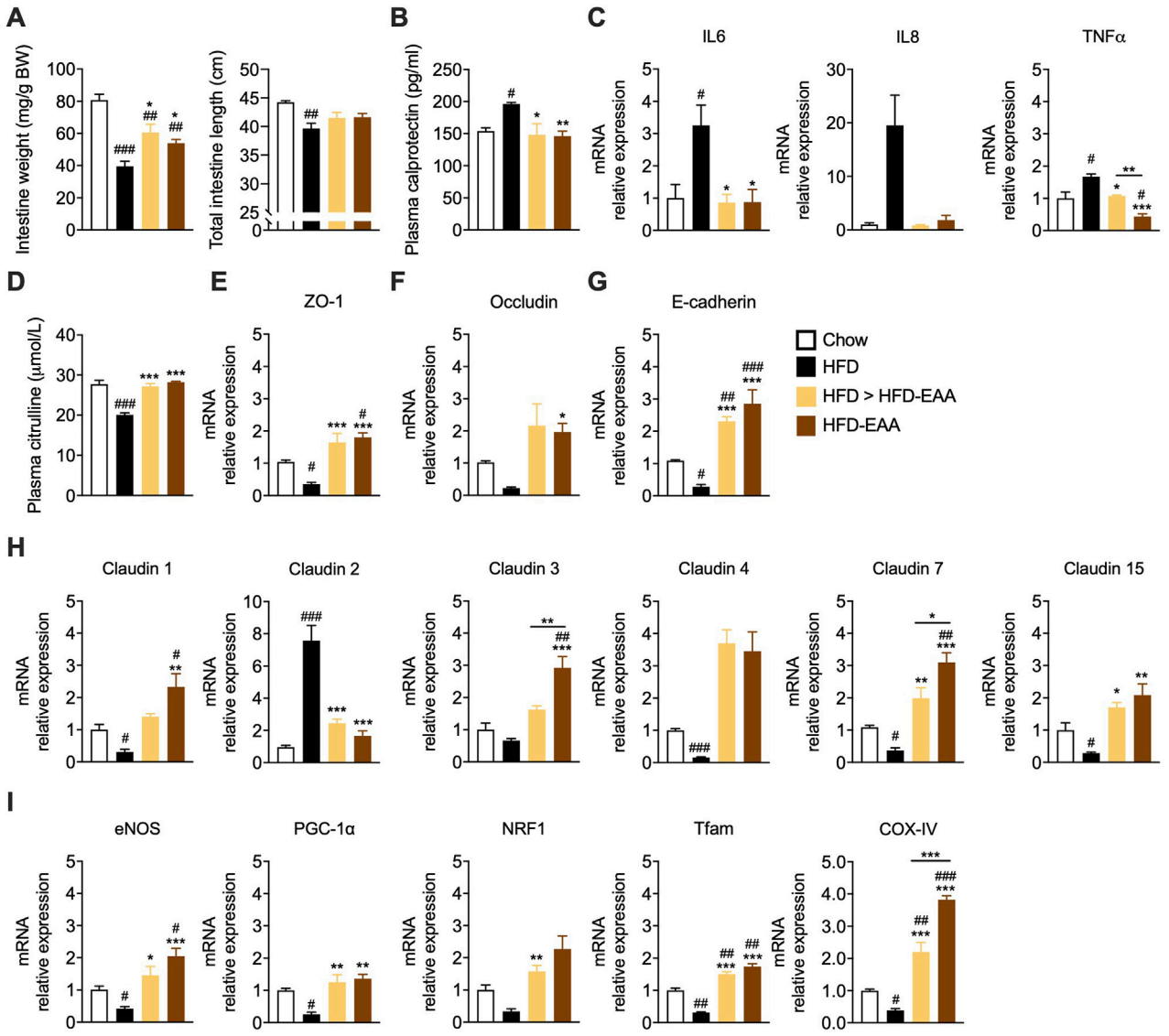

FIGURE 3
EAA-based diet preserves intestinal barrier integrity and reduces inflammation *via* mitochondrial pathways. **(A)** Intestine weight normalized to BW (mg/g; *left*) and length (cm; *right*). **(B)** Plasma calprotectin levels (pg/mL) at week 33, as a marker of inflammation. **(C and E–I)** mRNA expression in jejunum at week 33. Inflammatory markers (IL6, IL8, TNFα) **(C)**. Intestinal barrier markers (ZO-1, occludin, E-cadherin, claudins) **(E–H)**. Mitochondrial biogenesis markers **(I)**. Transcript levels were normalized to GAPDH and expressed relative to control (CTRL) mice (set as 1.0). **(D)** Plasma citrulline levels (µmol/L) at week 33, as biomarker of gut permeability and enterocyte mass. Data are mean ± SEM (**A**: n = 6–10/group; **B** n = 4–6/group; **C and E–I**: n = 3–4/group; **D** n = 3–6/group). One-way ANOVA **(A–I)** or Kruskal–Wallis test (**C** - IL8, **F**, **H** Claudin 4, and **I** NRF1). #p < 0.05, ##p < 0.01, ###p < 0.001 vs. CTRL; *p < 0.05, **p < 0.01, ***p < 0.001 vs. HFD.

greater faecal energy loss and similar digestive efficiency compare to chow (Figures 2C,D). This suggests a selective modulation of nutrient absorption without impairing overall intestinal function. Elemental analysis of faeces supports this interpretation. EAA diets led to a higher faecal carbon content, indicating reduced absorption of carbohydrates and/or lipids, and a significant decrease in faecal nitrogen, consistent with improved protein/amino acid absorption (Figure 2E). Gene expression analysis aligns with these findings. HFD downregulated LAT1, the L-type amino acid transporter 1, a key amino acid transporter in IECs (Fraga et al., 2005), whereas HFD-EAA restored its expression, suggesting a likely increased intestinal EAA absorption following supplementation (Figure 2F). Similarly, HFD reduced SLC25A44, a mitochondrial amino acid transporter (Yoneshiro et al., 2019), which was normalized by EAAs, suggesting preserved mitochondrial integrity and amino acid utilization (markers of improved gut and metabolic efficiency) (Figure 2F). Plasma profiling confirmed increased levels of EAAs included in HFD-EAA diet (Supplementary Figure S3), while correlation analysis linked amino acids such as asparagine, phenylalanine, and tyrosine to impaired glucose homeostasis and visceral adiposity, both normalized by EAAs (Supplementary Table S2). In addition, arginine, histidine, lysine, and tryptophan correlated with faecal output, potentially indicating differences in absorption efficiency (Supplementary Table S2). Collectively, EAAs

selectively improved amino acid absorption and utilization, contrasting with the non-specific nutrient uptake seen in HFD-fed mice. This effect likely contributes to enhanced epithelial mitochondrial function and intestinal barrier integrity.

