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Gastrointestinal (GI) disorders and antioxidant therapeutics

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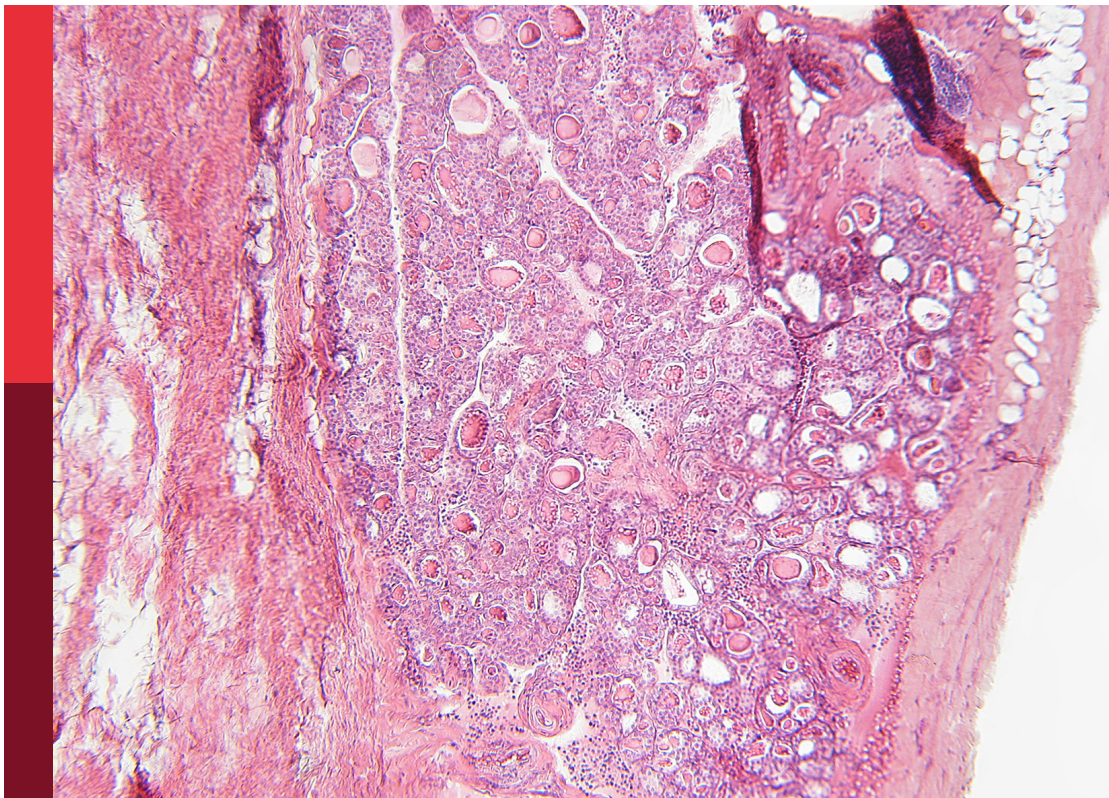

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Gastrointestinal (GI) disorders and antioxidant therapeutics

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Editorial: Gastrointestinal (GI) disorders and antioxidant therapeutics

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Editorial on the Research Topic
Gastrointestinal (GI) disorders and antioxidant therapeutics

Gastrointestinal (GI) disorders encompass diverse clinical conditions that impact the GI tract, such as inflammatory bowel disease (IBD), dyspepsia, and malignant tumors. Canine CIE exhibits several similarities with IBDs observed in human patients (1). Both acute and chronic GI disorders in humans and animal models are marked by a disruption in redox balance, which may result from increased production of reactive oxygen species (ROS) or weakened antioxidant defense systems (AODS). Oxidative stress (OS) denotes a disparity between oxidants and antioxidants, with a tendency towards the production of oxidants (2). This imbalance subsequently interferes with redox signaling and regulation, potentially leading to detrimental effects at the molecular level. Advancements in treatments for GI disorders, such as IBD, that are influenced by OS require greater comprehension of the cellular and molecular processes governed by reactive oxygen species (ROS). In the GI tract, oxidative stressors encompass infections and pro-inflammatory responses, which enhance the generation of ROS by stimulating the production of pro-inflammatory cytokines. Nuclear factor kappa B (NF-κB) and nuclear factor erythroid 2–related factor 2 (Nrf2) are two key signaling pathways in intestinal immune cells that govern various pathological processes, encompassing anti-inflammatory and antioxidant functions (3).

Hong et al. highlighted that Nrf2, mammalian target of rapamycin (mTOR), adenosine monophosphate-activated protein kinase (AMPK), and forkhead box protein O1 (FOXO1) could serve as significant targets that concurrently influence OS, inflammation, and autophagy, as studied in adipogenesis. The oxidative balance score (OBS) is frequently utilized to evaluate OS, offering a thorough assessment of dietary and lifestyle-related factors. Li et al. investigated the association between OBS and IBD. OBS demonstrated a negative correlation with IBD, particularly among female Crohn's disease (CD) patients. This research highlights the importance of an antioxidant diet and lifestyle, potentially offering improved health for female patients with CD. The study conducted by Chang et al. indicates that increased exposure to antioxidants, evaluated through OBS (diet and lifestyle rich in antioxidants), could potentially reduce the incidence of colorectal cancer (CRC). A significant negative correlation was identified between OBS and the likelihood of

developing CRC and its various subsites (proximal colon cancer, distal colon cancer, and rectal cancer). The correlation was especially evident among male CRC patients. Biomarkers are essential for the detection of diseases and for evaluating the effectiveness of treatments (4, 5). Serum albumin, uric acid, and the neutrophil count are biomarkers mediating the potential association between OBS and CRC.

Antioxidants have been suggested as a possible alternative treatment to anti-inflammatory/immunomodulatory medications used to treat GI disorders (Figure 1). There are various types of antioxidants, each exhibiting distinct mechanisms of action and offering application to specific clinical applications (6). Natural antioxidant compounds can scavenge ROS and enhance the body's antioxidant defense mechanisms (6–13), potentially inhibiting pro-oxidative enzymes aiding the treatment of IBD. When the antioxidant capacity of the damaged mucosa is diminished, the application of nontraditional therapies, including pharmaceuticals, natural or synthetic agents, hormones, and probiotics that neutralize reactive oxygen and nitrogen species, mitigate cellular damage (protein carbonylation, lipid peroxidation, and DNA modification) and enhance the function of antioxidant enzymes, are helpful when used in conjunction with anti-inflammatory medications or independently (14–17). Muro et al. provided a comprehensive overview of the involvement of OS in the pathophysiology of IBD, highlighting its diagnostic targets and exploring the potential use of antioxidant therapies to manage IBD. Markers of OS, including 8-hydroxy-2'-deoxyguanosine, malondialdehyde, and serum-free thiols, were increased in the blood and stool of patients and correlated with disease severity. Consequently, markers of OS can serve a dual purpose, aiding in both the diagnosis and the assessment of IBD treatment efficacy. The treatment of IBD can also focus on the use of antioxidants, such as glutathione, vitamin C, vitamin E, and N-acetylcysteine. In their review, Sahoo et al. examined a range of polyphenolic substances, including curcumin, resveratrol, quercetin, caffeic acid phenethyl ester, green tea flavonoids, luteolin, genistein, xanthohumol,

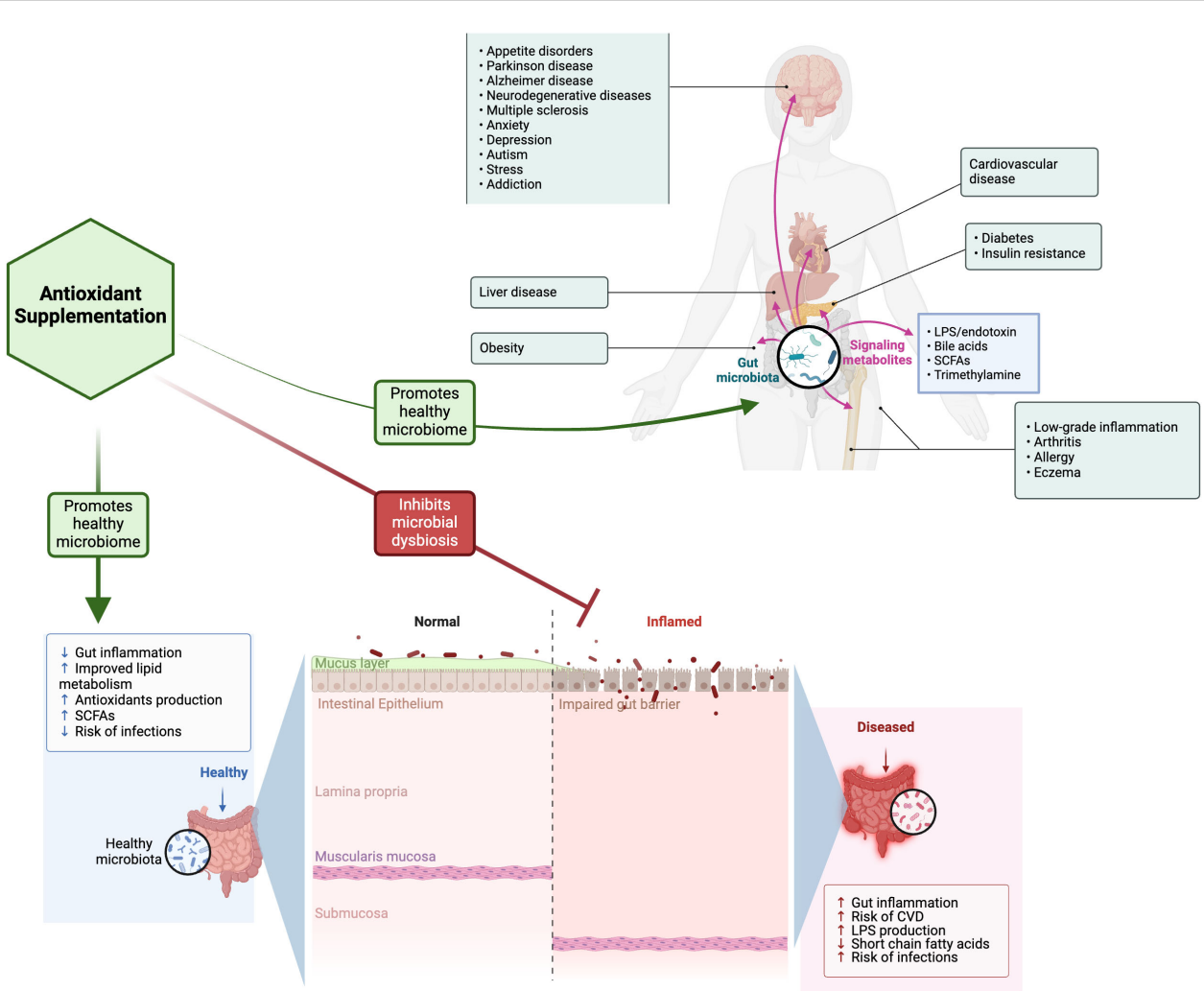

FIGURE 1
A schematic depiction of the influence of gut microbiota in nutrition and health with the potential protective effect of antioxidant supplementation in preserving gut health. This figure was created in BioRender (https://BioRender.com/u24c341). LPS, Lipopolysaccharide; CVD, cardiovascular disease; SCFAs, Short-chain fatty acids.

alpinetin, silymarin, proanthocyanidins, anthocyanins and phenolic compounds such as thymol, alkaloids like berberine, storage polysaccharides including tamarind xyloglucan, and various phytochemicals represented by isothiocyanate sulforaphane and food/spices such as ginger, flaxseed oil, along with antioxidant hormones like melatonin, all of which target cellular signaling pathways to mitigate intestinal inflammation associated with IBD. He et al. evaluated the relationship between dietary carotenoid intake and soluble Klotho (S-Klotho) plasma levels in elderly adults. The overall consumption of carotenoids showed a positive correlation with plasma concentrations of S-Klotho among the elderly population, especially with ingestion of α-carotene, β-carotene, and lutein with zeaxanthin.

The gut microbiota (GM), which includes bacteria, archaea, fungi, protozoa, and viruses, significantly influences host health and disease (18, 19). The GM plays a crucial role in the intestinal epithelial barrier, aids in host metabolism, offers protection against pathogens, and impacts the development of the mucosal immune system (19). Numerous research findings illustrate the positive impacts of prebiotics, probiotics, postbiotics, and synbiotics on human and animal health, such as contributing to the maintenance of a healthy GI microbiota, enhancing the intestinal mucosal barrier, promoting immune tolerance, and helping regulate the pro-inflammatory response (18, 20–22). The underlying mechanisms of functional GI disorders encompass alterations in the gut microbiota/gut hormone axis, which play a crucial role in GI motility. Hormone receptors have been discovered in inflamed regions, demonstrating the ability to trigger both pro- and anti-inflammatory responses. Dai et al. conducted a comprehensive review of the pathogenesis of cardiovascular disease (CVD), addressing factors such as inflammatory response, OS, mitochondrial dysfunction, pyroptosis, ferroptosis, and dysbiosis of GM. Research indicates that the GM functions as a "microbial organ" influencing cardiovascular health, and the "gut-heart" axis presents a promising pathway for preventing and treating CVD. A significant number of potent compounds derived from traditional Chinese medicine (TCM) have been identified, including flavonoids, phenolic acids, stilbenes, saponins, anthraquinones, terpenoids, alkaloids, polysaccharides, which all demonstrate therapeutic effects on various CVDs. TCM can potentially address CVD through various mechanisms, such as antioxidant properties, anti-inflammatory effects, enhancement of mitochondrial function, prevention of cell death (including apoptosis, autophagy, pyroptosis, and ferroptosis), and modulation of GM. Clinical trials have demonstrated the effectiveness and safety of TCM in reducing the symptoms associated with CVD. Studies indicate that numerous TCM monomers can potentially enhance blood sugar and lipid levels and early renal function via the "gut-liver-kidney axis" while mitigating pathological damage to kidneys. Xu et al. demonstrated that TCM exhibits a protective influence on the kidneys through its interaction with farnesoid X receptor (FXR). TCMs have the potential to modulate the intestinal microbiota-bile acid (BA) axis, thereby slowing the progression of DKD. The distinctive benefits of Chinese medicine in addressing kidney tissue damage, managing glucose and lipid metabolism, lowering urinary protein levels, and mitigating the inflammatory response have been determined. In patients with DKD, there is a reduction in both probiotics and prebiotics, and supplementation of these elements can enhance glucose metabolism, restore the function of the intestinal mucosa, and reverse structural damage to renal tissue in individuals with DKD. In TCM, the combination of *Salvia miltiorrhiza* and *Astragalus membranaceus* is an effective prescription for the treatment of DKD (23). *A. membranaceus* and *S. miltiorrhiza* enhanced the prevalence of *Akkermansia*, *A. muciniphila*, *Lactobacillus*, and *L. murinus*. While *Akkermansia* and *A. muciniphila* exhibited a positive correlation with the synthesis of arachidonic acid metabolites, *Lactobacillus* and *L. murinus* exhibit a negative correlation with glycerophospholipid metabolism, suggesting that TCM may contribute positively to anti-inflammatory effects and lipid reduction through the enhancement of intestinal flora distribution. Another TCM, astragaloside IV (AS-IV), the active component in astragaloside, facilitates the release of Nrf2 from Kelch-like ECH-associated protein 1 (Keap1)-Nrf2 complex and counteracts mitochondrial dysfunction (24). Huang-Lian-Jie-Du decoction, a TCM formulation, has the potential to improve blood glucose metabolism in DKD rats through the modulation of the advanced glycation endproducts (AGE)/receptor for AGE (RAGE)/protein kinase B (Akt)/Nrf2 pathway while also lowering triglyceride and low-density lipoprotein cholesterol levels to safeguard kidney function (25). Mycobacterial species possess a powerful immunomodulatory capability, primarily due to their complex cell wall composition. Abdallah et al. conducted a study to explore the possible prophylactic anti-diabetic effects of heat-killed *Mycobacterium aurum* (HK-*MA*) in mice with streptozotocin (STZ)–induced diabetes. Prophylactic administration of three doses of HK-*MA* to diabetic mice led to a notable decrease in their blood glucose levels compared to the control diabetic mice. Prophylactic treatment of diabetic mice with HK-*MA* notably normalized their altered protein expression levels of liver uncoupling protein 2 (UCP2), lactate dehydrogenase, and skeletal muscle UCP3. HK-*MA* could lower hyperglycemia and may support its potential application as a dietary supplement for improving diabetes management. The decrease in hyperglycemia might be partially linked to an anti-inflammatory effect brought about by HK-*MA*. However, the potential influence of HK-*MA* on inflammation is still a hypothesis that necessitates additional research. In contrast, Shi et al. conducted a comprehensive review of the mechanisms by which the gut microbiome has tumor-promoting and potential anti-tumor effects. They outlined various bacterial therapeutic strategies for addressing tumors, encompassing natural and engineered anti-tumor approaches. In specific conditions, particular bacteria, notably anaerobes or parthenogenetic anaerobes, accumulate and proliferate within the tumor environment. This occurrence triggers a series of responses in the body that eventually lead to anti-tumor effects. The GI microbiome plays a significant role in tumor development through various mechanisms, such as the release of metabolic by-products, the induction of inflammatory cascades, the modulation of immune responses, and the alteration of microbial abundance and colonization sites. The metabolite indole-3-propionic acid, associated with quercetin, functions as an aryl hydrocarbon receptor (AhR)

agonist. It reduces inflammatory responses in colonic epithelial cells, consequently hindering carcinogenesis and demonstrating anticancer effects. *Bifidobacterium* is classified among probiotics and has been linked to possible advantages in preventing IBD and CRC. The GM produces numerous metabolites that demonstrate anti-tumor effects by inhibiting the proliferation of tumor cells, regulating apoptosis, and suppressing inflammatory responses. It is important to highlight that butyrate, recognized as the most thoroughly researched short-chain fatty acids (SCFAs), has demonstrated a dual role, possessing the capacity to promote tumorigenesis and elicit anti-tumor effects. Butyrate demonstrates its ability to combat colon cancer effects by influencing Fas and p21 in animal tumor models and inhibiting enzymes associated with pro-carcinogenic activities in the intestine, including histone deacetylases. These actions reduce tumor cell proliferation and contribute to the inhibition of further CRC progression. Furthermore, butyrate plays a crucial role in preserving the integrity of the intestinal barrier, which is essential for the overall functioning of the intestines. A bidirectional two-sample Mendelian randomization (BTS-MR) study by Liu et al. thoroughly illustrated the intricate relationship between GM and thyroid dysfunction. This research underlines the importance of selecting more targeted probiotics to uphold thyroid–gut axis homeostasis, thereby aiding in the prevention, management, and reversal of thyroid dysfunction development. This BTS-MR study demonstrated that the genera *Intestinimonas*, *Eubacterium brachy* group, *Ruminiclostridium*, and *Ruminococcaceae* UCG004 were associated with an increased risk of decreased thyroid function. In contrast, the genera *Bifidobacterium* and *Lachnospiraceae* UCG008, along with the phyla Actinobacteria and Verrucomicrobia, exhibited protective effects.