3.3 EAAs preserve intestinal barrier integrity and reduce inflammation *via* mitochondrial pathways

To determine whether EAA supplementation protected intestinal barrier structure in obesity, we assessed gut morphometric parameters (weight and length), inflammation, and TJ integrity (Rohr et al., 2020). HFD-fed, mice exhibited reduced intestinal length and weight—hallmarks of intestinal dysfunction—accompanied by increased plasma calprotectin, an inflammation marker (Malham et al., 2019), and higher expression of pro-inflammatory cytokines (IL6, IL8, and TNFα) in jejunal tissue, all of which were attenuated or reversed by EAAs (Figures 3A–C). Accordingly, plasma levels of cystine, reduced in HFD-EAA-fed mice (therapeutic schedule), was positively correlated with intestinal weight, suggesting a potential link with gut structural changes (Supplementary Table S2). Plasma citrulline—a non-proteinogenic amino acid predominantly synthesized by small intestinal enterocytes and recognized as a biomarker of enterocyte mass and barrier function (Crenn et al., 2008)—was markedly reduced in HFD-fed mice and restored by EAAs (Figure 3D). Notably, citrulline positively correlated with branched-chain amino acids (BCAAs), linking EAA status to gut epithelial health (Supplementary Table S2). At the structural level, HFD induced TJ remodeling with downregulation of ZO-1, occluding, E-cadherin, and barrier-forming claudins (1, 3, 4, 7, 15), alongside upregulation of pore-forming claudin-2 (Figures 3E–H) (Ahmad et al., 2017). EAA supplementation preserved TJ and AJ expression and prevented claudin 2 induction, indicating intact epithelial integrity. Given the central role of mitochondria in epithelial renewal and barrier maintenance, we assessed mitochondrial markers. HFD markedly reduced jejunal expression of PGC-1α and other genes involved in mitochondrial biogenesis, consistent with mitochondrial dysfunction (Guerbette et al., 2022; 2025) (Figure 3J). EAA supplementation restored their expression, supporting the preservation of mitochondrial function (Figure 3J). These findings show that EAAs counteract HFD-induced intestinal inflammation and barrier dysfunction by modulating the expression of genes involved in mitochondrial biogenesis and epithelial integrity, suggesting a potential mechanism for reducing the 'leaky gut' phenotype typical of obesity.

3.4 E7 improves intestinal barrier integrity by stimulating mitochondrial biogenesis *in vitro*

To dissect whether EAAs act directly on IECs, we used differentiated Caco-2 cell monolayers as an *in vitro* intestinal barrier model (Fogh J and Trempe G, 1975; Engle et al., 1998; Haddad et al., 2023). To define the optimal experimental conditions, we performed dose- and time-response studies. Differentiated Caco-2 monolayers (14 and 21 days post-confluence) were treated with E7 for either 24 or 48 h. E7 treatment (0.1%–1.0%) did not reduce cell viability. Instead, the MTT assay indicated increased cell viability not detected by the TOX6 test (Figure 4A), suggesting enhanced mitochondrial activity, as MTT primarily reflects dehydrogenase activity, including mitochondrial succinate dehydrogenase (Rai et al., 2018). Under basal conditions, E7 upregulated TJ and AJ markers (ZO-1, occludin, claudin 1/3/4, E-cadherin), most effectively in differentiated cells (14 days) treated with 1% E7 for 48 h (Figures 4B–F; Supplementary Figures S4A–D). This was accompanied by increased TEER, indicating improved barrier function (Figure 4G).

E7 enhanced mitochondrial biogenesis mainly in differentiated Caco-2 cells (14 days), as shown by upregulation of PGC-1α, Tfam, Cytc, and COX-IV, and increased OCR (+44% vs. control) (Figures 5A,B; Supplementary Figure S4E). These effects were linked to elevated eNOS phosphorylation, consistent with known EAA-induced mitochondrial activation (Figure 5D) (Nisoli et al., 2008). Interestingly, E7 reduced phosphorylation of mTORC1 effectors, significantly decreasing S6 phosphorylation and showing a trend toward reduced p70S6K phosphorylation (Figure 5E). These data suggest that the observed barrier improvement and mitochondrial stimulation involve mTORC1 inhibition, a mechanism associated with improved gut permeability under chronic stress, in accordance with Kaur et al. (Kaur and Moreau, 2019; 2021).

To determine whether E7's barrier-protective action relies on mitochondrial function, we challenged Caco-2 monolayers with antimycin A, a complex III inhibitor. While antimycin A alone did not significantly alter TEER under our conditions, pre-treatment with E7 (1.0%) failed to enhance TEER in the presence of antimycin A (0.1–1.0 μM), indicating a loss of E7's barrier benefit during mitochondrial inhibition (Figure 5F). Importantly, cell viability remained unchanged across conditions (Figure 5G). These data support that E7 improves barrier integrity through mitochondrial activation rather than non-specific trophic effects.

In addition, we confirmed that the inclusion of Krebs cycle intermediates in E7 resulted in greater efficiency than the standard EAA mixture (*i.e.*, without Krebs cycle intermediates) used *in vivo* (Supplementary Figure S5) (Brunetti et al., 2020; Tedesco et al., 2020b). For this reason, we chose to use E7 for the *in vitro* experiments.

In summary, these findings indicate that E7 exerts a direct effect on enterocytes by promoting mitochondrial biogenesis, closely associated with improved intestinal barrier function. Preliminary data identified 14 days of differentiation as the optimal time point for evaluating the effects of E7, providing the basis for subsequent experiments under inflammatory conditions. Additionally, treatment with E7 at 1.0% proved most effective at both 24 and 48 h, consistent with our previous studies in other cell types (Tedesco et al., 2018; 2020a; 2022; Ruocco et al., 2020). Notably, the E7 concentrations used *in vitro* were lower than the peak luminal EAA levels estimated *in vivo* (Supplementary Figure S2D), yet still elicited significant protective effects. This observation strengthens the translational value of our *in vitro* model, indicating that even lower luminal concentrations of EAAs are sufficient to promote mitochondrial function and barrier integrity in IECs.