Exploring natural products (26, 27) and the production of essential therapeutics through molecular farming (28) have garnered significant interest in recent years due to their potential therapeutic advantages and reduced adverse effects compared to synthetic medications. Considering the current body of scientific evidence, it seems possible that forthcoming therapies may incorporate antioxidants alongside conventional treatments or potentially serve as an alternative medical approach for humans and animals suffering from IBD. However, additional research is necessary before these antioxidants can be utilized in clinical practice.

Author contributions

DS: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. GC: Conceptualization, Resources, Supervision, Writing – review & editing. AJ: Conceptualization, Investigation, Resources, Supervision, Writing – review & 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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Oxidative stress, hormones, and effects of natural antioxidants on intestinal inflammation in inflammatory bowel disease

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Inflammatory bowel disease (IBD) is a chronic, relapsing gastrointestinal (GI) disorder characterized by intestinal inflammation. The etiology of IBD is multifactorial and results from a complex interplay between mucosal immunity, environmental factors, and host genetics. Future therapeutics for GI disorders, including IBD, that are driven by oxidative stress require a greater understanding of the cellular and molecular mechanisms mediated by reactive oxygen species (ROS). In the GI tract, oxidative stressors include infections and pro-inflammatory responses, which boost ROS generation by promoting the production of pro-inflammatory cytokines. Nuclear factor kappa B (NF-κB) and nuclear factor erythroid 2–related factor 2 (Nrf2) represent two important signaling pathways in intestinal immune cells that regulate numerous physiological processes, including anti-inflammatory and antioxidant activities. Natural antioxidant compounds exhibit ROS scavenging and increase antioxidant defense capacity to inhibit pro-oxidative enzymes, which may be useful in IBD treatment. In this review, we discuss various polyphenolic substances (such as resveratrol, curcumin, quercetin, green tea flavonoids, caffeic acid phenethyl ester, luteolin, xanthohumol, genistein, alpinetin, proanthocyanidins, anthocyanins, silymarin), phenolic compounds including thymol, alkaloids such as berberine, storage polysaccharides such as tamarind xyloglucan, and other phytochemicals represented by isothiocyanate sulforaphane and food/spices (such as ginger, flaxseed oil), as well as antioxidant hormones like melatonin that target cellular signaling pathways to reduce intestinal inflammation occurring with IBD.

KEYWORDS

antioxidants, ulcerative colitis, Crohn's disease, IBD, oxidative stress, flavonoids, polyphenols, hormones

1 Introduction

Inflammatory bowel disease (IBD) in humans comprises at least two chronic inflammatory intestinal disorders, defined as Crohn's disease (CD) and ulcerative colitis (UC). Lesions of CD may occur in the small or large intestine but most commonly involve the colon and rectum as discontinuous areas of transmural inflammation. In contrast, UC affects only the colon and rectum continuously, with inflammation restricted to the mucosa (1). The clinical course of CD is associated with intestinal granulomas, strictures, and fistulae, while these lesions are absent in UC. The underlying mechanism for IBD is believed to result from dysregulated immune responses to environmental factors and the intestinal microbiota in genetically susceptible people (2). These disorders impact millions of people worldwide, with the prevalence of disease in Americans expected to rise by 229% by 2030, relative to the number of diagnoses in 2010 (3).

Scientific evidence that increased levels of reactive oxygen species (ROS) but decreased levels of antioxidants contribute to disease pathogenesis establishes a link between ROS and IBD (4, 5). The intestinal mucosa is lined with an epithelial cell monolayer which separates the anaerobic lumen from the highly metabolic lamina propria. Therefore, the intestinal epithelial cells function under a physiological oxygen gradient that is relatively steep (reaching from physiologic hypoxia to physioxia) compared to other cell types. Moreover, during active IBD, there is a significant metabolic shift towards hypoxia seen with mucosal inflammation (pathologic hypoxia). *In vitro* and *in vivo* studies have demonstrated that the activation of the transcription factor hypoxia-inducible factor (HIF) functions as a warning signal in several murine disease models. For example, HIF-1, which is increasingly stabilized in inflammatory lesions, protects against inflammation and IBD by triggering the transcription of several genes that allow the intestinal epithelial cells to operate as an efficient barrier (4). While HIF-1 facilitates adaptive responses to oxidative stress (OS) via nuclear translocation and gene expression regulation, it is well known that mitochondrial HIF-1α protects against OS-induced apoptosis. Several studies have shown that nuclear factor erythroid 2–related factor 2 (Nrf2) helps the anti-inflammatory process by coordinating the recruitment of inflammatory cells and regulating gene expression via the antioxidant response element (ARE) (Figure 1) (6). A decrease in the expression of antioxidant/phase II detoxifying enzymes, such as UDP-glucurosyltransferase 1A1, NAD(P)H-quinone reductase-1, heme-oxygenase-1, and glutathione S-transferase Mu-1 was linked to the increased severity of colitis in Nrf2-deficient mice (7), while *Nrf2* overexpression was reported to improve UC (8).

2 Concepts of cellular and ROS damage

The intestinal mucosa in people with IBD (e.g., CD) is typically infiltrated with numerous inflammatory cells, including

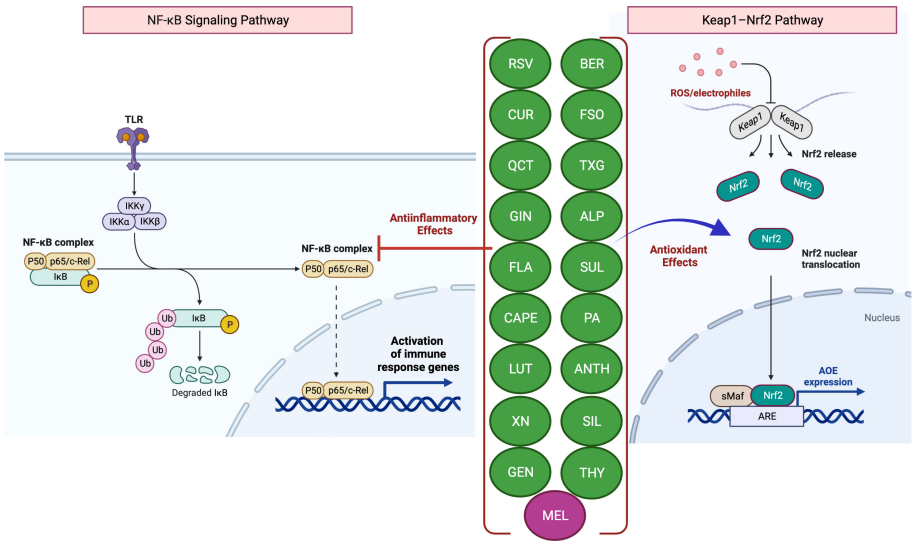

FIGURE 1
Antioxidant and anti-inflammatory effects of phytochemicals and hormones. Anti-inflammatory mechanisms involve the modulation of nuclear factor-kappa B (NF-κB) pathways, such as the downstream pro-inflammatory effects mediated by Toll-like receptor (TLR) activation. Activation (release of IκB inhibition) and nuclear translocation of NF-κB inhibition, which result in the transcription of several pro-inflammatory genes, can be inhibited by several phytochemicals and hormones (left panel). Kelch-like ECH-associated protein-1 (Keap1)-induced activation and nuclear translocation of nuclear factor erythroid 2-related factor 2 (Nrf2), resulting in an increased expression of antioxidant enzymes (AOEs), can also be inhibited by phytochemicals and hormones (right panel). In the presence of OS, Keap1 relinquishes its binding to Nrf2, thereby enabling the translocation of Nrf2 into the nucleus. Subsequently, Nrf2 forms a complex with small Maf (sMaf) proteins, resulting in the formation of Nrf2/sMaf heterodimer, which then binds to the Antioxidant Response Element (ARE) located on different stress-related gene targets. The figure was produced with BioRender (www.biorender.com; accessed on 17th July 2023). Resveratrol (RSV); Curcumin (CUR); Quercetin (QCT); Ginger (GIN); Flavonoids (FLA); Caffeic acid phenethyl ester (CAPE); Luteolin; (LUT); Xanthohumol (XN); Genistein (GEN); Berberine (BER); Flaxseed oil (α-linolenic acid) (FSO); Sulforaphane (SUL); Tamarind xyloglucan (TXG); Alpinetin (ALP); Proanthocyanidins (PA); Anthocyanins (ANTH); Silymarin (SIL); Thymol (THY); Melatonin (MEL); ubiquitin (Ub); phosphorylation (P); IκB kinase (Iκκ); Mammalian NF-κB family members: NF-κB1 (p50), c-Rel and RelA (p65).

neutrophils, macrophages, and lymphocytes (9, 10). Uncontrolled immune responses are driven by the excessive activity of effector T-lymphocytes and their increased production of pro-inflammatory cytokines (e.g., tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, and IL-6) and chemokines, which, along with other inflammatory mediators, cause tissue damage and perpetuate the inflammatory response (11). Typically, the equilibrium between proinflammatory cytokines (such as TNF-α, IL-1, IL-6, IL-8, IL-17, and IL-23) and anti-inflammatory cytokines, such as IL-5, IL-10, IL-11, and transforming growth factor β (TGF-β) is closely regulated within the GI mucosa (12). The pathogenesis of IBD is characterized by an imbalance between T helper (Th) cells and regulatory T cells, specifically the impaired tolerance of regulatory T cells. While CD is distinguished by inflammation mediated by Th1 cells, which results in the overproduction of IL-12, IL-17, and IL-23, UC is marked by cytokines such as IL-4, IL-5, IL-10, and IL-13, which are produced by Th2-type T cells (13).

The active inflammatory process is coupled with the generation and release of ROS from infiltrating immune cells. Principal ROS produced by inflammatory cells include superoxide ($O_2^{\bullet-}$), hydroxyl radical (·OH), hydroperoxyl radical (HO_2·), nitric oxide (NO), and singlet oxygen ($^1O_2$) (14). Furthermore, ROS upregulates genes involved in innate and adaptive immune responses to amplify mucosal inflammation (12). ROS and other inflammatory markers released in the inflamed mucosal environment cause progressive cellular and molecular damage, resulting in increased tissue destruction. The most common cellular targets for ROS include cell membrane lipids, proteins, and DNA which causes lipid peroxidation (LPx), enzymatic dysfunction, and DNA damage, respectively (15–17). OS in IBD occurs due to an imbalance between oxidant and antioxidant substances in affected tissues (18). This review also evaluates the role of antioxidants and hormones in the crosstalk between OS and inflammation in IBD.

3 Clinical studies in companion animals

Canine chronic inflammatory enteropathies (CIE) refer to a group of intestinal disorders characterized by persistent or recurrent gastrointestinal (GI) signs and variable intestinal inflammation (19, 20). The prevalence of CIE in referral veterinary practice is estimated at 2%, and it is generally subclassified by the response to different therapeutic trials (20). The different disease phenotypes include food-responsive enteropathy (FRE), antibiotic-responsive enteropathy (AE), steroid-responsive enteropathy (SRE), often termed idiopathic IBD, and nonresponsive enteropathy (NRE) (20–22). While the cause of canine CIE is unknown, it is also recognized as a multifactorial disorder resulting from a complex interplay among the environment (e.g., diet, microbiome), mucosal immunity, and host genetics that initiates and drives chronic intestinal inflammation, like human IBD (19).

There are few clinical studies evaluating the role of OS in dogs with CIE. In one case-control study, colonic lavage analytes as markers of mucosal inflammation were compared between healthy dogs and dogs with biopsy-confirmed idiopathic IBD (23). Polyethylene glycol solution was administered into the colon via rectal balloon catheter prior to colonoscopy and was then analyzed for total protein, IgG, and nitrite concentrations and myeloperoxidase (MPO) activity. Results showed that mean nitrite and IgG concentrations were higher in lavage samples from idiopathic IBD dogs compared with samples from healthy dogs. Serum metabolite profiles have also demonstrated a potential relevance of OS in the pathogenesis of dogs affected by idiopathic IBD (24). Using an untargeted metabolomic approach, gluconic acid lactone and hexuronic acid increased in the serum of idiopathic IBD dogs when compared to samples from healthy dogs. Gluconic acid is an oxidized derivative of glucose that can scavenge free radicals, and hexuronic acid is a biologically active form of vitamin C that functions as an antioxidant by donating electrons. Interestingly, there were no significant changes in serum metabolite profiles in dogs with idiopathic IBD following medical therapy, despite clinical improvement.

Several other studies have investigated serum biomarkers of OS in dogs with CIE at diagnosis and in response to medical treatment. In one study, trolox equivalent antioxidant capacity (TEAC), cupric reducing antioxidant capacity (CUPRAC), ferric reducing ability of the plasma (FRAP), total thiol concentrations, and paraoxonase-1 (PON1) activity were evaluated in serum to determine the antioxidant response in dogs with idiopathic IBD. Additionally, ferrous oxidation-xylenol orange (FOX), thiobarbituric acid reactive substances (TBARS), and ROS concentrations in serum were determined (25). The mean concentrations of all antioxidant biomarkers except FRAP were lower, and the oxidant markers were higher in the sera of dogs with idiopathic IBD than in healthy controls. Another study showed lower serum fatty acid concentrations in dogs with CIE than in healthy dogs, indicating dysregulation of both pro-inflammatory (arachidonic acid and cyclooxygenase pathways) and anti-inflammatory (omega-3 essential fatty acids) mediators (26). Perturbations in these mediators in the face of chronic intestinal inflammation are a recognized feature of IBD in people (27).

Differences in systemic phospholipids were reported in another study involving dogs with idiopathic IBD and FRE (28). Overall, disease severity and treatment (e.g., elimination diet alone for FRE versus elimination diet and immunosuppressive dose of prednisolone for idiopathic IBD) were the most significant variables affecting phospholipid profiles at diagnosis. After treatment, a shift of phospholipid species from phosphatidylcholine to lysophosphatidylcholine was observed for both disease groups, presumably caused by an increase in anti-inflammatory lipid mediators (lipoxins and resolvins). The effects of dietary supplements and diet therapy on metabolomic changes in dogs with CIE have been investigated in other treatment trials. In one controlled trial, dogs with idiopathic IBD were randomized to treatment with either a hydrolyzed diet alone or a hydrolyzed diet supplemented with prebiotics (PRE) and glycosaminoglycans (GAG) (29). Results indicated that the majority of metabolomic changes involved several different lipid classes (glycerophospholipids, sphingolipids, and di- and triglycerides) and that both treatments increased beneficial metabolites in serum lipid profiles. In addition, co-treatment with PRE + GAG was associated

with the greatest increase in lipid metabolites suggesting a possible additional beneficial effect in dogs with idiopathic IBD. Another randomized controlled trial in dogs with idiopathic IBD involved combination therapy with hydrolyzed diet and oral chondroitin sulfate + PRE versus hydrolyzed diet alone (30). The supplement group showed decreased serum levels of paraoxonase-1 after 60 days of treatment, whereas the placebo group showed reduced serum total antioxidant capacity after 120 days. A decrease in the intestinal histologic score was observed only in the supplement group post-treatment. Additionally, breed-specific changes in the fecal metabolomic profile have been reported in Yorkshire Terriers, which show an increased susceptibility to CIE and protein-losing enteropathy. Here, changes in bile acid, fatty acid, and sterol metabolism that only partially recovered with successful treatment were observed (31).

While most studies have investigated the pathomechanisms of OS in chronic gastroenteritis, other studies have evaluated the role of antioxidants in acute enteropathy and in mitigating OS induced by surgery. In one study, a comparison of the OS status of dogs with uncomplicated acute diarrhea (AD) was compared to healthy controls (32). Both cohorts were screened for clinical and laboratory abnormalities as well as routine redox indices (reactive oxygen metabolites [dROMs], serum antioxidant capacity [SAC], and the oxidative stress index [OSi]). Dogs with AD showed increased levels of dROMs and OSi values (calculated as the ratio between dROMs and SAC) as compared to control indices.

Summarizing, different metabolomic studies in dogs with CIE also show disturbances in serum and fecal metabolites reflective of OS at diagnosis. Notably, disturbances in lipid metabolism appear to be a common denominator across multiple studies. Moreover, treatment using a hydrolyzed diet with or without different dietary supplements improves several different measures of OS in most animals showing clinical remission. Given the importance of these metabolites in mediating chronic intestinal inflammation, additional well-designed and sufficiently powered clinical trials in dogs with CIE are warranted.

4 Oxidative stress and antioxidant defenses

The most prevalent antioxidant signaling pathway is the Kelch-like epichlorohydrin-related protein-1 (Keap1)/Nrf2-ARE signaling pathway (33, 34). As an inactive compound with its cytosolic repressor, Keap1, Nrf2 is sequestered in the cytoplasm. In response to OS that oxidizes two SH groups, Nrf2 is dissociated from the inhibitory protein Keap1 and translocated to the nucleus, where it binds ARE to activate the transcription of antioxidant genes (33, 34) (Figure 1).

The production of ROS is a natural consequence of biological metabolism (Figure 2). The beneficial effects of ROS are seen in a variety of physiological processes at low and moderate concentrations, including the killing of invading pathogens, the healing of wounds, and the repair of damaged tissues. Aerobic organisms possess a wide range of antioxidants that are critical to their survival. Antioxidants can be classified as either enzymatic or non-enzymatic, depending on their functions. While the antioxidant defense enzyme superoxide dismutase (SOD) converts the superoxide anion to hydrogen peroxide, catalase (CAT),

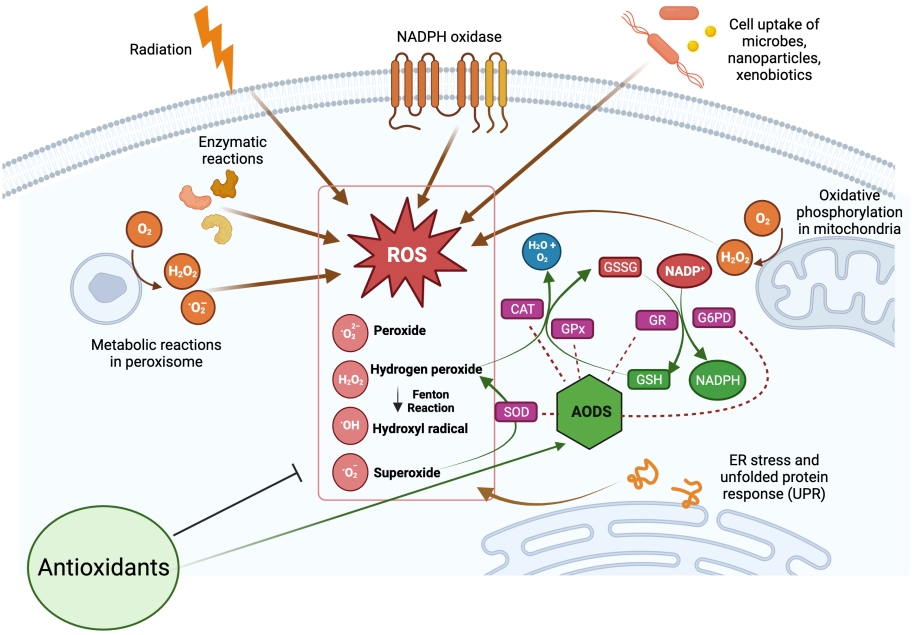

FIGURE 2
Overview of cellular reactive oxygen species (ROS) generation and their scavenging by antioxidant defense system (AODS). Supplemental antioxidants can help lower oxidative stress by scavenging free radicals, blocking enzymes that produce ROS, or stimulating AODS enzymes and molecules. The figure was produced with BioRender (www.biorender.com; accessed on 17th July 2023). Superoxide dismutase (SOD); catalase (CAT); glutathione peroxidases (GPx); glutathione reductase (GR); reduced glutathione (GSH); oxidized glutathione (GSSG); glucose-6-phosphate dehydrogenase (G6PD).

peroxiredoxins (Prxs), and glutathione peroxidases (GPx) are examples of antioxidant enzymes (AOEs) that catalyze the breakdown of hydrogen peroxide (35, 36). Recent studies investigating refined kinetic measurements show that Prxs remove more than 90% of cellular peroxides in comparison to other antioxidant defense enzymes like CAT and GPx (35). Copper and iron ion-sequestering molecules, heme oxygenase, lipoic acid, uric acid, coenzyme Q, and bilirubin are all examples of non-enzymatic antioxidants present *in vivo* (37). In the non-enzymatic antioxidant defense system, glutathione (GSH) plays a crucial function as the most abundant cytosolic thiol. Glutathione can protect cells from free radicals and pro-oxidant damage because it also serves as a cofactor for other antioxidant and detoxifying enzymes, including GPx, glutathione S-transferases (GST), and glyoxalases. Thioredoxin (Trx) serves as a co-substrate molecule for Prxs, and its reducing capabilities are essential to the antioxidative activities of Prxs. Trx and GSH need glutathione reductase (GR) and thioredoxin reductase (TR), in addition to NADPH, to retain their reducing capabilities (38). During the process of GST-mediated detoxification of electrophilic compounds and xenobiotics, GSH functions as a cofactor (39). Following detoxifying interactions of vitamin E with lipid peroxyl radicals (LOO·), GSH can replenish the vitamin E pool (40). Antioxidants that can regenerate their original qualities through interactions with other antioxidants are referred to as an "antioxidant network" (41). Growing research suggests that pathological states characterized by elevated ROS levels are associated with diminished enzymatic and non-enzymatic antioxidant activity (34, 39, 42–59). The signaling pathways of nuclear factor kappa B (NF-κB), mitogen-activated protein kinase (MAPK), and signal transducer and activator of transcription 3 (STAT3) are among the major targets that can be influenced by ROS. Therefore, these pathways play a crucial role in defending against the effects of OS and can be used to identify antioxidant food ingredients or to develop therapeutics for diseases such as IBD. Myeloperoxidase is frequently overexpressed in a variety of inflammatory disorders (60, 61), including chronic gastroenteritis. As a lysosomal enzyme, MPO is secreted into the phagosome of neutrophils after degranulation, where it catalyzes the formation of strong oxidants, such as hypohalous acid (HOX; X = Cl or Br) with potent antibacterial properties. When generated at an improper location, time, or concentration, HOX can potentially damage host tissue. MPO-mediated damage is associated with a number of disorders in people, including IBD (60, 62). High leukocyte infiltration in the inflamed mucosa generates high levels of ROS, which triggers OS and causes cellular and tissue damage seen with inflammation (63).

5 ROS generation in the gastrointestinal tract

The GI tract is one of the primary sources of ROS. Although epithelium acts as a physical and antimicrobial barrier, ingested materials and enteric pathogens can promote inflammation by stimulating the production of proinflammatory cytokines, which further contribute to OS. Oxidative stress is a contributing factor in the development of several GI pathological disorders, such as gastroduodenal ulcers, GI cancer, and IBD. Acute and chronic GI disorders in humans and animal models are characterized by increased ROS production or decreased counteracting antioxidant mechanisms, both of which disrupt redox homeostasis (12, 32, 64, 65).

Oxidative stress-induced damage in chronic intestinal disorders is associated with mucosal infiltration by activated leukocytes, which produce excessive ROS that overwhelm the tissue's antioxidant defenses and perpetuate or exacerbate mucosal inflammation. Several ROS generated by unstable types of oxygen, including the superoxide ion, hydrogen peroxide, and hydroxyl radicals, are the principal pro-oxidant molecules (12).

The intestinal epithelium has been acknowledged as a crucial factor in the development of IBD due to its dual nature of exhibiting both immune and organ-specific functions. In the context of mucosal inflammation, the activation of NADPH oxidase (NOX) and inducible nitric oxide synthase (iNOS) by inflammatory cytokines leads to the production of superoxide and nitric oxide by intestinal epithelial cells (IECs), neutrophils, and macrophages (12). IECs generate an increased amount of ROS/RNS through the activation of NOX and iNOS. The presence of excessive ROS has the potential to cause harm to cytoskeleton proteins, which may modify tight junctions to increase intestinal permeability. Ultimately, this disrupted intestinal epithelial barrier leads to further mucosal inflammation (66). Thus, the initiation of IBD can be attributed to inflammation of the GI tract caused by OS. The microvascular network encircling the epithelial cells can attract inflammatory mediators causing more tissue damage and an escalation of intestinal inflammation. Morphologic lesions associated with intestinal inflammation include goblet cell depletion, decreased production of mucous, development of ulcers and/or hyperplasia of colonic crypt cells (12, 67).

Both ROS and RNS have been implicated in IBD pathogenesis, with a particular role in CD initiation and progression (68). With inflammation, the production of ROS by leukocytes and monocytes increases along with prostaglandins and leukotrienes (e.g., eicosanoids derived from arachidonic acid metabolism) (69, 70). ROS in the GI tract is produced by infiltrating neutrophils and macrophages, as well as IECs. Elevated blood levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) may serve as a biomarker for OS in people with IBD (13). In murine colitis models, systemic depletion of macrophages or neutrophils results in decreased ROS/RNS production, reduced concentrations of proinflammatory cytokines, and mitigation of intestinal inflammation (71).

These ROS promote cell damage and harm tissue integrity by preventing the accumulation of antioxidant defenses in host cells. For example, oxidative damage is observed in the intestinal tissues and peripheral blood leukocytes of patients with CD (72). Moreover, CD patients have lower levels of antioxidant vitamins A, C, E, and beta-carotene in their blood and intestinal mucosa, as well as reduced activity of key cellular AOEs such as glutathione peroxidase and SOD (73). Oxidative stress and redox signaling pathways, especially that involving NF-κB, are also involved in active IBD (Figure 1). Since the redox status of mucosal glutathione

is associated with inflammation and disease progression, impaired mucosal antioxidant defenses likely contribute to the development of UC (74).

Chronic NF-κB stimulation promotes cellular infiltration and mucosal inflammation by increasing the transcription of proinflammatory cytokines (e.g., IL-6, IL-8, IL-16, and TNF-α) and by degrading the intestinal barrier through increased apoptosis of intestinal epithelial cells (Figure 3) (75), up-regulation of metalloproteinases which digest mucosal cells, and the release of ROS metabolites that activate NF-κB to further impair barrier stability (76). Matrix metalloproteinases (MMPs), a disintegrin and metalloproteinase (ADAMs), and tissue inhibitors of metalloproteinases (TIMPs) are involved in the regulation of the inflammatory response (77). The intestinal mucosa of IBD patients demonstrates an up-regulation of MMPs and ADAM17 (TNF-α converting enzyme; TACE), which is commonly correlated with disease severity but is not accompanied by an up-regulation of TIMP (78). It seems possible that the expression of different MMPs in IBD is affected by the imbalance between oxidants and antioxidants, given the importance of OS in the etiology of the disease (79). In addition to normalizing the intracellular redox state, antioxidants directly influence the regulation of MAPK and transcription factors and can reduce the production of MMPs, restoring their levels to normal (79).

One of the well-established signaling pathways that play a vital role in the modulation of mucosal immunological tolerance relevant to the pathogenesis of IBD is the Janus kinase/signal transducer and activator of transcription (JAK/STAT) system (80). To ensure effective intestinal immunity, the JAK/STAT pathway modulates the proportions of effector to regulatory T cell numbers, intestinal epithelial cells and myeloid cells in the mucosa. In IBD, pro-inflammatory cytokines deliver their signal through cytoplasmic JAKs, which, once phosphorylated, associate with another class of cytoplasmic proteins called STATs. Subsequently, STATs are phosphorylated and translocated into the nucleus, where they enhance the transcription of target genes (including *TGF-β*, *TNF-α*, *IL-2*, *IL-6*, *IL-8*, *MMP9*, *Intercellular Adhesion Molecule 1* (*ICAM-1*/*CD54*), *STAT1*, and *STAT3*) (81).

6 Role of antioxidants for IBD-related therapeutic applications and hormonal intervention

Oxidative/nitrosative stress is a significant pathophysiologic factor that plays a role in the initiation and progression of IBD. Overproduction of ROS is stimulated, and consequently, OS is triggered during inflammation because of the large number of cytokines and chemokines secreted by inflammatory cells. Considering this, therapeutic interventions incorporating substances possessing antioxidant and anti-inflammatory properties may be considered. Multiple antioxidant therapeutic

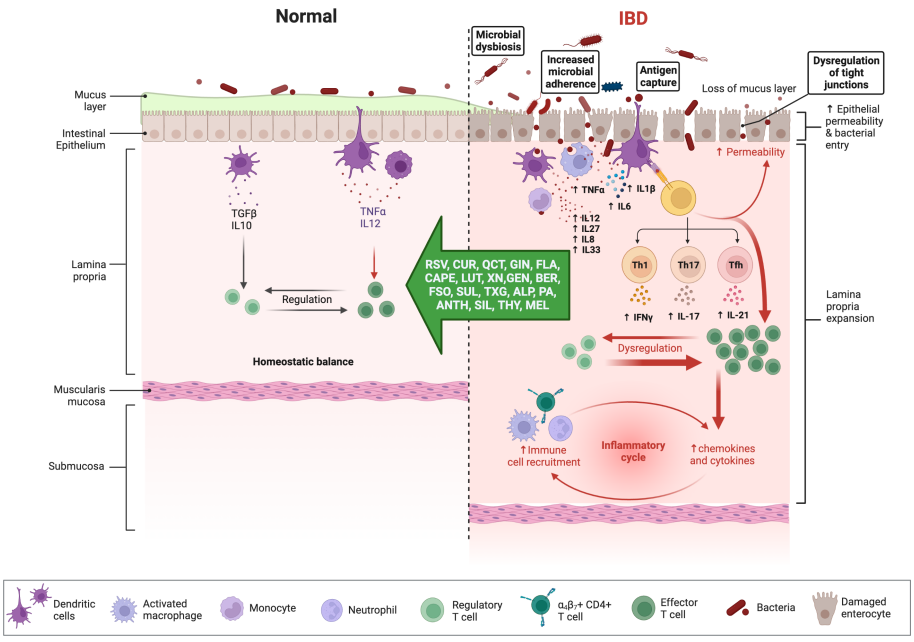

FIGURE 3
Immune responses in IBD and anti-inflammatory effects of phytochemicals and hormones. Whereas the physiologic state of the gastrointestinal (GI) immune system is dominated by immune tolerance, which maintains homeostatic balance, disturbances with IBD are associated with an exaggerated (i.e., pro-inflammatory) immune response, intestinal dysbiosis, and compromised intestinal barrier function. Pro-inflammatory mediators can perpetuate and exacerbate these dysregulated immune responses, while several phytochemicals and hormones can shift this imbalance toward homeostasis. The figure was produced with BioRender (www.biorender.com; accessed on 17th July 2023). Resveratrol (RSV); Curcumin (CUR); Quercetin (QCT); Ginger (GIN); Flavonoids (FLA); Caffeic acid phenethyl ester (CAPE); Luteolin; (LUT); Xanthohumol (XN); Genistein (GEN); Berberine (BER); Flaxseed oil (α-linolenic acid) (FSO); Sulforaphane (SUL); Tamarind xyloglucan (TXG); Alpinetin (ALP); Proanthocyanidins (PA); Anthocyanins (ANTH); Silymarin (SIL); Thymol (THY); Melatonin (MEL); interferon-gamma (IFNγ); tumor necrosis factor-alpha (TNF-α); transforming growth factor β (TGF-β); interleukin (IL). Naive CD4 T cells differentiated Th1, Th17, Tfh (follicular T helper), and Treg (T regulatory) subsets.

strategies are being investigated because of the importance of OS in the pathophysiology of IBD to eliminate ROS, enhance AOE activities, and inhibit abnormal redox signaling (Figure 2). Colonic malondialdehyde (MDA) level rises because of OS, LPx, and free radical chain reactions that damage the intestinal mucosal barrier and activate inflammatory mediators. The use of antioxidants in people with uncomplicated GI disorders has been proposed as a potential alternative therapy to the use of anti-inflammatory/immunomodulatory drugs (82). The aim of antioxidant therapies is to mitigate the adverse effects of traditional treatments and to enhance the patient's quality of life. However, the safety of synthetic antioxidants has been a subject of debate over time, despite their extensive utilization as a viable alternative to natural antioxidants (83–85). Numerous studies have documented a correlation between the prolonged consumption of synthetic antioxidants and certain health problems, such as GI disorders and increased cancer susceptibility (83–85). The utilization of natural antioxidants as a substitute for synthetic products is a noteworthy strategy, given that they are employed within the confines of regulatory thresholds (85).