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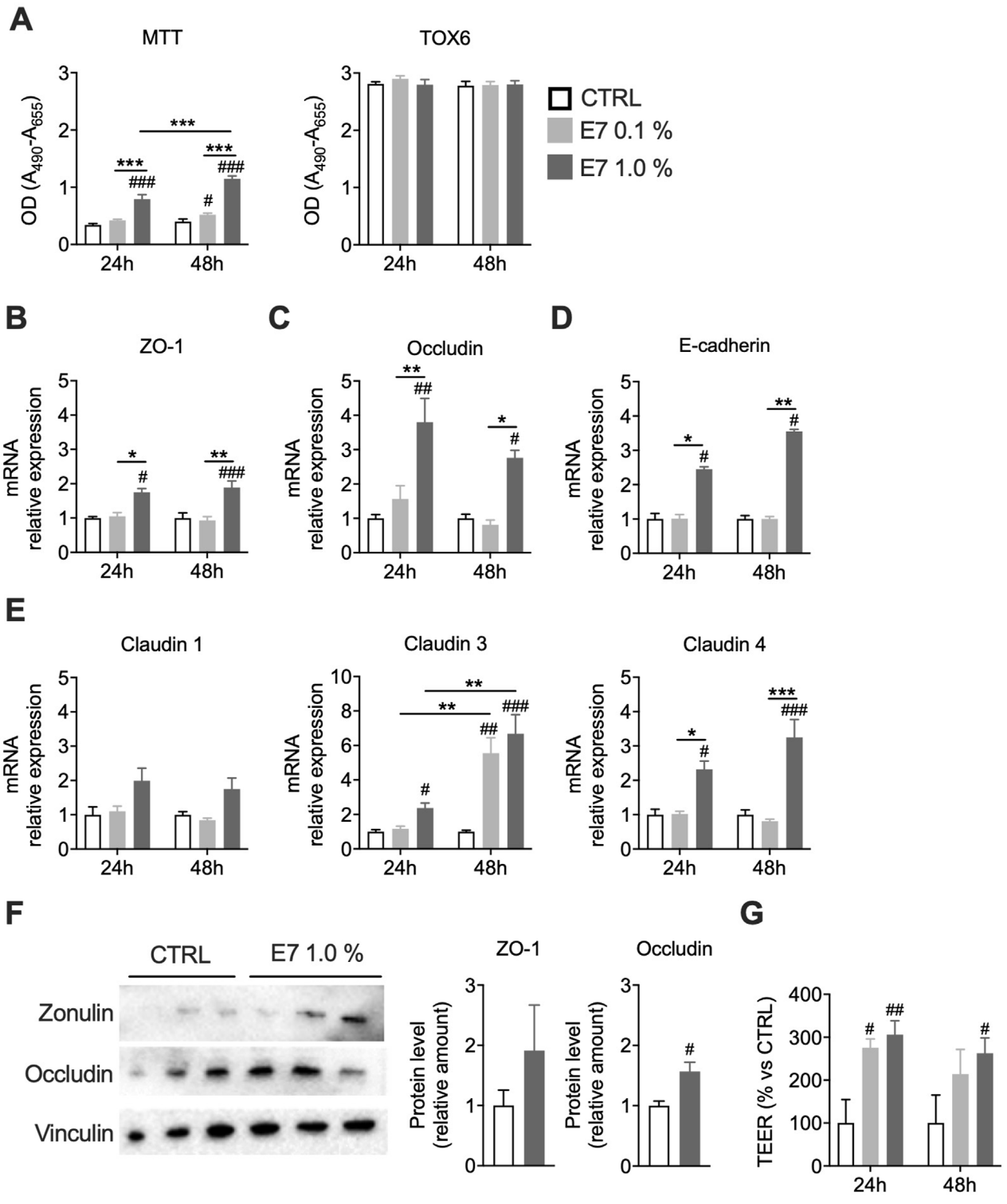

FIGURE 4
E7 improves intestinal barrier function in an *in vitro* Caco-2 model. Caco-2 cells differentiated for 14 days were treated with E7 (0.1 or 1.0%) for 24 h or 48 h. **(A)** Cell viability assessed by MTT and TOX6 assays. **(B–E)** mRNA levels of intestinal permeability markers. Transcript levels were normalized to GAPDH and expressed relative to untreated control (CTRL) cells (set as 1.0). **(F)** Western blot analysis of ZO-1 and occluding protein levels in cells treated for 48 h. Data are presented as relative amounts normalized to CTRL (set as 1.0); representative blots from three independent experiments are shown. **(G)** Transepithelial electrical resistance (TEER) across the monolayer, expressed as percentage relative to CTRL. Data are mean ± SEM (**A–E**: n = 4/group; **F, G** n = 3/group). Two-way ANOVA **A–E and G**) or unpaired Student's t-test **(F)**. #p < 0.05, ##p < 0.01, ###p < 0.001 vs. CTRL.

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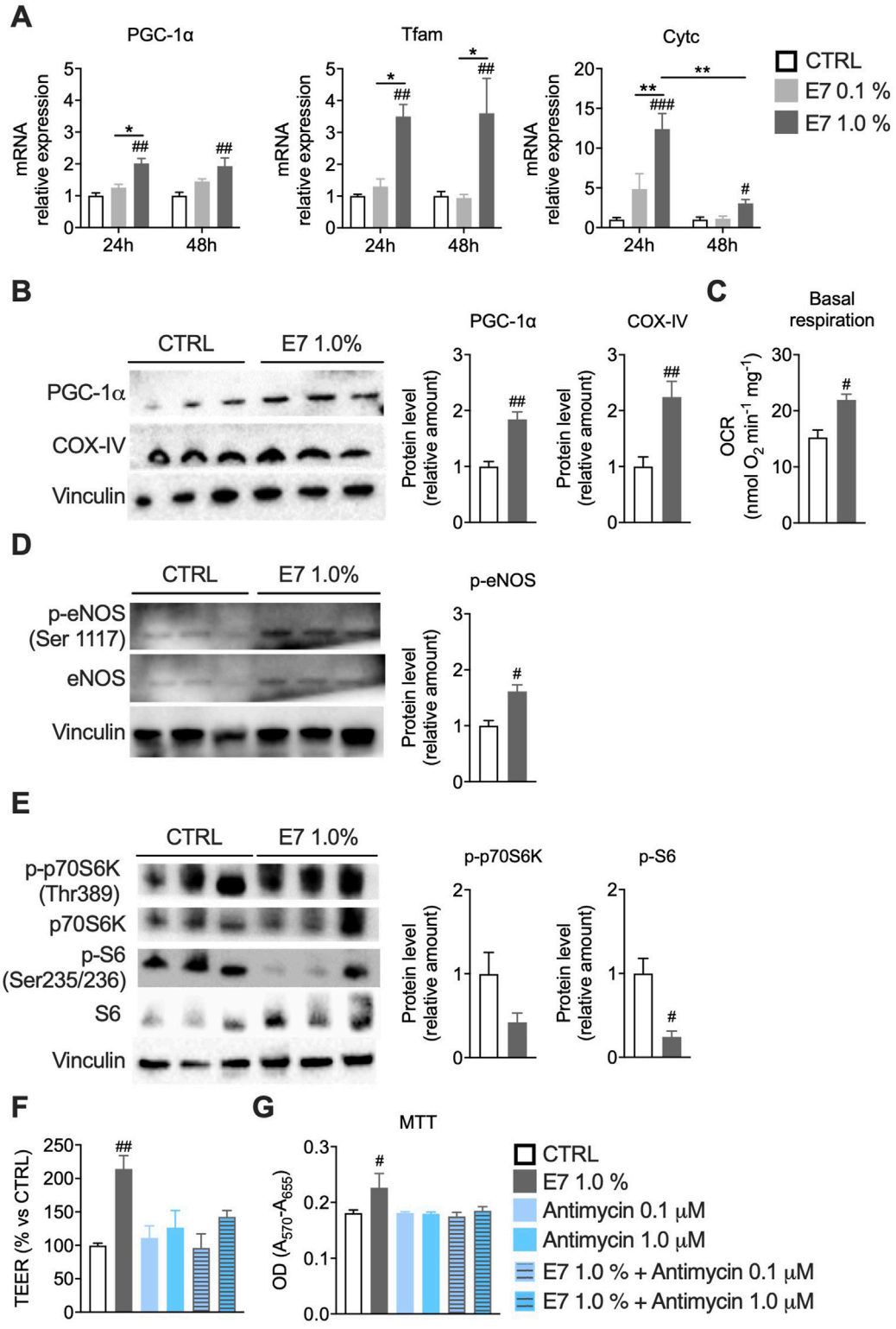