6.1 Phenolic and polyphenolic compounds

Flavonoids, phenolic acids, lignans, and stilbenes are examples of polyphenols, a family of phytochemicals found in many plant diets. A growing body of research has demonstrated that natural polyphenols effectively mitigate the severity of intestinal inflammation and OS in the early stages of IBD (82, 85–89). Polyphenol-rich diets may ameliorate the pathophysiology of disorders where excessive production of ROS plays a role in disease progression (82). The phytochemicals that inhibit Toll-like receptor 4 (TLR4) activation were shown to cause reduced lipopolysaccharide (LPS)-mediated expression of cyclooxygenase-2 (COX-2), NF-κB, and pro-inflammatory cytokines (Figure 1; Supplementary Table 1). Numerous studies have shown the efficacy of phytochemicals against TLR4-mediated inflammation (Figure 1) (137, 138). Flavonoids such as quercetin, catechin, and silymarin have proven therapeutic efficacy in the treatment of IBD (Figure 3) (139, 140). Here, they operate as effective antioxidants and cellular modulators of the protein kinase and lipid kinase signaling pathways that drive chronic intestinal inflammation (139, 140). Dogs with idiopathic IBD also have up-regulated activity of the JAK/STAT pathway marked by phosphorylated STAT3 (pSTAT3) overexpression (141). Numerous studies have demonstrated that consuming antioxidants found naturally in plants can neutralize harmful free radicals and protect against certain diseases.

6.1.1 Resveratrol

The polyphenol resveratrol (RSV, trans-3,5,4'-trihydroxystilbene) is found in grapes, soybeans, berries, nuts, and pomegranates, among other natural sources (142). In rodent models of IBD, the antioxidant potential of RSV has been investigated. In one trinitrobenzene sulphonic acid (TNBS)-induced colitis study, pretreatment of rats with RSV reduced histologic inflammation and MDA levels but increased GPx activity compared to markers in the TNBS and vehicle groups (91) (Supplementary Table 1).

MPO is responsible for tissue damage in IBD and is inhibited effectively by resveratrol and its derivatives (143). Inhibition of IL-1, IL-6, and TNF-α release from macrophages, iNOS expression and subsequent NO production, prostaglandin production, cyclooxygenase (COX) enzyme activity, apoptosis, and MPO activity are the potential mechanisms by which RSV exerts its anti-inflammatory effect (90, 91) (Supplementary Table 1). The action of resveratrol on ROS generation may involve a direct radical scavenger effect or an effect on the activation of NADPH oxidase (90).

It has been shown that the NF-κB pathway is linked to both colitis and colon cancer development as a consequence of chronic intestinal inflammation (144). Additionally, inflammation in the colon inhibits the activity of the silent information regulator 1 (*SIRT-1*) gene and increases the activity of NF-κB. By activating SIRT-1 and down-regulating NF-κB activation, RSV plays a vital role in the regulation of inflammation that mediates colitis and colon cancer (145) (Supplementary Table 1).

6.1.2 Curcumin

Curcumin, a polyphenol extracted from *Curcuma longa* rhizomes, has a wide variety of beneficial antioxidant, anti-inflammatory, immunomodulatory, neuroprotective, hepatoprotective, anti-cancer, antiproliferative, and chemopreventive properties (34, 49, 50, 146–148). Curcumin reduces OS and improves intestinal barrier integrity and mitochondrial functions by inducing Parkin-dependent mitophagy via AMPK (adenosine 5'-monophosphate activated protein kinase) activation and TFEB (transcription factor EB) nuclear translocation (149). As a result of its ability to inhibit the expression of transcription factors and pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-8, IL-12, IL-1β, and monocyte chemoattractant protein-1 (MCP-1), curcumin has demonstrated anti-inflammatory properties (Supplementary Table 1). Curcumin can also compete with LPS for TLR4 receptor activation, thereby inhibiting the TLR4/myeloid differentiation 88 (MyD88)/NF-κB signaling pathways (150, 151). Curcumin can reduce LPS-induced inflammation in vascular smooth muscle cells via TLR4-MAPK/NF-κB pathways by inhibiting ROS generation (93). This effect is mediated through curcumin's effects on TLR4 as it specifically prevents the LPS-induced generation of MCP-1, TNF-α, and NO (93). Curcumin reduces TNBS-induced colitis in rats by inhibiting the TLR4/NF-κB signaling pathway and pro-inflammatory IL-27 expression (94).

Oral supplementation with curcumin reduces OS generated by hyperthyroidism in rats, as shown by decreased LPx and protein carbonyl (PC) levels and increased SOD and CAT activities in tissues (34, 39, 49). *In vitro* studies show that curcumin can boost cellular glutathione levels by stimulating the transcription of two Gcl genes (*Gclc* and *Gclm*) encoding glutamate cysteine ligase, which is the rate-limiting enzyme in glutathione synthesis (49, 152). It has been reported that curcumin may reduce OS by modulating Nrf2 and KEAP1 function in the rat heart during altered thyroid states (153). Oral delivery of nanoparticles

containing curcumin in dextran sulfate sodium (DSS)-induced colitis in Guinea pigs was associated with a substantial drop in tissue LPx and PC levels, leukocyte infiltration, and TNF-α production (95). Antioxidant balance is also regulated by curcumin in rats with UC by lowering both colonic MPO activity and total NO content as well as increasing colonic GST activity and GSH contents when administered prior to the DSS challenge (154). Curcumin has been shown to decrease OS markers MPO and MDA levels and cell apoptosis in different animal models of colitis (155–158) (Supplementary Table 1).

6.1.3 Quercetin

Quercetin (QCT), a member of the flavonols (a subclass of flavonoids), is a polyphenolic molecule found in plants and has demonstrated anti-inflammatory, antioxidant, and antitumoral effects. Several studies have shown that quercetin can suppress the expression of inflammatory mediators and cytokines such as COX-2, NO, NF-kB, prostaglandin E2 (PGE2), iNOS, TNF-α, IL-1β, and IL-6 that are generated through the LPS-TLR4 pathway (159) (Supplementary Table 1). LPS-induced inflammation is suppressed by quercetin-rich *Myrsine seguinii* ethanolic extract, which inhibits Src- and Syk-mediated phosphoinositide 3-kinase (PI3K) tyrosine phosphorylation and the TLR4/MyD88/PI3K signaling pathways (160). This extract also suppressed iNOS, a high-output Ca^{++}-independent NOS stimulated by cytokines (161), and COX-2 gene expression through reduced NF-κB and activator protein (AP-1) stimulation (160).

Modulation of the stress response genes, including the antioxidant enzyme GPX1, was detected after LPS stimulation of the IBD enteroids and colonoids (57). Organoids have more advantages than conventional models and have been employed in fundamental and clinical research, such as for genetic and infectious diseases, regenerative medicine, and accurate and reliable drug screening (162–168). Murine colitis-derived intestinal organoids stimulated by LPS show reduced mRNA expression of inflammatory mediators such as TNF-α and lipocalin-2 (LCN2) when treated with quercetin (96). The anti-inflammatory action of quercetin was also accompanied by a decrease in the expression of C/EBP-β, a transcription factor that induces the expression of several inflammatory mediators, including TNF-α (169). Human *apoB* promoter analysis shows that a CCAAT enhancer-binding protein (C/EBP)-response element is critical for the action of quercetin. Through its interaction with C/EBPβ, quercetin has the potential to inhibit the recruitment of co-activators. Quercetin also suppresses apolipoprotein B (apoB) expression by inhibiting the transcription of C/EBPβ (169). Several polyphenols, including quercetin, affect the expression of tight junction (TJ) proteins of the intestinal epithelium (88). Using a DSS-colitis murine model, quercetin restored the expression of zonula occludens-1, occludin, junctional adhesion molecule-A, and claudin-3 (88).

There have been several studies on animal models of UC that provide evidence for the use of quercetin to treat IBD (92, 170). Oral quercetin (doses ranging from 25 to 100 mg/kg for 11 days) was associated with decreases in loss of body weight loss, rectal bleeding, and macroscopic and biochemical intestinal damage. It is possible that the antioxidant activity of QCT reduces LPx and OS by regulating nitrites and nitrates, increasing glutathione (GSH), and decreasing MPO activity in the colonic mucosa (92, 170) (Supplementary Table 1). Additional research using animal models demonstrates that QCT activity is mediated through the inhibition of TNF-α expression. By regulating the anti-inflammatory effects and bactericidal activity of macrophages through heme oxygenase-1 (HO-1)-dependent pathway, QCT may reduce the severity of experimental colitis (170, 171). Dietary delivery of QCT to restore intestinal homeostasis and intestinal normobiosis is a potentially promising treatment for IBD (171). Quercetin inhibits the apical efflux of N-acetyl 5-aminosalicylic acid (Ac-5-ASA) from Caco-2 cells, which was mediated by multidrug resistance-associated protein 2. This suggests that using QCT as an additional therapy may contribute to reduced dosages of sulfasalazine required for therapeutic action while reducing adverse drug effects (172). In another murine colitis model study (173), quercetin-loaded microcapsule-treated mice showed significantly more 2,2'-Azino-bis (3-ethylbenzothiazole-6-sulfonic acid) (ABTS) radical cation scavenging and ferric reducing activity as compared to colitis control mice. Results indicated that the QCT-treated mice had significant reduction in neutrophil influx (MPO activity), edema, and colonic macroscopic and histologic inflammation. Finally, this treatment reduced the levels of the pro-inflammatory cytokines IL-1β and IL-33 while maintaining anti-inflammatory cytokine IL-10 levels, and maintained the levels of endogenous antioxidants in the colons of colitic mice (173) (Supplementary Table 1).

6.1.4 Green tea flavonoids

Currently, there is no established tolerable upper limit for flavonoids in the Dietary Reference Intake framework (174). However, the consumption of flavonoids in quantities naturally present in foods does not pose any toxicity concerns (175). A high consumption of flavonoids may increase the likelihood of iron deficiency in populations with suboptimal iron levels (e.g., the elderly). Nonetheless, in Western societies where sufficient intake of heme iron and ascorbic acid occurs, the likelihood of developing anemia is minimal (175). The bioavailability of flavonoids is limited, as only a small fraction (i.e., less than 10% of the ingested quantity) can attain peak concentrations in the bloodstream within a few hours The bioavailability of flavonoids may be subject to various factors, such as ingestion of dietary fiber, macro and micronutrients, the duration of GI transit times, and composition of the gut microbiota (176, 177).

Flavonoids in green tea have been shown to regulate the expression of pro-inflammatory genes that target TLRs and inhibit downstream MyD88- and Toll/IL-1R domain-containing adaptor-inducing IFN-β (TRIF)-dependent signaling pathways (89). Epigallocatechin-3-gallate (EGCG), a flavonoid present in green tea, reduces TNF-α and also gene expression and the effects of nitric oxide synthase (NOS) and COX in murine RAW 264.7 macrophages (97). Treatment with EGCG-docosapentaenoic acid (DPA) esters has been shown to reduce the production of the pro-inflammatory mediators like nitric oxide (NO) and PGE2 via down-regulation of

iNOS and COX-2 gene expression. Other EGCG ester derivatives (stearic acid, eicosapentaenoic acid, and docosahexaenoic acids) also have anti-inflammatory activity in murine RAW 264.7 macrophages (97). The mechanism of action is believed to be the inhibition of downstream TLR signaling affecting the MyD88- and/or TRIF-dependent pathways with activation of NF-κB. A study also demonstrated that EGCG blocks both the MyD88-dependent and TRIF-dependent signaling pathways of TLRs in RAW264.7 cells (89). In TRIF-dependent signaling pathways of TLR3 and TLR4, the molecular target of EGCG is TANK-binding kinase1 (TBK1), resulting in the decrease of interferon regulatory factor 3 (IRF3) activation, as TBK1 is the downstream kinase of TRIF and phosphorylates IRF3 resulting in its activation (89).

The combination of EGCG and piperine (piperine for enhancing the bioavailability of EGCG) significantly decreased weight loss, improved the clinical course, and increased overall survival in the DSS-murine model of colitis (87). Reduced severity of the colitis was linked to improved histology scores and reduced colonic MDA and MPO activity (Supplementary Table 1). The combination of EGCG and piperine improved SOD and GPx expression and suppressed the generation of proinflammatory cytokines *in vitro*. It appears that the powerful antioxidative potential of EGCG is responsible for its anti-inflammatory effects in the DSS-murine model of colitis. Lipid peroxidation occurs during IBD inflammation when ROS are not neutralized, altering the permeability and selectivity of the cell membrane and the activity of transmembrane transporters, receptors, and enzymes (87). DSS-colitis mice that had been administered EGCG and piperine had increased levels of AOEs (SOD and GPx), indicating that the antioxidant capacity had improved (87) (Supplementary Table 1).

6.1.5 Caffeic acid phenethyl ester

The anti-inflammatory, anti-cancer, and antioxidant effects of caffeic acid phenethyl ester (CAPE) have been studied extensively (98, 99, 178–181). Caffeic acid phenethyl ester inhibits LPS-induced IL-12 production and NF-κB activation in monocyte-derived dendritic cells (178). Caffeic acid phenethyl ester prevents the activation of TLR4 by interfering with the interaction between the TLR4/MD2 complex (180). In LPS-induced breast cancer cells, CAPE can down-regulate the expression of TLR4, NF-kB p65, TRIF, MyD88, and IRAK4 while stimulating cell apoptosis and autophagy (181). In gingival fibroblasts, CAPE suppresses LPS-induced production of IL-6, IL-8, iNOS, COX-2, TLR4/MyD88 mediated NF-κB, and phosphorylation of PI3K and protein kinase B (PKB, or Akt) (179).

FA-97 (caffeic acid phenethyl ester 4-O-glucoside) is a new synthetic CAPE derivative shown to attenuate body weight loss, colon length shortening, increased colonic inflammatory cell infiltration, and pro-inflammatory cytokine production (99). While FA-97 increased overall antioxidant capacity in DSS-treated mice and LPS-treated BMDMs and RAW 264.7 cells, it also decreased ROS and MDA production. The mechanism of action of FA-97 is believed to be the activation of the Nrf2/HO-1 pathway in both *in vivo* and *in vitro* models. FA-97 activates Nrf2 and promotes its nuclear translocation to increase expression of its downstream target proteins HO-1 and NAD(P)H:quinone oxidoreductase (NQO-1), which reduces ROS. It also inhibits the NF-κB and AP-1 signaling to suppress the expression of pro-inflammatory cytokines IL-1, IL-6, TNF-α, and IL-12 (Supplementary Table 1). In addition to ameliorating DSS-induced colitis, FA-97 promotes normal epithelial barrier function (98, 99).

6.1.6 Luteolin

The flavonoid compound luteolin, found in various plant extracts, has been shown to have anti-inflammatory, antioxidant, anti-metastasis, and apoptosis-inducing properties. Luteolin has been reported to reduce LPS-stimulated expression of NF-κB, TNF-α, and ICAM-1 and TBK1-kinase activity via the MyD88-independent signaling pathway (182). Luteolin inhibited the expression of target genes IL-6, IL-12, IL-27, TNF-α, IP-10, IFNβ, CXCL9 (C-X-C motif chemokine ligand 9) in macrophages by inhibiting IRF3 and NF-κB activation (Supplementary Table 1). Luteolin was able to suppress the ligand-independent activation of IRF3 or NF-κB triggered by TLR4, TRIF, or TBK1. Luteolin also reduces the amount of TBK1-dependent gene expression by inhibiting TBK1-kinase activity, and IRF3 dimerization and phosphorylation. Moreover, luteolin structural analogs, such as quercetin, chrysin, and eriodictyol, also inhibit TBK1-kinase activity and TBK1-target gene expression. These findings indicate that TBK1 is a unique target of anti-inflammatory flavonoids resulting in the inhibition of the TRIF-dependent signaling pathway (182).