FIGURE 5
E7 enhances intestinal barrier function by stimulating mitochondrial activity. **(A)** mRNA levels of mitochondrial biogenesis genes. Transcript levels were normalized to GAPDH and expressed relative to untreated control (CTRL) cells (set as 1.0). **(B)** Western blot analysis of PGC-1α and COX-IV protein levels in Caco-2 cells treated for 48 h. Data are presented as relative to CTRL (set as 1.0); representative blots from three independent experiments are shown. **(C)** Basal OCR measured with a Clark's electrode in Caco-2 cells treated for 48 h; OCR normalized to total protein content. **(D)** Western blot analysis of phosphorylated-eNOS (p-eNOS) normalized to total eNOS. **(E)** Western blot analysis of phosphorylated p70S6K normalized to total p70S6K and phosphorylated S6 normalized to total S6. **(F)** TEER in Caco-2 monolayers (14 days) pre-treated with E7 (1.0%) for 24 h and then exposed to antimycin A (0.1–1.0 μM) for other 24 h; TEER expressed as % vs. CTRL. **(G)** Cell viability (MTT) in differentiated Caco-2 cells pre-treated with E7 (1.0%) and exposed to

(*Continued*)

FIGURE 5 (Continued)
antimycin A (0.1–1.0 μM) for 24 h. Data are mean ± SEM (**A**: n = 4/group; **B–F**: n = 3/group; **G** n = 5/group). Two-way ANOVA **(A, F, G)**, unpaired Student's t-test **(B–E)**, Mann-Whitney test (B–right). #p < 0.05, ##p < 0.01, ###p < 0.001 vs. CTRL.

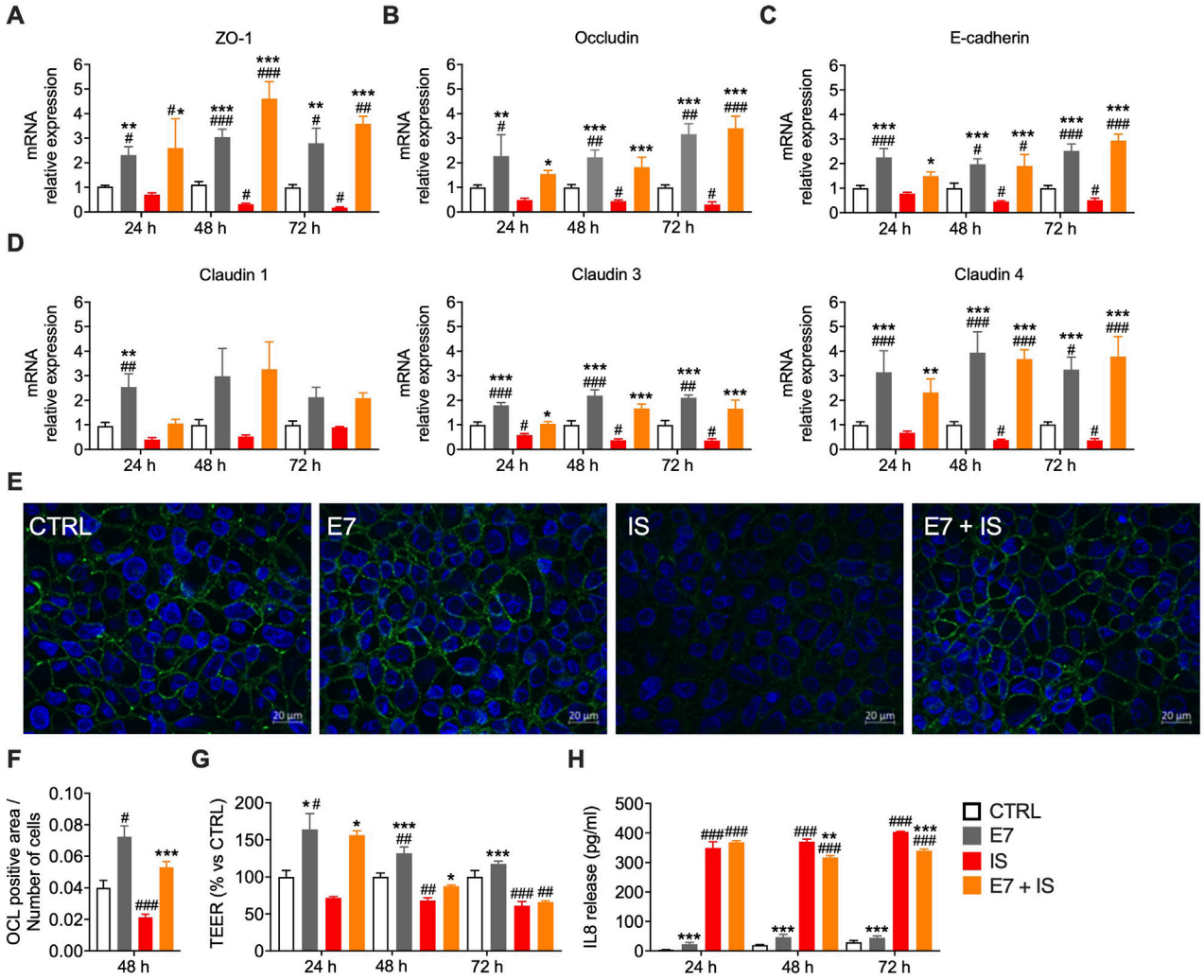

FIGURE 6
E7 preserves intestinal barrier integrity and exerts anti-inflammatory effects in an *in vitro* model of gut inflammation. **(A–D)** mRNA levels of intestinal permeability markers in Caco-2 cells differentiated for 14 days and treated with E7 (1.0%) ± inflammatory stimuli (IS) for 24 h, 48 h, or 72 h. Transcript levels were normalized to GAPDH and expressed relative to untreated control (CTRL) cells (set as 1.0). **(E,F)** Immunofluorescence analysis of occluding in Caco-2 cells differentiated for 14 days and treated with E7 (1.0%) ± IS for 48 h. Representative images of occludin staining (green); cell nuclei were stained with DAPI; scale bar = 20 μm **(E)**. Quantification of occludin-positive area (n = 25 fields) **(F)**. **(G)** TEER in Caco-2 monolayers (14 days post-differentiation) pre-treated with E7 (1.0%) for 24 h, then exposed to IS for 24–72 h. TEER is expressed as percentage relative to CTRL. **(H)** IL8 secretion in culture medium from Caco-2 cells pre-treated with E7 (1.0%) for 1 h, then exposed to IS for 24–72 h; values expressed in pg/mL. Data are mean ± SEM (**A–D**: n = 6/group; **F**: n = 3/group; **G**: n = 5/group). Two-way ANOVA **(A–D, G, H)** or one-way ANOVA **(F)**. #p < 0.05, ##p < 0.01, ###p < 0.001 vs. CTRL; *p < 0.05, **p < 0.01, ***p < 0.001 vs. IS.

3.5 E7 preserves intestinal barrier integrity and reduces inflammation *in vitro*

We next investigated whether E7 protects IECs under inflammatory stress. Differentiated Caco-2 monolayers, a well-established model of gut epithelium, respond to IS by producing cytokines such as IL8 (Hollebeeck et al., 2012; Rodríguez-Ramiro et al., 2013; Ponce de León-Rodríguez et al., 2019). Cells were exposed to IL-1β, TNF-α, and LPS (*i.e.*, IS) for 24–72 h, with or without E7 pre-treatment (1.0%). Since cytokine release typically represents an early response (12–24 h), whereas epithelial barrier disruption—manifested by reduced TEER and TJ protein expression—requires prolonged exposure (≥48 h) (Al-Sadi et al., 2014), we evaluated effects at 24, 48, and 72 h. As previously

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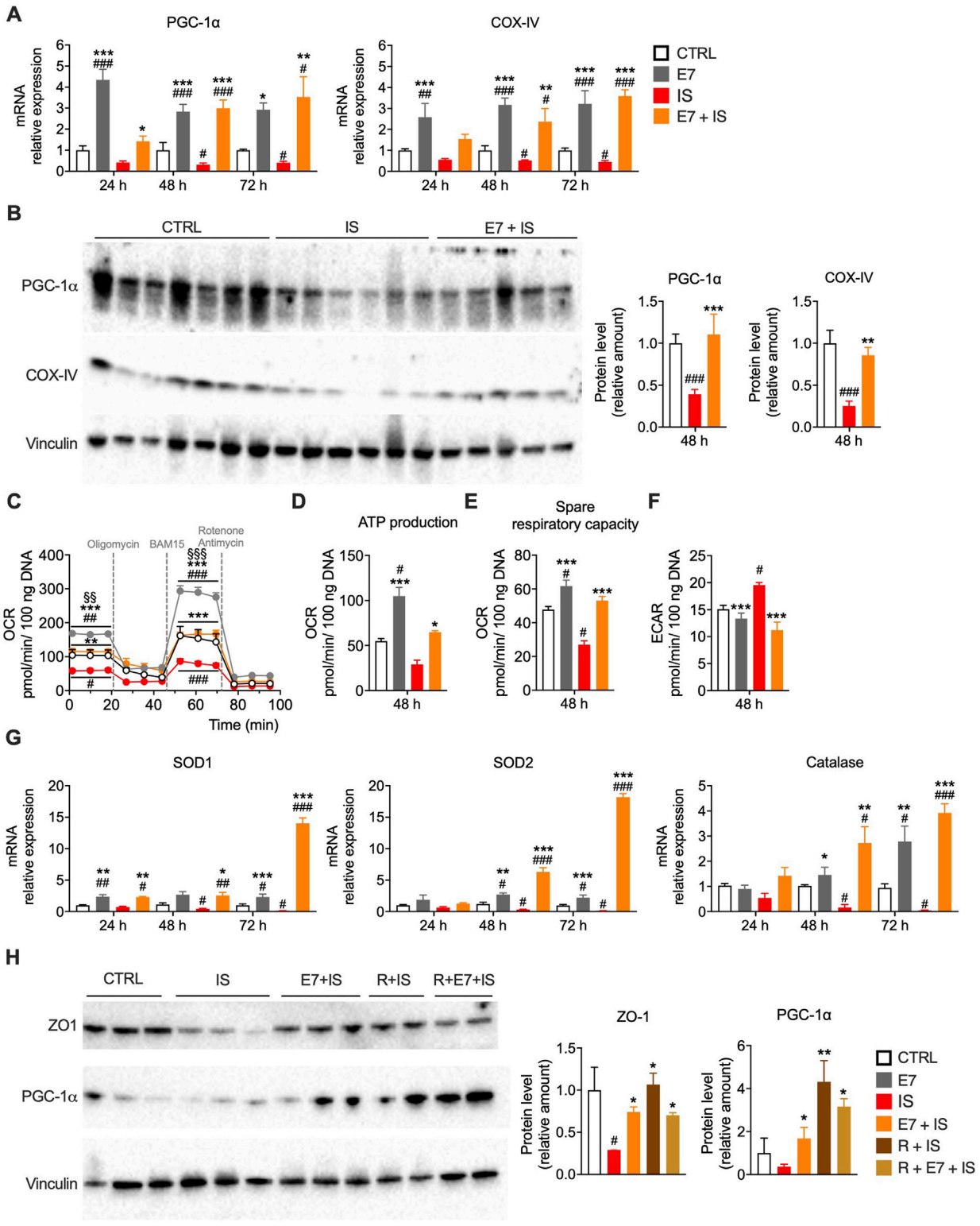

FIGURE 7
E7 prevents mitochondrial dysfunction and metabolic shift during inflammation in Caco-2 cells. **(A)** mRNA levels of mitochondrial genes in Caco-2 cells differentiated for 14 days and treated with E7 (1.0%) ± IS for 24, 48, or 72 h. **(B)** Western blot analysis of mitochondrial protein markers in cells treated for 48 h. Data are presented as relative amounts normalized to CTRL (set as 1.0); representative blots from three independent experiments. **(C–F)** Mitochondrial respiration analysis using Seahorse XFe24 in Caco-2 cells treated for 48 h: oxygen consumption rate (OCR) **(C)**, ATP production **(D)**, spare respiratory capacity (maximal respiration minus basal respiration) **(E)**, and extracellular acidification rate (ECAR) as a measure of glycolysis **(F)**. **(G)** mRNA levels of antioxidant enzyme genes, normalized to GAPDH and expressed relative to CTRL (set as 1.0). **(H)** Western blot analysis of ZO-1 (gut

(*Continued*)

FIGURE 7 (Continued)
permeability marker) and PGC-1α (mitochondrial biogenesis marker) in cells treated for 48 h with E7 ± IS, with or without rapamycin (R, 50 nM). CTRL values were set as 1.0. Data are as mean ± SEM (**A, G**: n = 6/group; **B**: n = 6–7/group; **C–F**: n = 5/group; **H**: n = 2–3/group). Two-way ANOVA **(A,C and G)**, one-way ANOVA **(B,E,F and H)** or or Kruskal–Wallis test **(D)**. #p < 0.05, ##p < 0.01, ###p < 0.001 vs. CTRL; *p < 0.05, **p < 0.01, ***p < 0.001 vs. IS.

observed, neither E7 nor IS compromised cell viability (Supplementary Figure S6).

Under basal conditions, E7 enhanced mRNA and protein levels of TJ and AJ components (ZO-1, occludin, E-cadherin, claudins), with effects sustained up to 72 h (Figures 6A–F), confirming our prior findings. Exposure to IS markedly disrupted barrier integrity, reducing both gene and protein expression, whereas co-treatment with E7 prevented these alterations, preserving TJ architecture (Figures 6A–F). TEER measurements confirmed functional protection. E7 increased TEER under basal conditions, although this effect declined over time (Figure 6G). IS reduced TEER beginning at 48 h (−30% vs. CTRL at 72 h), but E7 significantly mitigated this decline, improving TEER by +26% vs. IS at 24 h and preserving barrier integrity at 48 h (−17% vs. CTRL; +12% vs. IS) (Figure 6G). At 72 h, however, protection diminished, likely reflecting amino acid depletion (Figure 6G).