LPS-induced inflammatory responses are controlled at the transcriptional level through the MAPK and NF-κB pathways (100, 183, 184). Toll-like receptors trigger NF-κB and MAPK cascades responses to LPS stimulation, causing the production of ROS, increased MPO expression, and expression of pro-inflammatory molecules and chemokines. In mice with LPS-induced acute lung injury (ALI), luteolin reduced the activation of ERK, p38MAPK, and JNK in lung tissue. The protective action of luteolin is due to its ability to block MAPK pathways, which prevents the activation of NF-κB and the degradation of IκB. Upon administration of LPS, luteolin pretreatment prevents these inflammatory processes from developing (100). In addition, the results in mice indicate that the activities of SOD and CAT increased following pretreatment with luteolin versus LPS treatment alone. MPO levels are reduced in LPS-induced acute lung injury (ALI) when pretreated with luteolin. This mechanism of action is believed to be due to luteolin suppression of LPS-induced ALI-related MDA generation in the lungs (100) (Supplementary Table 1).

6.1.7 Xanthohumol

Xanthohumol (XN), a compound derived from hop plants, is a prenylated chalcone with, antioxidant, anti-cancer, and anti-inflammatory activities via inhibition of TLR4/MD-2 complex (185). Downstream, XN suppresses macrophage iNOS expression and NO, and interferon-gamma (IFN-γ) production (186, 187). Pretreatment with XN in DSS-treated mice decreased the severity of diarrhea, hematochezia, rectal bleeding, and reduced colon length. Moreover, XN protected against epithelial cell injury, cellular

infiltration, pro-inflammatory cytokines, and crypt changes following the DSS challenge (101). Pre-treatment with XN prior to DSS exposure resulted in decreased MDA levels and COX-2 expression suggesting a protective effect against experimental colitis (101) (Supplementary Table 1). Moreover, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) phosphorylation, nuclear translocation of p65, p50, and p105 NF-κB subunits, and NF-κB DNA-binding transcriptional activity were suppressed by XN treatment of DSS-treated mice and H_2O_2- or LPS-treated IEC-6 cells (101).

XN protects mice from DSS-induced colitis and H_2O_2- or LPS-treated IEC-6 injury, possibly by the interaction between the α, β-unsaturated carbonyl moiety of XN and Cys99 in IKKβ, and thus by its ability to inhibit the IKKβ/NF-κB signaling pathway. Additionally, XN inhibited the activation of the canonical NF-κB pathway, as evidenced by the downregulation of NF-κB target genes, like *A1a*, *A20*, *Bcl-xL*, and *c-myc* (101) (Supplementary Table 1). These collective results indicate that XN may be a promising therapeutic agent for the prevention or treatment of colitis (188).

6.1.8 Genistein

A soy isoflavone, genistein, is a powerful antioxidant and anti-inflammatory agent (102–105, 189). Following treatment with genistein, reduced levels of IL-6, TNF-α, and NF-κB activation have been observed in *in vitro* and *in vivo* studies (104, 189). Genistein also inhibited BV2 microglia LPS-induced NO production, prostaglandin E2 release and expression of inflammatory cytokines (IL-1β, TNF-α), TLR4, and MyD88 expression (102). Expression of interferon beta (IFN-β), IL-1α, IL-1β, IL-6, IL-10, TNF-α, colony-stimulating factor 2 (CSF-2), colony-stimulating factor 3 (CSF-3), chemokines CCL2 (chemokine ligand 2), and CXCL10 (C-X-C motif chemokine ligand 10), transcription factor NF-κB, IκBα, and COX-2 are all decreased in genistein-pretreated LPS-induced RAW264.7 macrophages (103) (Supplementary Table 1). In humans, six months of daily oral administration of genistein has been shown to decrease TNF-α levels decreased in obese postmenopausal women (106). Dietary supplementation with genistein lowered the expression of vascular adhesion molecule-1 (VCAM-1), a major cell adhesion molecule involved in inflammation (190), and F4/80 positive macrophages in the aorta of TNF-α-treated C57BL/6 mice (104). Human umbilical vein endothelial cells (HUVECs) in culture showed significant increases in the activities of GR, GPx, GST, NQO-1, SOD, and CAT when treated with 50 μM genistein (104) (Supplementary Table 1). Due to its ability to stimulate IL-1β secretion, the NLRP3 inflammasome has been linked to IBD in experimental models as well as human studies (191). In the DSS murine colitis model, treatment with genistein significantly slowed weight loss and reduced colon shortening, infiltration of inflammatory cells as well as the production of pro-inflammatory mediators in both serum and colon (105). Genistein's protective properties may stem from the ubiquitination of NLRP3 caused by the interaction of cAMP with the protein. It has been shown that genistein can inhibit the NLRP3 inflammasome via TGR5-cAMP signaling in human macrophages (105).

6.1.9 Alpinetin

Alpinetin, a naturally occurring dihydroflavone (192), ameliorates clinical severity (disease activity index; DAI), colonic shortening, histological scores, and MPO activity in DSS-treated mice (108). The expression of occludin and zonula occludens-1 and SOD activity were increased, while the MDA amount was decreased by alpinetin in DSS-treated mice. Also, Nrf2/HO-1 signaling pathways were activated by alpinetin in DSS-treated mice (108). Alpinetin exhibits its anti-inflammatory activities by suppressing TLR4/IκBα/NF-κB signaling (107). In that study, alpinetin decreased the expression levels of IL-1β, IL-6 and TNF-α (Supplementary Table 1) in LPS-treated RAW 264.7 macrophages *in vitro* and in the *in vivo* LPS-induced acute lung injury murine model (107) by disrupting ERK/p38MAPK signaling (193).

6.1.10 Proanthocyanidins

Grape seed proanthocyanidin extract (GSPE) is composed of approximately 90% proanthocyanidins, including oligomers (74.8%), dimers (6.6%), trimers (5.0%) and tetramers (2.9%) (194). Inhibiting the expression of NF-κB-targeted genes is a primary mechanism by which GSPs exert their antioxidant and anticarcinogenic actions (109, 195, 196). It is proposed that GSPs inhibit NF-κB activation by inhibiting the activation of IκK (inhibitor of κB kinase) to prevent phosphorylation-induced degradation of IκBα (196). The effect of GSPs on enhancing antioxidant enzymatic defense was confirmed by their ability to boost colonic SOD activity (109, 195).

Nutritional and pharmacological dosages of proanthocyanidins protect against endotoxin (LPS)-induced intestinal inflammation, OS, and intestinal permeability in colitic rats (197). Notably, area-specific LPS-induced inflammation in the intestine has been identified, and a unique gene signature may be useful to determine the affected intestinal region (57). Treatment with GSPE reduces MPO and COX-2 activity, modifies the gene expression of ileal inflammatory and permeability proteins, and reduces ROS, MDA, and NO levels, and iNOS activities (Supplementary Table 1) in the large intestine (109, 197). Also, GSPE treatment enhances SOD, GPx activity, and glutathione levels in colon tissues and serum of TNBS-induced colitis in rats (109) (Supplementary Table 1).

6.1.11 Anthocyanins

The antioxidant properties of anthocyanins, as well as their ability to influence gut microbiota and to down-regulate the immune response, have significant implications for reducing intestinal inflammation (198, 199). In human monocytic THP-1 cells, treatment with bilberry extract (BE), rich in anthocyanins, inhibited IFN-γ activation of STAT1 and STAT3 and lowered mRNA expression and/or secretion of MCP-1, TNF-α, IL-6, and ICAM-1 (110). The effects of genetically engineered tomato extracts (enriched in anthocyanins) (200) showed that the extract reduced the activation of epithelial cells SAPK/JNK (stress-activated protein kinase/c-Jun NH (2)-terminal kinase) and p38MAPK signaling pathways. Furthermore, pro-inflammatory cytokines and chemokines, including TNF-α and IL-10, were inhibited by anthocyanin-rich tomato extracts (200). It has

been shown that anthocyanin-rich fractions from red raspberries (112) and purple carrots (111) decrease the production of iNOS, COX-2, IL-1β, and IL-6 in LPS/IFN-γ activated macrophages *in vitro*. In murine colitis, anthocyanins extracted from the Chinese plant *Dioscorea alata* L (201). decreased DAI (body weight loss, fecal occult blood, and fecal consistency), increased the gene expression of tight junction proteins and reduced OS markers (MPO and iNOS) along with IFN-γ and TNF-α levels. Red raspberry berry (RB) powder (202) can attenuate the effects of DSS treatment by preventing weight loss, neutrophil infiltration, colon shortening by inhibiting IL-1, IL-6, IL-17, and TNF- α and COX-2 levels in inflamed tissues (Supplementary Table 1). Supplementation with RB restored CAT levels to normal, decreased xanthine oxidase (XO) levels and expression of MCP-1, and thus reduced neutrophil infiltration and ROS formation (202). Similarly, when an extract of blueberry anthocyanins (BBA) (203) was used, disease activity, MPO and MDA levels were reduced in murine colitis. In addition to decreasing serum prostaglandin E2 levels, BBA reduces OS marked by increased SOD and CAT levels. Moreover, mRNA expression of NF-κB, IFN-γ, COX-2, IL-1β, and iNOS was reduced, indicating that blueberry's protective effect is at least partially mediated by the inhibition of inflammatory mediators (203). Similarly, it has been shown that colitis symptoms (colon shortening, DAI, loss of appetite, and weight gain), decreased SOD, and increased MPO can be mitigated and that GPx and GR activities (Supplementary Table 1) can be increased by administering grape pomace extract (GPE) (86). Anti-inflammatory effects of GPE were observed in DSS-treated mice as evidenced by reduced IL-1β, IL-1α, IL-6, and IFN-γ and also TNF-α activities. Gene expression for the p65 subunit of NF-κB, TNF-α, and COX-2 was reduced, which down-regulates the production of pro-inflammatory cytokines by GPE (86). Similarly, cocoa (anthocyanidins/anthocyanins) supplementation (204) decreased MDA, increased SOD, CAT, GPx, and GRx, and reduced iNOS and COX-2 gene expression in murine colitis. Moreover, cocoa is able to stimulate Nrf-2 transcription factor-activated expression of NQO1 and UDP-GT to reduce OS. Cocoa supplementation can also lower $CD68^+$ neutrophils, MPO, TNF-α, IL-1β and IL-17 and suppress STAT-3 activation in colitic tissues (204) (Supplementary Table 1).

One randomized, placebo-controlled trial involving healthy humans found that eating anthocyanins-rich, purple-fleshed potatoes for six weeks reduced the levels of inflammatory markers like IL-6 and C-reactive protein (CRP) and the oxidative DNA-damage marker 8-hydroxydeoxyguanosine (8-HdG) (205). In another study, patients with mild to moderate UC were given an anthocyanin-rich bilberry preparation for six weeks and showed improvements in endoscopic Mayo score, histologic Riley index, and fecal calprotectin levels for intestinal inflammation (206).

6.1.12 Silymarin

Silymarin (SM), an extract from milk thistle (*Silybum marianum*), has been shown to have anti-inflammatory and antioxidant properties. Silymarin decreases inflammation by inhibiting NF-κB pathways and by optimizing the redox balance in the cell through activating AOEs and non-enzymatic antioxidants via Nrf2 activation (207). Studies show that SM increases the total antioxidant capacity of colonic tissue to reduce LPx levels, neutrophilic infiltration and pro-inflammatory cytokine production in TNBS-induced colitic rats. Also, treatment with SM in TNBS-colitic rodents dramatically decreased colonic NF-κB activity, levels of IL-1β, TNF-α, TBARS and MPO activity (113) (Supplementary Table 1). In one randomized, double-blind, placebo-controlled clinical trial, 35/38 UC patients administered SM for 6 months achieved remission. Moreover, remission was accompanied by improved patient biochemical parameters such as decreased erythrocyte sedimentation rates, increased hemoglobin levels, and decreased DAI scores (208). However, the bioavailability of oral SM appears to vary widely between species and is impacted by preparation methods (209–211). Thus, care should be taken when designing experimental trials and extrapolating results between species and preparations.

6.1.13 Thymol

Essential oils from Thymus, Origanum, and Lippia species are rich in thymol (2-isopropyl-5-methylphenol), a monoterpene phenol derivative of cymene (212). Thymol has demonstrated a wide range of biological and pharmacological activities, such as anti-inflammatory, antioxidant, anti-tumor, and antimicrobial effects (213). Thymol exhibits gastroprotective effects on both acute and chronic ulcer models by modulating the enhancement of mucus production, prostaglandin synthesis, and activation of ATP-sensitive K(+) channels (212). Thymol mitigates the reduction of trans-epithelial electrical resistance (TEER) in a porcine IPEC-J2 monolayer cell model stimulated with LPS by increasing ZO-1 and actin expression (118). In the DSS-induced murine colitis model, thymol mitigates intestinal damage induced by DSS by up-regulating the expression of tight junction protein claudin-3 (119). Similarly, thymol enhances the tight junction integrity and induces up-regulation of cyclooxygenase-1 (COX1) activity in Caco-2 cells (115).

Along with improving barrier function, thymol can decrease the production of ROS and the expression of pro-inflammatory cytokines following stimulation with LPS (118). Thymol exhibits the ability to attenuate increased MPO and MDA levels induced by LPS, as well as the expression of NF-κB, in a murine model of acute lung injury (214) and in colonic homogenates in murine colitis (215). Thymol exhibits inhibitory effects on the expression of TLR4 and the activation of NF-κB signaling in mice treated with acetic acid to induce colitis (117, 120). Thymol has been found to exhibit inhibitory effects on p38 phosphorylation, and it disrupts the activation of the MAPK signaling pathway, thereby contributing to the maintenance of immune homeostasis (216). Moreover, thymol exhibits inhibitory effects on the activation of p-p38, p-JNK, and p-ERK induced by LPS in RAW264.7 cells (116). Consequently, thymol effectively suppresses the production of various inflammatory cytokines such as NO, IL-6, TNF-α and COX-2 (116), thus exhibiting the ability to attenuate the inflammatory response through the inhibition of MAPK signaling pathway. Similarly, thymol had the potential to mitigate inflammatory responses by regulating the expression of JNK, AP-1, STAT-3, and nuclear factors of activated T-cells (NFATs) in LPS-stimulated J774.1 mouse macrophages (117).

6.2 Alkaloids

6.2.1 Berberine

Berberine, an isoquinoline alkaloid from *Berberis aristata*, has been used for decades to treat intestinal parasites and enteropathogenic diarrhea due to its bactericidal activity, its ability to inhibit protozoan growth, and also to inhibit enterotoxin-induced intestinal electrolyte secretion (217–219). Several *in vivo* studies confirm its anti-inflammatory role in decreasing the expression of IL-1β, TNF-α, iNOS, ICAM-1, IL-6, and NF-κB activation (220) (Supplementary Table 1). Berberine reduces damage, inflammation scores, MPO activity, and colon shortening caused by oral DSS ingestion. Moreover, berberine has been shown to decrease levels of the pro-inflammatory cytokines IFN-γ, TNF-α, KC (keratinocyte chemoattractant or CXCL1), and IL-17 and to maintain colon epithelial barrier function in DSS-treated mice. Additionally, berberine enhanced apoptosis of colonic macrophages and decreased proinflammatory cytokine production in colonic macrophages and epithelial cells of DSS-treated mice. Berberine suppresses Src activation and TLR4-mediated cell motility in LPS-stimulated macrophages (121). Berberine also inhibits the activation of MAPK and NF-κB signaling pathways that stimulate proinflammatory cytokine production in both colonic macrophages and epithelial cells from DSS-treated mice (122). Multiple cellular kinases and signaling pathways, including AMPK, MAPKs, and Nrf2 and NF-κB pathways, are involved in berberine's antioxidant and anti-inflammatory activities (221). By suppressing the expression of NADPH oxidase, a key enzyme in the generation of ROS in cells, berberine can mitigate OS (222). Superoxide anion production in LPS-stimulated macrophages was suppressed, while SOD activity normally was restored following berberine treatment. Since it can suppress gp91phox (a plasma membrane subunit of NADPH oxidase) expression and boost SOD activity, berberine is able to restore cellular redox activity (222) (Supplementary Table 1). In this instance, it is possible that berberine enhances SOD expression through the silent mating type information regulation 2 homolog 1 (SIRT1)/Forkhead Box Class O (FOXO) pathway (221). In other experiments, berberine has been shown to directly bind to PLA2G4A and inhibit the MAPK/JNK signaling pathway to suppress PLA2G4A activity in murine colitis (223). A double-blind placebo-controlled phase I trial (123) demonstrated that berberine reduced colonic tissue inflammation (Geboes score) (224) in mesalamine-treated UC patients. However, it had no effect on inflammatory biomarkers in other tissues or blood (123).