To assess anti-inflammatory effects, IL8 secretion was quantified. As expected, IS induced a robust IL8 response (+99-fold vs. CTRL at 24 h), persisting through 72 h (Figure 6H). E7 reduced IL8 release at 48 h (−15% vs. IS), and 72 h (−16% vs. IS), even when TEER protection was waned (Figures 6G,H). Together, these findings demonstrate that E7 preserves barrier integrity and function while attenuating cytokine secretion under inflammatory stress, with efficacy sustained for up to 48 h.

3.6 E7 prevents mitochondrial dysfunction and metabolic shift during inflammation *in vitro*

Mitochondria are central to epithelial energy metabolism, survival, and immune regulation (Rath et al., 2018). Mitochondrial dysfunction is common in gastrointestinal diseases characterized by barrier disruption, including obesity-associated gut inflammation (Wang et al., 2014; Guerbette et al., 2024). It involves downregulation of electron transport chain (ETC.) genes, impaired oxidative phosphorylation, reduced respiration, altered membrane potential, and excessive ROS production (Chernyavskij et al., 2023), all of which compromise TJ integrity and increase epithelial permeability. While basal mitochondrial ROS support epithelial renewal, excessive ROS coupled with diminished antioxidant defenses (*e.g.*, catalase, SOD2) drives oxidative damage and apoptosis (Chernyavskij et al., 2023).

To determine whether E7 mitigates inflammation-induced mitochondrial dysfunction, we assessed mitochondrial biogenesis and respiration in Caco-2 cells exposed to IS ± E7. IS significantly reduced PGC-1α and COX-IV expression (mRNA and protein) at 48 h, whereas E7 prevented this decline at all time points (Figures 7A,B). Mitochondrial function, assessed *via* OCR, was markedly impaired by IS, with reductions in basal (~43%), ATP-linked respiration (~47%) and spare respiratory capacity (~44%) (Figures 7C–E), consistent with prior reports of cytokine-induced mitochondrial dysfunction (Crakes et al., 2019). IS also induced a metabolic shift toward glycolysis, evidenced by increased ECAR values (Figure 7F), similar to findings in endothelial cells (Xiao et al., 2021). E7 restored oxidative phosphorylation and attenuated this glycolytic shift, promoting a more energy-efficient phenotype. Inflammation also downregulated antioxidant enzymes, suggesting excessive ROS involvement (Figure 7G). E7 prevented this downregulation and enhanced antioxidant enzyme expression, indicating an antioxidant effect that may contribute to its anti-inflammatory action (Figure 7G).

To assess the role of mTORC1, we treated differentiated Caco-2 cells with rapamycin (an mTORC1 inhibitor, 50 nM) ± E7 (1.0%, 48 h) under IS. Both rapamycin and E7 individually preserved ZO-1 and PGC-1α expression, while their combination showed no additive effect (Figure 7H), suggesting that E7 may act partly through mTORC1 inhibition. This result is consistent with previous studies in which Caco-2 cells treated with rapamycin exhibited improved gut permeability in association with mTORC1 inhibition (Xu et al., 2024). Together, these findings link inflammation-induced gut barrier disruption to mitochondrial dysfunction. E7 counteracts the IEC damage by preserving mitochondrial biogenesis, restoring respiration, reducing glycolytic shift, and enhancing antioxidant defenses. Its overlap with rapamycin implicates mTORC1 modulation as part of its mechanism. Thus, E7 exerts anti-inflammatory effects through integrated mitochondrial and signaling pathways that sustain epithelial barrier integrity.

4 Discussion

Unhealthy diets compromise the gut barrier, driving chronic inflammation that contributes to IBDs, obesity, diabetes, autoimmune, and aging-related diseases (Martel et al., 2022). High-fat food intake, in particular, disrupts barrier function by inducing mucosal inflammation and dysbiosis, which contribute to systemic endotoxemia and metabolic complications (Genser et al., 2018; Mouries et al., 2019). Mitochondria in IECs are crucial for maintaining barrier homeostasis (Rath et al., 2018). However, chronic inflammation and HFD impair mitochondrial function, weaken mucosal integrity, and disrupt key proteins regulating permeability (Guerbette et al., 2022; 2024; 2025).

Lifestyle modification remains one of the most effective strategies to prevent and reverse obesity and its complications. A critical question is whether dietary interventions can improve conditions associated with increased gut permeability. Evidence from both human and animal studies suggests that several approaches, including dietary fiber reintroduction (Krawczyk et al., 2018), caloric restriction (Ott et al., 2017), intermittent fasting and fasting-mimicking diets (Rangan et al., 2019; Liu

et al., 2020) and supplementation with bioactive compounds such as berberine (Amasheh et al., 2010), curcumin (Feng et al., 2019), quercetin (Suzuki and Hara, 2009), and resveratrol (Mayangsari and Suzuki, 2018), enhance barrier integrity and attenuate inflammation. However, despite its robust health benefits in preclinical models, caloric restriction and intermittent fasting are notably difficult to sustain in humans over time, and frequently result in weight regain, with high heterogeneity across participants (Langeveld and DeVries, 2015; Wang et al., 2025). In parallel, most bioactive compounds are characterized by poor bioavailability and rapid metabolism, which significantly limits their translational potential and often necessitates advanced formulations to achieve clinically meaningful efficacy (Smith et al., 2011; Pannu and Bhatnagar, 2019; Ai et al., 2021; Hegde et al., 2023).

Here, we propose a nutritional approach based on the administration of EAAs. Dietary EAA supplementation has been extensively shown to regulate metabolism and energy balance by directly modulating peripheral tissues such as muscle, adipose tissue, heart and liver. EAAs promote mitochondrial biogenesis (Nisoli et al., 2008; D'Antona et al., 2010; Valerio et al., 2011; Ragni et al., 2023), protect against oxidative damage (Corsetti et al., 2014; D'Antona et al., 2016; Tedesco et al., 2020a), enhance protein synthesis and physical endurance (Yamamoto et al., 2010; Nisoli et al., 2015), reduce body weight and improve glucose and lipid metabolism (Cota et al., 2006; Binder et al., 2013; 2014; Ruocco et al., 2020), stimulate energy expenditure (Ruocco et al., 2020; 2023), and strengthen immune function (Bassit et al., 2002; Aquilani et al., 2011). Collectively, these effects contribute to improved metabolic health and lifespan (Ruocco et al., 2021). Notably, the action of EAAs is context-dependent, in catabolic states they serve primarily as energy substrates, whereas in anabolic conditions they fuel protein synthesis and cell growth (Bifari and Nisoli, 2017). Clinical data further highlight the therapeutic potential of amino acid supplementation. EAAs enhances physical and cognitive performance in the elderly, improving mitochondrial biogenesis in peripheral blood mononuclear cells (Buondonno et al., 2019), preserves muscle mass during weight loss (Brunani et al., 2023), reduces infection risk (Aquilani et al., 2011), and is safe with no reported side effects. Furthermore, amino acids play a critical role in gut homeostasis, regulating structural integrity, epithelial turnover, redox balance, immune responses, and microbial composition. Inflammatory conditions, such as LPS challenge, disrupt amino acid metabolism and reduce digestibility, whereas supplementation with tryptophan, phenylalanine, or tyrosine improves amino acid sensing and exerts anti-inflammatory effects (Duanmu et al., 2022). Glutamine has also been shown to reduce gut permeability, endotoxemia, and inflammation in postoperative patients and to improve barrier function in malnourished children (Quan, 2004; Lima et al., 2005). In elderly patients with chronic kidney disease, EAAs reduced intestinal inflammation and improved barrier function (Aquilani et al., 2022).