6.3 Storage polysaccharides

6.3.1 Tamarind xyloglucan

Tamarind xyloglucan (TXG), a nanofiber extracted from tamarind, is a novel antioxidant that prevents DSS-induced colitis in mice (124, 125). TXG protects the colon by reducing the total inflammatory index, infiltration of inflammatory cells, submucosal edema, goblet cell loss, epithelial erosion, granulation tissue, epithelial hyperplasia, and crypt irregularity, abscesses and loss (124, 125). While TXG reduces inflammation by reducing TNF-α and increasing anti-inflammatory IL-10, it reduces OS by lowering levels of MDA, superoxide anion production, and the expression of iNOS, NOX, COX-2, and p47 (125) (Supplementary Table 1).

6.4 Other phytochemicals

6.4.1 Sulforaphane

Sulforaphane, found in cruciferous vegetables, contains a wide range of antibacterial, antioxidant, anti-inflammatory, and immunomodulatory properties (225, 226). Treatment of rats with colitis using sulforaphane reduces NO and MDA levels, accompanied by increased GPx and reduced glutathione levels, demonstrating its therapeutic antioxidant properties (8). Treatment with sulforaphane increases the levels of Nrf2 and HO-1 (8) (Supplementary Table 1). It has been observed that HO-1, an antioxidant defense protein downstream of Nrf-2, prevents oxidative damage to colonic tissue (99). Sulforaphane possesses anti-inflammatory characteristics by decreasing TNF-α and IL-6 levels, inhibiting COX-2 expression, TLR4 oligomerization, TLR4/MyD88 pathway and blocking the degradation of IL-1R-associated kinase-1, NF-κB, and IFN regulatory factor 3 activation (227, 228).

6.5 Food/spices

6.5.1 Flaxseed oil (α-linolenic acid)

Flaxseed oil is an herbal product with a high α-linolenic acid content, and it has been shown to reduce colonic damage in DSS-induced colitis by modulating inflammatory factors, oxidative state, and the cecal microbiota imbalances. DSS-colitic rat colon showed low SOD activity and GSH levels, increased MPO activity and MDA levels, and flaxseed oil dose-dependently alleviated this condition. Compared to the DSS-treated group, flaxseed oil treatment for six weeks raised SOD activity and GSH levels while decreasing MDA levels and MPO activity (127) (Supplementary Table 1). A metabolite of linoleic acid, 10-hydroxy-cis-12-octadecenoic acid (HYA), inhibits TNF-α expression and DSS-induced alterations in the expression of TJs such as occludin, zonula occludens-1, and myosin light chain kinase. The metabolite HYA partially restores the integrity of the intestinal epithelial barrier via the GPR40-MEK-ERK pathway, as reported in human Caco-2 cells and the murine DSS-colitis model (126).

6.5.2 Ginger

Ginger rhizomes, a rich source of many active compounds, including gingerols, shogaols, gingediols, zingerone, dehydrozingerone, gingerinone, and diarylheptanoids, have a wide range of anti-inflammatory, analgesic, antioxidant, and anti-cancer effects (129, 151, 229). S-[6]-gingerol inhibits the expression of the inflammatory mediators IL-6, IL-8, and serum amyloid A1 (SAA1) in cytokine-stimulated human HuH7 hepatocyte cells by inhibiting the NF-κB/COX-2 pathway to reduce OS (129).

Concentration-dependent inhibition of LPS-induced IL-1β, IL-6, TNF-α and PGE2 levels was demonstrated with 6-shogaol, the most bioactive component of ginger (230). 6-Shogaol also reduces the phosphorylation and nuclear translocation of NF-κB p65, thus preventing LPS-induced NF-κB activation. In LPS-induced murine RAW 264.7 macrophages, 6-shogaol was reported to inhibit protein and mRNA expression of iNOS and COX-2 (128). By inhibiting the phosphorylation of inhibitor κB (IκB)α and p65 and the subsequent degradation of IκBα, 6-shogaol decreased the LPS-induced activity of NF-κB. PI3K/Akt and extracellular signal-regulated kinase 1/2 activation by LPS is also blocked by 6-shogaol, although the p38MAPK activation is not (128). Since TLR4 dimerization mediated by LPS is necessary for the activation of downstream signaling pathways, including NF-κB, 6-shogaol blocks this process and prevents NF-κB activation (231). Also, 6-shogaol can block TLR-mediated signaling pathways directly at the receptor (231). In LPS-induced macrophages, 6-shogaol suppresses the MyD88-dependent signaling pathway by inhibiting IκB kinase activity and TRIF-dependent signaling pathways that target TBK1 (232). Compounds extracted from ginger were tested for their ability to stimulate phagocytosis and suppress nitric oxide generation in the RAW 264.7 cell line induced by LPS (229). Significant reductions in both LPS-induced nitric oxide production and inducible nitric oxide synthase expression were observed when 6-shogaol, 1-dehydro-10-gingerdione, and 10-gingerdione were applied (229). Macrophages are essential components of the immune system as they are responsible for the elimination of necrotic cells. This function is critical as it prevents the release of harmful contents from these cells, thereby minimizing a proinflammatory response (233). RAW 264.7 cells treated with 1-dehydro-10-gingerdione (1D10G) showed increased phagocytic activity similar to stimulation by LPS. Also, RAW 264.7 cells treated with 6-gingerol, 8-gingerol, 10-gingerol, 6-paradol, 10-gingerdione, 1,7-bis-(4' hydroxyl-3' methoxyphenyl)-5-methoxyhepthan-3-one, and methoxy-10-gingerol exhibited increases in phagocytic activity (229). 1D10G directly decreased the catalytic activity of cell-free IκB kinase β (IKKβ) in RAW 264.7 macrophages activated with the TLR4 agonist LPS. In LPS-activated macrophages, 1D10G inhibited TLR4-mediated expression of TNF-α, NF-κB, IL-1β, IRF3, IFN-β and IP-10 (234). Furthermore, in macrophages triggered by the TLR agonists LPS or TNF-α, D10G irreversibly blocks cytoplasmic IKKβ-catalysed IκBα phosphorylation and IKKβ vector elicited NF-κB transcriptional activity to minimize inflammatory signals (235). Substitution of Cys (179) with Ala in the activation loop of IKKβ abrogates these effects suggesting a direct interaction site of D10G. Lastly, D10G reduced NF-κB activation in LPS-stimulated macrophages and decreased the expression of iNOS, COX-2, and IL-6 (235).

In mice with DSS-induced colitis, ginger alleviates the pathological lesions and reduces the expression of IL-6 and iNOS (131). This study also showed that ginger has an anti-inflammatory effect like that of the anti-inflammatory medication sulfasalazine (SASP), reducing DAI and preventing further weight loss (131). Ginger reduces IBD activity by targeting IL-17, IFN-γ, and TNF-α, while increasing the anti-inflammatory cytokines IL-10, IL-22, and TGF-β (236) (Supplementary Table 1).

Ginger extract exhibited the antioxidant effects in IL-1β-mediated OS in human C28I2 chondrocyte cells by stimulating the expression of several AOEs, decreasing IL-1β-induced ROS production, LPx, and by reducing apoptosis (Bax/Bcl-2 ratio, and caspase-3 activity) (130) (Supplementary Table 1). Patients with active mild to moderate UC who ingested 2000 mg/day of dried ginger powder in a randomized, placebo-controlled clinical trial had lowered MDA without affecting serum total antioxidant capacity (132).

6.6 Hormones and their anti-inflammatory and antioxidant roles in IBD

There is growing recognition that the endocrine system plays an important role in the pathogenesis of IBD through various mechanisms (237). Hormone actions influence seemingly every phase of inflammatory and immunological responses, and the intestinal tract is the largest endocrine gland of the body, secreting a vast amount of peptides with paracrine or endocrine function. In sites of inflammation, several hormone receptors have been found to be present in the reactive structures, which are known to have both pro- and anti-inflammatory effects. Signals are generated upon the binding of hormone molecules to specific hormone receptors; these receptors are found on the surface of endothelial and inflammatory cells and play a role in both pro- (such as insulin receptors) (238, 239) and anti-inflammatory (glucocorticoid receptors) (240–242) responses, respectively. The activity of the adrenal cortex is responsible for mediating the indirect anti-inflammatory effects that are caused by glucagon and thyroid hormones (243). Therefore, inflammation is not only a local response but also a hormone-controlled process that occurs locally (paracrine regulation) and throughout the body (endocrine modulation).

One study involving 1,203 females (64% diagnosed with CD,34% diagnosed with UC) reported increased symptoms during their menstrual cycle. Symptoms were comparable among CD and UC cohorts except for pregnant women, where symptoms worsened (244). Women with UC have increased symptoms as compared to women with CD. Understanding the significance of hormones in the context of IBD is crucial for identifying potential approaches to managing hormonal-associated symptoms in women with IBD. The primary endocrine manifestations of IBD include growth failure, metabolic bone disease, alterations in lipid and carbohydrate metabolism, pubertal delay, and hypogonadism (237). These manifestations are interrelated, and their complex development is influenced by intestinal inflammation and the individual's nutritional status.

6.6.1 Sex hormones

Estrogens have been shown to increase AOEs by upregulating their expression (34, 147, 245). The antioxidant impact of estrogen is thought to be the principal method by which this hormone protects various tissues from oxidative damage (246–248). It has been postulated that sex hormones such as estrogen, progesterone, and androgen contribute to the pathophysiology of sexual dimorphism in human IBD (249). These control the behavior of

many different types of immune cells, including lymphocytes, macrophages, granulocytes, and mast cells. Multiple clinical features of the disease, including intestinal barrier disintegration and mucosal immune activation, may be modulated by sex hormones, as shown in both clinical and experimental models (249). There is growing interest in the potential regulation of the intestinal microbiota by sex hormones. Estrogens have an effect on the microbicidal activity that is carried out by MPO in polymorphonuclear leukocytes (250). In CD, the G protein-coupled estrogen receptor (GPER) appears to be a powerful therapeutic target in maintaining remission because it promotes anti-inflammatory effects. Reducing mortality, improving macroscopic and microscopic scores, and lowering CRP levels were all achieved with GPER activation in a TNBS-induced CD murine model (251). Immunohistochemistry verified that estrogen signaling inhibits intestinal inflammation. Genes involved in signal transduction and immunological response, as well as the expression of certain miRNAs (miR-145, miR-148-5p, and miR-592), were shown to be altered in tandem with GPER activation, as was the extracellular-signal-regulated kinase (ERK) signaling pathway (251).

Increased expression of tight junction proteins was a mechanism by which progesterone and estrogen facilitated wound healing and epithelial barrier function in intestinal epithelial cells, as reported using 2D cell lines and IBD patient-derived inflammatory organoid models (252). These sex hormones also inhibited the generation of pro-inflammatory cytokines in intestinal epithelial models and greatly decreased endoplasmic reticulum (ER)-stress. Pregnancy hormones like estrogen and progesterone have been shown to have beneficial effects on disease activity by positively modulating the intestinal epithelial lining (252).

6.6.2 Glucagon

Glucagon decreased iNOS expression and plasma levels of nitrite/nitrate in LPS-treated rats (253). Glucagon is responsible for inducing the antioxidant response by increasing GSH levels and reducing both protein carbonyl and 3-nitrotyrosine (254) or by upregulating AOEs as indicated by high levels of CAT expression in the α cells of diabetic vs. non-diabetic murine models (255).

6.6.3 Enterohormones

Glucagon-like peptide-1 (GLP-1) and GLP-2 are important enterohormones that mediate local and systemic effects (gut-brain-periphery axis), contribute to glucose homeostasis, and modulate GI functions such as the intestinal absorption of lipids and antioxidant defense (256). The effects of these molecules critically depend upon nitric oxide synthase (NOS) in the enteric nervous system (ENS) and the intestinal microbiome (257).

Activation of the GLP-1 receptor has anti-inflammatory, antioxidant, and anti-apoptotic effects, which include a reduction of the pro-inflammatory actions of advanced glycation end products (258) and of the accumulation of intracellular ROS, the release of NO, and GPx and SOD production (259). Moreover, it has been proposed that GLP-1 plays a critical role in the production of NO (260), and intestinal GLP-1 production can be increased by the ingestion of grape-seed procyanidin extract (GSPE), resveratrol, and curcumin (261–264). GSPE also increases intestinal peptide YY (PYY) and varied cholecystokinin (CCK) secretion, which modulates food intake (261, 265, 266).

The enterotrophic peptide hormone glucagon-like peptide 2 (GLP-2) was shown to abrogate OS and improve intestinal antioxidant capacity by reducing LPS-induced increases in intestinal IL-1β and oxidized glutathione levels (267) and increasing intestinal SOD activity and reduced-glutathione levels (268, 269) (Supplementary Table 1). Additionally, GLP-2 has a protective effect on the function of the intestinal barrier (270), intestinal IgA production (270), NO-regulated intestinal perfusion (271), and stabilizes the expression and function of intestinal xenobiotic transporters (267). Because intestinal barrier function is critically impaired in IBD and CIE, complex neutraceuticals that provide antioxidants and can enhance intestinal GLP-2 secretion, and thus support enterocyte tight junctions and intestinal barrier integrity (e.g., berberine, soy flavonoids, pre-/probiotics, soluble fiber, glutamine), may be useful to restore and maintain intestinal health (272).

Given the effects of GLP-1 and GLP-2 in maintaining intestinal homeostasis, including cross-talk with the immune and central nervous systems (273, 274), they appear to present potentially beneficial treatment targets in IBD and CIE.

Reduced levels of serum motilin and gastrin, together with lower pro-inflammatory cytokines and reduced tissue NF-κB and COX-2 levels, and increased serum somatostatin, vasoactive intestinal peptide, and tissue SOD, NO, and MDA levels were associated with genistein- and daidzein-rich fermented soy (shuidouchi) in an experimental animal model (275). Similar antioxidative, anti-inflammatory, and enterohormone signature-modulating effects have been demonstrated in experimental mice receiving unsaturated fatty acid-rich silkworm pupa oil (276).

6.6.4 Glucocorticoids

The presence of glucocorticoid receptors in endothelial and inflammatory cells (240–243), as well as increased concentrations of circulating glucocorticoids at the onset of inflammation downregulating inflammatory responses, is proof that glucocorticoids are modulators of inflammatory responses (243, 277). Adrenalectomized animals also showed greater microvascular responses to inflammatory mediators and cell migration to inflamed regions (243, 278). Glucocorticoids have powerful anti-inflammatory and immunosuppressive effects, such as lowering cytokine production or activity, reducing microvascular responses to inflammatory mediators, preventing leukocyte accumulation at inflamed sites, impeding phagocytic functions and microbicidal capacity of polymorphonuclear leukocytes, preventing the recruitment of mononuclear phagocytes to injured areas, and interfering with immune function (243, 278–280).

6.6.5 Thyroid hormones

Thyroid hormones are associated with the oxidative and antioxidative status of an organism because of their role in regulating oxidative metabolism and in the formation of ROS (34,

39, 50–55, 281). Rats with thyroid dysfunction, produced by hormone injection or thyroidectomy, were evaluated for their ability to respond to noxious stimuli in an effort to determine the processes through which thyroid gland activity can influence the development of inflammatory reactions (243, 282, 283). Rats with hypothyroidism exhibit typical inflammatory responses, whereas animals with a sustained excess of circulating thyroid hormones exhibit consistently suppressed inflammatory responses (282, 283).

6.6.6 Corticotropin releasing hormone

A study was conducted to investigate the impact of administering Corticotropin releasing hormone (CRH) to induce psychosocial stress in DSS-treated colitic mice, specifically by examining the potential enhancement of autophagy in intestinal macrophages. The inflammatory challenges associated with IBD led to increased autophagy in both intestinal macrophages and murine bone marrow-derived macrophages, and these effects were further increased by CRH (284).

6.6.7 Adipokines

IBD is distinguished by symptoms such as reduced appetite, inadequate nutrition, changes in body composition, and the enlargement of mesenteric white adipose tissue (mWAT) (285, 286). Adipokines, namely leptin, adiponectin, and resistin, play a significant role in anorexia, malnutrition, changes in body composition, and hypertrophy of mWAT (285, 286). Studies have demonstrated that there is an increased expression of leptin, adiponectin, and resistin in mWAT in individuals diagnosed with CD.

6.6.7.1 Leptin

Leptin serves as a regulator of diverse immune and inflammatory reactions in addition to its metabolic and endocrine roles. Leptin has the ability to initiate activation and alter the pattern of cytokine production, favoring a Th1 response by promoting the release of IL-2 and IFN-γ while inhibiting the secretion of IL-4 (287). Additionally, it directly stimulates the expression and release of IL-1a, IL-1b, IL-6, and TNFα by T-cells (288). Leptin was found to be expressed and released into the intestinal lumen by inflamed colonic epithelial cells in patients diagnosed with UC. Leptin, in turn, elicited damage to the epithelial wall and infiltration of neutrophils (289). There was a higher level of leptin mRNA expression in the mesenteric white adipose tissue (mWAT) of patients with IBD compared to the control group suggesting that leptin may play a role in the inflammatory process by increasing the expression of mesenteric TNFα (290). The study found that individuals with IBD had lower levels of serum leptin compared to a control group of healthy individuals. This difference was observed regardless of factors such as sex, age, CRP levels, years since diagnosis, and disease activity and localization. The study found that individuals with a BMI greater than or equal to 25 exhibited significantly lower levels of serum leptin compared to individuals with a BMI below 25 (286). The administration of infliximab, an anti-TNFα biologic agent, to individuals diagnosed with CD resulted in an elevation in leptinaemia levels. This suggests that TNFα plays a significant role in suppressing the production of leptin in patients with CD (291).

6.6.7.2 Adiponectin

Adiponectin production and TNFα are mutually suppressed, and their actions are antagonistic in the target tissues (292). Additionally, *in vitro* studies have shown that IL-6 reduces adiponectin levels (292). The induction of adiponectin resulted in the production of IL-10 and interleukin-1 receptor antagonist (IL-1Ra) in human peripheral blood mononuclear cells (PBMC), macrophages, and dendritic cells (DC). Additionally, it hindered the production of IFN-γ in macrophages. The macrophages treated with adiponectin demonstrated a decreased ability to engulf particles and a diminished immune response to cells from a different individual (293). Previous studies have suggested a potential protective role of adiponectin against OS (294). Conversely, it has been observed that OS leads to a reduction in adiponectin secretion in 3T3-L1 adipocytes (295). Adiponectin can inhibit the enhanced cytotoxic activity of natural killer (NK) cells that are induced by IL-2, as well as the production of IFN-γ (296). Studies conducted using the adiponectin knock-out (KO) murine model revealed that adiponectin KO mice exhibited a notably more severe form of colitis in comparison to their wild-type counterparts. However, the severity of colitis was significantly reduced when adiponectin was supplemented. Additionally, it was observed that adiponectin exhibited inhibitory effects on the production of IL-8 in HT-29 cells stimulated with LPS, suggesting that adiponectin may possess a direct anti-inflammatory impact on intestinal epithelial cells (297). However, other studies showed contradictory results on the effect of adiponectin on the development of colitis and restoration of inflammation (298). There is an elevated secretion of adiponectin from mWAT in patients with CD who have undergone surgery, in comparison to patients with diverticulitis and colon carcinoma. Also, serum adiponectin levels are increased in patients with IBD versus healthy controls (286).

6.6.7.3 Resistin

The secretion of resistin from mWAT in patients who underwent surgery for colon cancer or diverticulitis was observed to be significantly lower when compared to the secretion from the adipose tissue adjacent to the affected intestine in patients with CD (285). It was noted that the administration of steroids led to a reduction in resistin production in CD patients (299). The levels of serum resistin in patients with IBD (with active disease and also with quiescent disease) are elevated in comparison to healthy controls.

6.6.7.4 Ghrelin

In chronic DSS-induced colitis in ghrelin KO mice, due to the absence of endogenous ghrelin, the DAI was reduced, and the infiltration of neutrophils was delayed compared to wild-type (300). The introduction of ghrelin either at the onset of the disease or a few days after colitis had developed resulted in ameliorating both the clinical and histopathologic severity of the disease. This therapeutic effect was observed alongside the suppression of inflammatory and Th1-driven autoimmune response, as well as elevated levels of IL-10

(301). Ghrelin mRNA expression and its receptor were found to be increased in TNBS-induced colitic murine models. Serum ghrelin levels were increased in patients with IBD, regardless of whether the disease was active or in remission and were higher in male versus female patients. Ghrelin levels were higher in patients with ileal CD compared to those with colonic CD. In contrast, Peracchi et al. (302) demonstrated that individuals with active IBD exhibited elevated levels of circulating ghrelin in comparison to both healthy controls and patients in a state of remission.

6.6.8 Type 2 diabetes and IBD

Previous research conducted on animals has indicated that the presence of peroxisome proliferator-activated receptor γ (PPAR γ) in the intestinal epithelial cells, macrophages, and T cells of mice with experimental IBD demonstrates immunoregulatory effects and potentially plays a role in the prevention of intestinal inflammation (303, 304). The administration of the gamma subtype of peroxisome proliferator-activated receptors (PPARγ) ligands has been demonstrated to reduce the production of inflammatory cytokines, such as IL-1β and TNF-α, as well as inhibit the proliferation of inflammatory cells and the expression of specific adhesion molecules (305). The thiazolidinedione (TZD) antidiabetic medications for the treatment of type 2 diabetes function as ligands for the gamma subtype of PPARs. Several trials with TZD in the context of IBD have yielded intriguing outcomes (237, 306, 307). In a multicenter randomized, double-blind, placebo-controlled clinical trial, the effectiveness of rosiglitazone in managing UC with mild to moderate activity has been demonstrated (306). Here, 17% of patients who received treatment with rosiglitazone were able to achieve remission. Significant clinical improvement was observed in as short as 4 weeks, with enhanced quality of life by the 8th week (306). In another study, it was determined that TZD does not confer any discernible benefits in comparison to alternative oral antidiabetic drugs with regard to the prevention of UC-related flares. Nevertheless, the utilization of TZD may diminish the probability of experiencing more severe disease flares that necessitate oral steroid treatment (307).

6.6.9 Melatonin

Melatonin (N-acetyl-5-methoxytryptamine) has several unique scavenging effects making it an exceptionally potent direct free radical scavenger and multifunctional antioxidant (34, 308, 309). Melatonin is an electron-rich molecule that can react with free radicals to produce stable compounds that are excreted in the urine (310). Melatonin is frequently referred to as a suicidal or terminal antioxidant because it eliminates free electrons from the system throughout its chemical rearrangement, and each of the rearrangement products is again a potent antioxidant. Melatonin acts as an indirect antioxidant, increasing AOEs, including SOD, CAT, GPx, GR (311, 312), and glucose-6-phosphate dehydrogenase (G6PD) (313). Increased intracellular levels of the antioxidant GSH are generated as a consequence of melatonin stimulating γ-glutamyl cysteine synthetase (the rate-limiting enzyme in GSH formation) and GR (the enzyme converting GSSG to GSH) (314, 315). Melatonin also potentiates the function of the mitochondrial electron transport chain, which reduces free radical generation and electron leakage (316). Melatonin inhibits the production of free radicals by acting as a negative modulator of several pro-oxidant enzymes including 5-lipoxygenase, 12-lipoxygenase, and NO synthase (309). It also prevents mitochondrial damage and reduces NF-κB signaling, suggesting a repair mechanism for intestinal injury caused by OS (317). Decreased levels of hydroxyl radical (HO•), peroxynitrite ($ONOO^-$), $RO_2^•$, and singlet oxygen, and decreased expression of COX-2 and iNOS and NF-kB activation are all antioxidant effects of melatonin (318, 319), pointing to the possibility that melatonin is beneficial in UC by lowering inflammation and regulating OS. Pineal-derived melatonin and *de novo* synthesis in the GI tract both produce melatonin which helps regulate gut immunity and intestinal barrier integrity. Due to its antiapoptotic action and ability to decrease bacterial translocation across epithelia, melatonin can limit mucosal damage (73). Animal studies have demonstrated that melatonin treatment reduces inflammation by blocking the production of IL-10, IFN-γ, TNF-α, IL-6, and $NO^•$ (320) (Supplementary Table 1). In murine colitis, melatonin reduces the generation of ROS and reactive nitrogen species (RNS), characterized by lowering colonic MDA levels and MPO activity and improved antioxidant defenses with increased GSH and SOD levels in the colon (134).

By modulating autophagy and Nrf2 signaling pathways, melatonin slows the progression of colitis-associated colon cancer in mice. In this colon cancer model, melatonin decreased the levels of inflammatory markers IL-6, IL-17, TNF-α, NF-κB, COX-2 and STAT3, as well as reduced DNA damage and OS indicated by reduced TBARS and increased GSH levels (Supplementary Table 1). The decrease in the expression of Beclin-1 and the LC3-II/LC3-I ratio, along with an increased expression of p62, indicate melatonin-inhibited cancer-associated autophagy. These results are like those showing upregulation of Nrf2 and AOEs NQO-1 and HO-1 in melatonin-treated mice with colon cancer (321). Another study investigated the role of oral melatonin therapy on OS in dogs before and after ovariohysterectomy (OHE) (135). The levels of SOD, GPx, and CAT were increased, and MDA decreased in ovariohysterectomized dogs (Supplementary Table 1) that received melatonin compared to those of the control group. These preliminary findings suggest that melatonin administration may reduce OS induced by OHE in dogs. Adjuvant melatonin treatment may aid in maintaining remission in patients with UC (136). In this clinical trial, patients receiving melatonin adjuvant treatment-maintained remission (e.g., lower disease activity scores and inflammatory biomarkers such as CRP levels with increased hemoglobin concentrations) over 12 months of observation (136).

7 Conclusion

Oxidative stress plays a crucial role in the pathogenesis of IBD and is closely associated with the development of intestinal inflammation in humans and animals. Therapeutic strategies involving natural antioxidant products have shown benefit in numerous human studies and animal models. An improved comprehension of the free radical biology mediating GI disease is essential for developing effective future treatments to reduce intestinal inflammation and improve the quality of life in affected

individuals. Also, hormonal intervention holds potential importance in the context of IBD and cannot be disregarded. In this review, we have discussed the major antioxidant and anti-inflammatory properties of various phytochemicals and hormones in IBD. We have also discussed significant evidence-based observations, including the results from clinical trials using natural antioxidants or their modified formulations. Based on the existing scientific evidence, it appears likely that future therapies will include antioxidants with standard treatments or even as an alternative medical option in humans and animals with IBD.

Author contributions

Writing—original draft preparation, revisions, figures, review, and editing DKS, Writing—review, and editing RMH, BP, AP, VKY, DW, AEJ. All authors contributed to the article and approved the submitted version.

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Conflict of interest

AEJ is one of the founders of a biopharmaceutical start-up company, 3D Health Solutions, Inc.

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1217165/full#supplementary-material

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Aging and antioxidants: the impact of dietary carotenoid intakes on soluble klotho levels in aged adults

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Objectives: The association between dietary carotenoid intake and Soluble Klotho (S-Klotho) levels among the elderly population requires further evaluation. The purpose of this study is to evaluate the relationship between the dietary carotenoid intake and the S-Klotho plasma levels in older adults.

Methods: Eligible participants aged 60 years or above were selected from the National Health and Nutrition Examination Surveys (NHANES) data, collected between 2007 and 2016. The consumption of carotenoids was determined through two 24-hour dietary recall assessments. Moreover, the S-Klotho levels in the serum were measured using an Enzyme-Linked Immuno-Sorbent Assay (ELISA).

Results: A total of 5,056 participants were included in the study having a median total carotenoid intake of 9775.25 μg (95% confidence interval (CI): 8971.30–10579.21) and a median S-Klotho concentration of 815.59 pg/mL (95% CI: 802.59–828.60). The multivariable regression analysis showed that a single standard deviation increase in total carotenoid intake was significantly associated with an 8.40 pg/mL increase in S-Klotho levels (95% CI: 0.48–16.31). When the carotenoids were divided into quartiles, participants in the third ((4963.5μg/day,11662.5μg/day]) and fourth quartiles ((11662.5μg/day,377178μg/day]) showed higher S-Klotho levels compared to those in the first quartile. Among carotenoid subtypes, increased intake of α-carotene, β-carotene, and lutein with zeaxanthin was associated with elevated S-Klotho levels. These observed associations between carotenoid subtypes and S-Klotho levels remained consistent across male participants, having a normal weight, and a moderate physical activity based on stratified analysis.

Conclusion: The total carotenoid intake was positively related to plasma levels of S-Klotho in the elderly population, particularly for α-carotene, β-carotene, and lutein with zeaxanthin. However, further research is needed to confirm these findings and explore the underlying mechanisms behind this relationship.

KEYWORDS

carotenoids, antioxidants, klotho, NHANES, aging

Introduction

Aging is a multifactorial process that is characterized by its complexity. One of the proposed mechanisms underlying this process is the changes in oxidative stress and inflammation that occur with advancing age (1). Klotho is a type-I membrane protein that is linked to β-glucuronidases and is produced by the klotho gene (2). This protein is a regulator for aging, discovered by Kuro-o and his colleagues in a mouse model with genetic defects, resulting in several premature aging syndromes and shortened lifespans (3). Moreover, Soluble Klotho (S-Klotho) is produced through the cleavage of the membrane-bound Klotho and can be detected in the blood, cerebrospinal fluid, and urine (4). It is also involved in the regulation of oxidative stress, inflammation, and aging, and a low level of S-Klotho is associated with various metabolic and aging-related diseases, such as type 2 diabetes, Non-Alcoholic Fatty Liver Disease, cardiovascular disease, and cognitive regression (5–10). Furthermore, a low level of S-Klotho yields in an increase in the risk of all-cause and, most importantly, the cardiovascular disease-related mortality (11–13). The levels of systemic inflammation and S-Klotho in the plasma are inversely correlated; the decrease of the S-Klotho levels in the plasma is associated with systemic inflammation increase and vice versa (14).

In opposite, carotenoids are organic pigmented molecules that arise from fungi, bacteria, and plastids of algae and plants (15). They are well-known for their antioxidant properties and their ability to protect the human being against oxidative stress and inflammation (16). These molecules play a crucial role in sustaining human health and increasing the lifespan of animal models (17–19). Since the human body cannot synthesize carotenoids, these latter must be acquired from dietary sources (20) where, among the most well-known carotenoids, one can find the β-carotene, lycopene, lutein, and zeaxanthin. Finally, due to their antioxidant properties, it is believed that carotenoids can assist in reducing the severity of chronic illnesses (21).

Despite the numerous benefits that carotenoids offer in terms of combating oxidative stress, inflammation, and their general positive influence on human health (17), the connection between the consumption of dietary carotenoids and S-Klotho plasma levels in aged adults remains largely unexplored. Therefore, the purpose of this study is to explore the link between carotenoid intake in the diet and the S-Klotho plasma levels among aged adults. The obtained results could provide valuable information regarding the potential impact of carotenoid-rich diets on aging and aging-related diseases. Furthermore, understanding the associations between dietary carotenoid intakes and S-Klotho levels in aged adults could have important implications for the development of dietary interventions yielding to promote healthy aging and prevent aging-related diseases.

Abbreviations: S-Klotho, Soluble Klotho; BMI, Body Mass Index; NHANES, National Health and Nutrition Examination Surveys; ELISA, Enzyme-Linked Immuno-Sorbent Assay; CI, Confidence Intervals.

Methodology

Study population

The National Health and Nutrition Examination Survey (NHANES) is a comprehensive study that evaluates the health and nutritional conditions of the non-institutionalized population in the USA. The survey uses a multi-stage and stratified probability design to obtain a representative sample of the US population. The protocols and the investigation plan used in the NHANES have been approved by the National Center for Health Statistics Ethics Review Board (https://www.cdc.gov/nchs/nhanes/irba98.htm) where all participants have to provide their informed consent in the survey. The data used in this study was obtained from five independent cross-sectional waves of NHANES (2007-2008, 2009-2010, 2011-2012, 2013-2014, and 2015-2016) and it was freely available. Participants over the age of 60 years were included in the study, and those who declined consent to participate or who had missing data on carotenoid intake, serum S-Klotho level, demographic information, or health status were excluded.