Based on these concepts, our study extends the focus to EAAs, which—unlike single amino acids—simultaneously target both epithelial bioenergetics and nutrient-sensing pathways. We hypothesized that EAAs could be particularly beneficial in the context of intestinal damage, where mitochondrial dysfunction in IECs is well documented (Guerbette et al., 2022). Our results demonstrate that dietary EAAs provide substantial protection against obesity-induced intestinal barrier dysfunction by preserving mitochondrial function in IECs. Using a combined *in vivo* and *in vitro* approach and building on our previous work (Ruocco et al., 2020), we show that EAAs both prevent and reverse HFD-induced barrier impairment.

We show that dietary supplementation with EAAs prevents and reverses obesity and metabolic dysfunction in DIO mice by restoring intestinal barrier integrity, reducing inflammation, and sustaining mitochondrial biogenesis. EAA-fed mice exhibited reduced visceral fat, particularly in mesenteric depots, which likely contributes to attenuated systemic and intestinal inflammation, in line with previous evidence linking adiposity to gut barrier dysfunction and IBD pathogenesis (Kredel and Siegmund, 2014; Ha et al., 2020). EAAs also modulated nutrient absorption and gut metabolism. In HFD-fed mice, reduced fecal output was associated with increased caloric absorption, suggesting non-selective nutrient uptake driven by impaired barrier function (Murphy et al., 2015). In contrast, EAA-fed mice displayed enhanced protein and amino acid absorption, evidenced by decreased fecal nitrogen, upregulation of LAT1 and mitochondrial SLC25A44, and elevated plasma amino acid levels. These findings support a model in which EAAs promote selective nutrient uptake, likely preserving mitochondrial activity in intestinal epithelial cells and supporting intestinal homeostasis.

Correlation analyses further underscore the role of EAAs in nutrient sensing and metabolic regulation. Asparagine, phenylalanine, and tyrosine, elevated in HFD mice and normalized by EAA treatment, were positively associated with impaired glucose homeostasis and increased eWAT mass, while arginine, histidine, lysine, and tryptophan correlated with fecal output, suggesting differences in absorption efficiency. By promoting amino acid uptake while limiting non-specific absorption of carbohydrates and lipids, EAAs may reduce net caloric intake while preserving mitochondrial function and energy expenditure, thereby enhancing metabolic efficiency and limiting fat accumulation. This interpretation agrees with our previous findings showing that EAAs stimulate energy expenditure through BAT thermogenesis, contributing to weight loss and improved glycemic control (Ruocco et al., 2020). Nonetheless, part of the beneficial metabolic effect may also derive from reduced net caloric intake. Importantly, EAAs protected against HFD-induced intestinal barrier remodeling. HFD feeding reduced intestinal length and weight, increased pro-inflammatory cytokines (IL6, IL8, TNFα), altered TJ and AJ expression, lowered enterocyte-derived citrulline (a marker of enterocyte mass and barrier integrity) (Crenn et al., 2008), and elevated calprotectin (a marker of inflammation) (Malham et al., 2019), collectively indicating barrier damage and chronic inflammation. This aligns with Cani et al., who demonstrated that HFD-induced elevations in circulating LPS trigger metabolic disturbances through TJ disruption and microbial translocation (Cani et al., 2007). EAA supplementation prevented and reversed these alterations by restoring TJ and AJ, normalizing barrier-forming claudins, and suppressing pore-forming claudin-2. These effects were accompanied by preserved mitochondrial biogenesis markers and electron transport chain gene expression, reinforcing

the link between gut barrier integrity and mitochondrial function (Rath et al., 2018; Crakes et al., 2019). Furthermore, increased eNOS gene expression suggests sustained mitochondrial biogenesis (Nisoli, 2003; Nisoli et al., 2005; 2008). This is consistent with our previous work demonstrating that replacing dietary protein with an EAA mixture can both prevent and reverse obesity and glucose intolerance. In mice fed a low-fat diet, EAA supplementation further improved gut homeostasis by enhancing microbiota composition, accelerating intestinal transit, promoting villus elongation, and maintaining fecal energy efficiency despite a reduction in excreted mass (Ruocco et al., 2020).

In vitro, E7 directly preserves epithelial barrier integrity and exerts anti-inflammatory effects. However, its direct action on IECs and the differences with *in vivo* treatment require careful interpretation. *In vivo*, the intestinal epithelium is exposed to EAAs only transiently. Plasma EAA levels rise within 30–150 min after ingestion, with luminal concentrations peaking immediately after feeding and then rapidly declining due to absorption, bacterial metabolism, and intestinal transit (Rondanelli et al., 2017). Free amino acid concentrations in the intestinal lumen are typically 50–300 μM after a standard meal, but can increase to 0.6–6 mM following a protein-rich meal (Bröer, 2023). Under our experimental conditions, we estimated peak luminal concentrations at ~ 6 M immediately post-ingestion. By contrast, *in vitro*, Caco-2 cells were continuously exposed to E7 (1.0%; 66 mM) for 24–72 h. In this setting, amino acids were gradually metabolized but remained available for an extended period. Thus, although the initial concentrations *in vitro* were nearly 100-fold lower than peak luminal levels *in vivo*, sustained exposure was sufficient to elicit robust protective effects. This highlights the translational value of our model, continuous exposure of Caco-2 cells to sub-luminal EAA concentrations avoids transient peaks and enables a more realistic assessment of the long-term benefits of amino acids on epithelial barrier function.

Mechanistically, E7 promoted mitochondrial biogenesis, enhanced eNOS phosphorylation and respiratory function, and prevented inflammation-induced metabolic reprogramming toward glycolysis (Xiao et al., 2021). In addition, E7 upregulated antioxidant enzymes such as SOD2 and catalase, thereby reducing ROS-mediated epithelial injury (Chernyavskij et al., 2023). These results are consistent with and further support findings obtained *in vivo*. Our results also implicate mTORC1 as a mediator of these effects. Leucine-driven mTORC1 activation is known to support enterocyte proliferation and nutrient transport in intestinal porcine enterocytes, particularly following acute exposure of up to 10 h (Zhang et al., 2014). However, while transient activation of mTORC1 promotes epithelial renewal and barrier integrity, chronic activation has been linked to exacerbated intestinal inflammation, whereas inhibition under pathological conditions can restore barrier function (Kaur and Moreau, 2019; 2021; Kotani et al., 2020). In our study, E7 reduced phosphorylation of p70S6K and S6, consistent with mTORC1 inhibition. Moreover, rapamycin reproduced the protective effects of E7 on ZO-1 and PGC-1α in inflamed cells, as also reported by Xu et al. in ulcerative colitis (Xu et al., 2024). No additive effect was observed with combined treatment, supporting mTORC1 may be a shared target. Together, these findings suggest that E7 mediates barrier protection directly in IECs, stimulating mitochondrial biogenesis *via* the eNOS/PGC-1α axis and reducing mTORC1 activity under inflammatory stress. In addition, our data indicate a catabolic state in intestinal epithelial cells, in which EAAs may be preferentially utilized as energy substrates. This metabolic shift further supports their role in sustaining epithelial homeostasis under stress conditions. Our *in vitro* studies showed further that the inclusion of Krebs cycle intermediates in the E7 formulation enhances mitochondrial biogenesis and supports epithelial homeostasis more effectively than the standard EAA mixture without intermediates, suggesting a synergic interaction between amino acids and citrate succinate and malate. This is consistent with our previous work, in which E7 was more effective than the EAA mixture in promoting mitochondrial biogenesis in cardiomyocytes (Tedesco et al., 2020b). Conceptually, these intermediates can fuel anaplerosis, sustain NADH/$FADH_2$ generation, and improve redox balance, thereby reinforcing oxidative phosphorylation and junctional homeostasis. Consistent with this rationale, succinate can counteract HFD-induced barrier dysfunction by influencing epithelial permeability, immune signalling, goblet cell differentiation, and microbiota composition (Li et al., 2019; 2023). Citric acid has been shown to strengthen TJs and improve mucosal immunity in porcine models of enterotoxaemia (Liu et al., 2021; Hu et al., 2024), and malic acid supplementation reduced oxidative stress and inflammatory signatures *via* microbiota–metabolite modulation (Chen et al., 2024). Mechanistically, in addition to their metabolic role, succinate–SUCNR1 (succinate receptor 1) signalling may contribute to epithelial and immune crosstalk, while improved mitochondrial flux can secondarily modulate mTORC1 and promote the eNOS–PGC-1α program we observed with E7. However, while supportive studies exist across species and models (Li et al., 2019; 2023; Liu et al., 2021; Chen et al., 2024; Hu et al., 2024), direct evidence in human HFD-associated gut leak is limited and represents a priority for translational validation.

We acknowledge several limitations in our work. Although we demonstrated e superior effect of E7 compared with EAA mixture *in vitro*, the specific contribution of each intermediate remains to be quantified. Future work will include add-back/subtraction experiments, pharmacologic receptor modulation (*e.g.*, SUCNR1 antagonism), and *in vivo* comparisons under HFD feeding. Moreover, while our data strongly suggest that preservation of mitochondrial function is central to maintaining gut barrier integrity and preventing inflammatory damage, a direct causal demonstration is still lacking and will require further investigation. Furthermore, although we observed direct effects of EAAs on IECs, potential interactions with other intestinal cell type (*e.g.*, goblet cell), gut microbiota and the immune system cannot be excluded. Amino acids can be utilized by enterocytes or metabolized by intestinal bacteria generating NO and short-chain fatty acids (acetate, propionate, butyrate) which influence epithelial barrier function and immune responses (Bifari et al., 2017). Additionally, microbial metabolites can modulate mitochondrial activity and mitochondrial function shapes the microbial niche. Disruption of this bidirectional signaling contributes to gut inflammation (Jackson and Theiss, 2020).

Amino acids are key modulators of immune function. Dietary amino acid restriction (*i.e.*, during malnutrition) impairs cytotoxic T lymphocyte and natural killer cell activity and contributes to immune-senescence (Lesourd, 2009). Conversely, EAA supplementation

preserves immune competence in several pathological conditions (*e.g.*, cirrhosis, surgical recovery, rehabilitative), *via* effects on lymphocyte and macrophage activation and cytokine production (Nuwer et al., 1983; Tsukishiro et al., 2000; Kakazu et al., 2009; Aquilani et al., 2011; 2021; Boselli et al., 2012). Among individual amino acids, phenylalanine can influence immunity directly, by regulating NO synthesis in leukocytes, and indirectly through conversion to tyrosine, which supports catecholamine synthesis and immune-cell signalling (Shi et al., 2004). Likewise, leucine and other BCAAs modulate immune activity by tuning mTORC1 signalling in T cells (Powell and Delgoffe, 2010; Sinclair et al., 2013). Specific amino acids also contribute to intestinal integrity by shaping gut-immune responses (Ruth and Field, 2013). For example, tryptophan metabolism by commensal microbiota generates indole derivatives that activate the aryl hydrocarbon receptor, promoting IL22 production by group 3 innate lymphoid cells and Th17 cells, enhancing antimicrobial defence and epithelial repair (Qiu et al., 2012; Zelante et al., 2013). In addition, threonine serves as an essential substrate for goblet-cell mucin biosynthesis, thereby supporting the mucus barrier and limiting paracellular permeability (Faure et al., 2005). These mechanisms align with emerging evidence that Th17–IL22 signalling influences epithelial lipid absorption and barrier integrity in response to HFD feeding (Gao et al., 2025). We further hypothesize that increased NO bioavailability and mTORC1 modulation converge with enhanced mitochondrial activity in immune cells, consistent with our previous findings that the EAA mixture containing phenylalanine and BCAAs stimulated mitochondrial biogenesis in PBMCs (Buondonno et al., 2019). Finally, growing evidence indicates that Krebs cycle intermediates, such as citrate and succinate, modulate macrophage activation, cytokine production, and gut epithelial barrier function (Li et al., 2019; 2023; Liu et al., 2021; Hu et al., 2024), suggesting potential synergy with EAAs in sustaining gut and immune homeostasis. Although we did not assess mucosal immune cells here, this framework provides a biologically plausible immune–epithelial crosstalk that could contribute to the benefits observed with EAA/E7. Accordingly, future studies should comprehensively investigate the direct effects of EAAs and Krebs cycle intermediates on microbiota dynamics and immune regulation, and determine whether activation of these pathways complements the epithelial, mitochondria-dependent effects observed in the present study.

Taken together, our study identifies EAAs as a promising nutritional strategy to preserve intestinal barrier integrity and counteract obesity-related gut dysfunction. EAAs support mitochondrial biogenesis, modulate mTORC1 signaling, and reduce epithelial inflammation, providing a mechanistic basis for restoring gut barrier function in metabolic and inflammatory disorders. While preclinical and some clinical data are encouraging, validation in human obesity cohorts is needed. Overall, EAAs offer a strategy to target interconnected mechanisms—mitochondrial dysfunction, barrier disruption, and systemic metabolic complications—within integrated lifestyle and therapeutic approaches.

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 animal study was approved by Italian Ministry of Health (Protocol No. 15/2024-PR). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

LS: Formal analysis, Investigation, Writing – original draft, Writing – review and editing. MR: Investigation, Writing – review and editing. AS: Formal analysis, Investigation, Writing – review and editing. AVe: Project administration, Writing – review and editing. GM: Investigation, Writing – review and editing. LC: Investigation, Writing – review and editing. MC: Writing – review and editing. RA: Writing – review and editing. GC: Investigation, Validation, Writing – review and editing. AVa: Funding acquisition, Investigation, Resources, Supervision, Validation, Writing – review and editing. EN: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review and editing. CR: Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Visualization, Writing – original draft, Writing – review and editing.

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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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