Measurement of the dietary total carotenoid and subgenus intake

In the NHANES study, the dietary information of the participants was obtained through two 24-hour recall interviews. The protocol and data collection methods for the interviews are outlined in the NHANES dietary interviewers' procedure manual, which can be found at this link: https://www.cdc.gov/nchs/nhanes/measuring_guides_dri/measuringguides.htm.The initial dietary recall interview was conducted face-to-face at the Mobile Examination Center. During this interview, participants were asked to provide a detailed description of all the food and beverages consumed within the preceding 24 hours, including the type and quantity of each item. Following the first interview, a second interview was conducted via telephone after a gap of three to ten days. During this second interview, participants were asked to provide a comprehensive breakdown of the specific types and amounts of each food they had consumed over the past 24 hours. The accuracy and reliability of the technique applied in this study have been ascertained by other investigations that also used NHANES data (22–24). Intakes of carotenoids and other nutrient data were derived from the US Department of Agriculture Food and Nutrient Database for Dietary Studies of NHANES (25). The total carotenoids considered in the study included α-carotene, lycopene, β-cryptoxanthin, β-carotene, and lutein with zeaxanthin. To calculate the intake of these compounds, the results from the two 24-hour recall periods were averaged. For each NHANES cycle, dietary intakes of lutein, zeaxanthin, and lycopene from supplements were documented during two 24-hour recall periods (26). In this study, the average intake of lycopene, lutein, and zeaxanthin supplements was taken over two days, and the total intake of these compounds was determined by summing both dietary and supplemental intakes. Finally, the total dietary carotenoid intake was determined by summing up the intake of

α-carotene, lycopene, lutein, and zeaxanthin, β-carotene, and β-cryptoxanthin from food sources, while the total carotenoid intake was ascertained by summing up the carotenoid intake from both dietary sources and supplements. The selection of two 24-hour recall interviews was based on the need to obtain more precise and representative dietary information from participants. Having interviews on different days enables to take into account the variations in dietary intake within an individual and offers a more comprehensive evaluation of the typical dietary habits of the participants.

Determination of S-Klotho concentrations

Specimens were collected from the antecubital vein of the participants who were in a horizontal position. Before the sample was taken, participants were instructed to fast for 12 hours, avoid drugs and caffeine, to have a specific dinner, and to refrain from moderate physical activity within 24 hours and vigorous activity within 48 hours. The S-Klotho levels were ascertained by employing a solid-phase sandwich Enzyme-Linked Immuno-Sorbent Assay (ELISA) kit from Demeditec (Kiel, Germany), as per the manufacturer's instructions (https://wwwn.cdc.gov/Nchs/Nhanes/2015-2016/SSKL_I.htm). To evaluate the accuracy of the test, the intra- and inter-assay coefficients of variation were calculated to get two doses of purified S-Klotho.

Assessment of covariates

The following important covariates were included for adjustment in this study: the year of the NHANES cycle, age, gender (male and female), ethnicity (non-Hispanic white, Mexican American, non-Hispanic black, and other ethnicities), marital status (unmarried and married), educational level (grade or less, high school, some college, and college or more), family income-to-poverty ratio, Body Mass Index (BMI), smoking status (never, former, and now smoking), drinking status (never, former, mild, moderate, and heavy drinking), energy intake, estimated Glomerular Filtration Rate (eGFR), physical activity, use of medication, systemic immune-inflammation index (SII), serum 25-hydroxyvitamin D, and self-reported chronic diseases including hypertension, diabetes, stroke, heart attack, congestive heart failure, coronary heart disease, and cancer. Unmarried individuals regroup those who were never married, divorced, widowed, living with a partner, and separated. As for the physical activity, it was categorized as either with or without moderate physical activity. Finally, the BMI variable was divided into four categories in the stratified analysis: underweight (<18.5 kg/m2), normal weight (18.5 to <25 kg/m2), overweight (25 to <30 kg/m2), and Obesity (≥30 kg/m2).The systemic immune-inflammation index (SII) is a new indicator of systemic inflammation, calculated by multiplying the platelet count and neutrophil count and dividing the result by the lymphocyte count (27). This index offered a more comprehensive and balanced representation of a person's immunological and inflammatory responses (28).

Statistical analysis

Using sample weights, strata, and primary sampling units, the analysis was conducted to ensure the accurate national estimates. The sample characteristics were displayed as mean values with 95% Confidence Intervals (CIs) for continuous variables and as percentages for categorical variables. Carotenoids were analyzed both as a continuous variable and as a categorical variable split into four quartiles, with the lowest quartile considered as the basis for comparison. Quartiles of carotenoid intakes (including the total carotenoid and its subgroups) were established according to the distribution among the study population. We have taken into consideration whether adjustments were necessary for multiple testing, if applicable. Multivariable linear regression models were employed to evaluate the association between carotenoids and the plasma level of S-Klotho. Moreover, three statistical models were constructed: a crude model with no adjustments (Model I), an adjusted model that takes into account the year and the age of the participant (Model II), and a further adjusted model that incorporated the year, age, gender, ethnicity, marital status, education level, family income-to-poverty ratio, smoking status, drinking status, eGFR, BMI, total energy intake, physical activity, medication use, SII, serum 25-hydroxyvitamin D, and self-reported chronic diseases (Model III). In addition to total carotenoids, the effects of the carotenoid subgroups on the S-Klotho level were also assessed. To examine if the association between the carotenoid subgenera and S-Klotho level was modified by some variables, such as sex, BMI, and physical activity, stratified analyses sorted by sex (male or female), BMI (normal weight, overweight, obesity), and physical activity (with or without moderate physical activity) were also conducted. Finally, to evaluate the robustness of our findings, we executed a sensitivity analysis that incorporated several macronutrients (carbohydrates, proteins, fat, and sugars) into our multivariable linear regression models. All analyses were performed using R (version 4.20) software. A two-tailed P value lower than 0.05 was considered to be a statistically significant result.

Results

Baseline characteristics of included participants

A total of 5,056 elderly individuals were recruited for this study, and the selection of the study cohort has been demonstrated in Figure 1. Table 1 shows the detailed characteristics of the included elderly population based on quartiles of dietary carotenoid intake. The average age of participants was 67.5 years, and their average annual family income was more than three times the poverty level (mean income/poverty ratio = 3.20). The daily energy intake was recorded to be an average of 1899.36 kcal, with a mean eGFR of 75.94 mL/min/1.73m^2, BMI of 29.62 kg/m^2, total carotenoid intake of 9775.25 μg per day, and S-Klotho concentration of 815.59 pg/ml. Individuals who consume a greater amount of carotenoids in their diet were more likely to exhibit certain demographic and lifestyle

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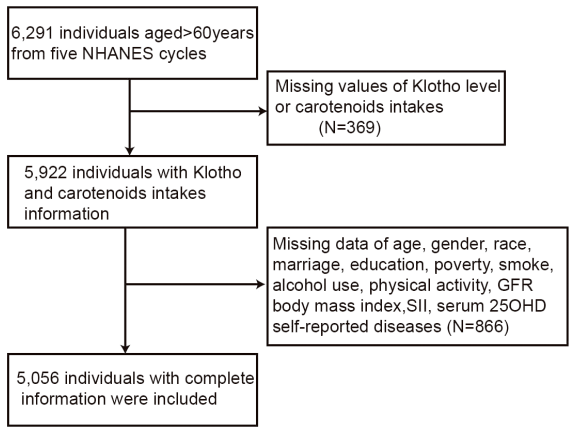

FIGURE 1
Flowchart of included participant selection.

characteristics. Specifically, individuals with high intake of carotenoids tended to be younger, female, non-Hispanic White, and possess a higher socioeconomic status (including higher educational levels, marriage, and income). Additionally, those with high intake of carotenoids had higher serum 25-hydroxyvitamin D levels, lower SII, and were less likely to smoke, while engaging in moderate drinking, physical activity, and medication use, and consuming more energy overall. Furthermore, participants with a large consumption of carotenoids displayed a higher serum Klotho level than those who consumed a smaller amount, although the difference was not statistically significant. Table S1 provided a summary of the characteristics exhibited by the elderly participants who were included in the study, based on the quartiles of S-Klotho concentration. In terms of carotenoid subgroups, the average daily consumption of α-carotene, β-carotene, β-cryptoxanthin, lycopene, and lutein with zeaxanthin was 477.3, 2,548.41, 88.4, 4,943.17, and 1,717.97 micrograms, respectively.

TABLE 1 Characteristics of included population based on quartile of dietary carotenoid intake in the NHANES (N =5,056).

		Quartile of dietary carotenoid intake (μg/day)				
Characteristic	Overall, N = 5056 (100%)	Q1 N = 1265 (25%)	Q2 N = 1263 (25 %)	Q3 N = 1264 (25%)	Q4 N = 1264 (25%)	P Value
Age (years)	67.50(67.27,67.73)	67.93(67.45,68.41)	67.25(66.89,67.62)	67.69(67.30,68.08)	67.20(66.79,67.60)	0.04
Gender %						<0.01
Female	52.83	54.36	56.01	53.73	47.79	
Male	47.17	45.64	43.99	46.27	52.21	
Ethnicity %						<0.01
Non-Hispanic White	80.35	75.53	80.31	82.32	82.29	
Mexican American	4.11	4.48	3.72	4.68	3.62	
Non-Hispanic Black	7.53	11.28	7.3	5.87	6.38	
Other ethnicities	8.02	8.71	8.67	7.12	7.71	
Education %						< 0.01
Grade or less	16.57	22.86	16.83	15.36	12.53	
High school	22.94	28.42	22.41	21.62	20.39	
Some college	31	31.26	33.01	32.92	27.06	
College or more	29.5	17.46	27.75	30.1	40.02	
Married status %	66.73	60.37	65.27	68.95	70.98	<0.01
Poverty income ratio	3.20(3.09,3.30)	2.67(2.52,2.82)	3.19(3.06,3.32)	3.34(3.18,3.49)	3.49(3.34,3.64)	< 0.01
Smoking status %						< 0.01
Never	12.66	14.49	14.28	11.7	10.63	
Former	40.61	36.36	39.68	43.05	42.49	
Now	12.34	20.02	11.5	10.69	8.65	
Drinking status %						< 0.01

(Continued)

TABLE 1 Continued

Characteristic	Overall, N = 5056 (100%)	Quartile of dietary carotenoid intake (μg/day)				P Value
		Q1 N = 1265 (25%)	Q2 N = 1263 (25 %)	Q3 N = 1264 (25%)	Q4 N = 1264 (25%)	
Never	12.66	14.49	14.28	11.7	10.63	
Former	22.25	28	24.38	19.83	18.03	
Mild	45.54	36	45.02	47.42	51.73	
Moderate	12.71	12.96	11.29	13.68	12.92	
Heavy	6.84	8.55	5.03	7.37	6.69	
Body Mass Index (kg/m2)	29.62(29.31,29.93)	29.85(29.35,30.36)	29.84(29.22,30.46)	29.80(29.33,30.26)	29.07(28.57,29.56)	0.08
Energy intake (kcal/day)	1899.36 (1867.60,1931.12)	1670.82 (1612.52,1729.12)	1836.50 (1769.30,1903.71)	1907.94 (1859.43,1956.45)	2129.89 (2069.76,2190.02)	< 0.01
Physical activity (Yes) %	70.66	63.59	72.36	72.77	72.64	<0.01
Glomerular filtration rate (ml/min/ 1.73m^2)	75.94 (75.12,76.77)	74.81 (73.36,76.25)	75.96 (74.71,77.21)	75.86 (74.30,77.42)	76.90 (75.69,78.11)	0.16
Serum 25-hydroxyvitamin D (nmol/ L)	78.31 (76.58,80.05)	74.02 (71.47,76.58)	77.76 (75.04,80.48)	81.04 (78.88,83.21)	79.60 (76.87,82.34)	< 0.01
Systemic immune-inflammation index	553.67 (539.27,568.08)	581.23 (546.18,616.29)	566.62 (535.64,597.61)	551.73 (527.65,575.80)	521.71 (501.85,541.57)	0.01
Use of medication %	85.67	85.33	85.54	87.59	84.23	0.41
S-Klotho level (pg/ml)	815.59 (802.59,828.60)	790.82 (773.13,808.51)	819.91 (797.43,842.39)	814.93 (794.51,835.34)	831.67 (806.78,856.56)	0.06
Total Carotenoid (μg/day)	9775.25 (8971.30,10579.21)	823.71 (790.56, 856.86)	3227.55 (3162.43, 3292.68)	7711.46 (7576.70, 7846.22)	24929.65 (22767.87,27091.44)	< 0.01
α-Carotene (μg/day)	477.30 (389.28,565.32)	33.87 (28.19, 39.55)	159.88 (141.04, 178.73)	526.14 (481.39, 570.89)	1077.25 (783.97,1370.54)	< 0.01
β-Carotene (μg/day)	2548.41 (2255.66,2841.16)	225.47 (210.47, 240.46)	847.46 (789.32, 905.60)	2329.24 (2207.83,2450.64)	6180.60 (5251.01,7110.19)	< 0.01
β-Cryptoxanthin (μg/day)	88.40 (80.42,96.39)	36.67 (31.14, 42.21)	76.61 (66.35, 86.86)	98.34 (81.90,114.79)	130.67 (108.21,153.12)	< 0.01
Lycopene (μg/day)	4943.17 (4524.48,5361.86)	136.74 (112.71, 160.78)	1293.31 (1198.89, 1387.74)	3378.04 (3194.07, 3562.01)	13642.37 (12444.97,14839.76)	< 0.01
Lutein with zeaxanthin (μg/day)	1717.97 (1536.96,1898.97)	390.96 (362.57, 419.34)	850.29 (787.99, 912.59)	1379.69 (1281.66,1477.73)	3898.76 (3350.39,4447.14)	< 0.01
Self-reported chronic diseases						
CHD %	8.83	9.47	7.81	8.99	9.13	0.73
Cancer %	23.64	24.13	22.95	24.46	23.11	0.89
CHF %	5.67	6.26	6.17	6.19	4.25	0.20
Stroke %	5.87	6.69	7.06	4.1	5.79	0.04
Heart attack %	7.34	8.26	7.1	7.51	6.68	0.72
Hypertension %	56.73	59.5	56.12	56.84	55.03	0.51
Diabetes mellitus %	19.43	18.91	20.29	19.85	18.64	0.34

Continuous variables are described as means ± 95%CI, and categorical variables are presented as percentages. All estimates accounted for complex survey designs. CHF, Congestive heart failure; CHD, Coronary heart disease. Quartile of dietary carotenoid intake (μg/day) with Q1 [0,1745], Q2 (1745,4963.5], Q3 (4963.5,11662.5], Q4 (11662.5,377178].

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Association between total and carotenoid subgroups intake and S-Klotho concentration

The total carotenoid intake was positively and significantly associated with S-Klotho concentration among the elderly population in the fully adjusted model, both as a continuous and categorical variable (Table 2). The fully adjusted model was adjusted with consideration of various factors, such as year, age, gender, ethnicity, marital status, education level, family income-to-poverty ratio, smoking, drinking status, eGFR, BMI, total energy intake, physical activity, medication use, SII, serum 25-hydroxyvitamin D, and self-reported chronic diseases. It was observed that individuals with the highest intake of total carotenoids had significantly increased S-Klotho levels compared to those with the lowest intake in the fully adjusted model (Table 2). Among the carotenoid subgroups, only intakes of α-carotene, β-carotene, and lutein with zeaxanthin were significantly correlated with increased S-Klotho concentration, while the relationship was not significant for β-cryptoxanthin and lycopene (Table 3).

Subgroup and sensitivity analysis

In stratified analyses, significant associations between α-carotene, β-carotene, lutein with zeaxanthin, and S-Klotho concentration were consistent among aged males, normal-weight individuals, and those with moderate physical activity (Table 4). For obese individuals, only β-cryptoxanthin intake was positively and significantly associated with S-Klotho concentration, while there was no significant relationship for α-carotene, lycopene, β-carotene, and lutein with zeaxanthin (Table 4). To further validate the robustness of our findings, we conducted sensitivity analyses that incorporated several macronutrients (carbohydrates, proteins, and sugars) into our multivariable linear regression models. The relationship between total carotenoids and S-Klotho concentrations did not change significantly. (Table S2).

Discussion

This cross-sectional analysis investigated the correlation between the dietary total carotenoid intake (including five carotenoid subgroups) and the S-Klotho plasma level in aged adults. Despite the emerging evidence of a relationship between dietary pattern and Soluble Klotho (S-Klotho) levels (23, 24, 29–33), further evaluation is needed to assess the association between dietary carotenoid intake and S-Klotho levels among the elderly population. Therefore, we demonstrated that the total carotenoid intake was associated with the increased S-Klotho level, even after adjusting for potential confounding factors. The fact that the β-coefficient was shown to be significant whether total carotenoid intake was examined as a continuous or quartile variable suggests that this finding may have important clinical implications. The positive relationship between α-carotene, β-carotene, and lutein with zeaxanthin intake and the S-Klotho level was still evident. In subsequent analyses stratified by gender, BMI, and physical activity, the positive associations of α-carotene, β-carotene, and lutein with zeaxanthin were statistically significant among male aged participants as well as those with normal weight, and with moderate physical activity.

As life expectancy increases, the challenges of an ever-growing segment of the population facing aging and age-related illnesses continue to emerge. Elderly individuals are more susceptible to oxidative stress due to weakened endogenous antioxidant systems. As antioxidants, carotenoids are thought to help protecting the body from oxidative damage caused by free radicals and reactive oxygen species, which can be accumulated over time and contribute to age-related diseases. Numerous studies have found a correlation between the carotenoid intake and the reduced oxidative stress (20). As a result, the role of carotenoids in promoting healthy aging through countering oxidative stress is a topic that deserves further attention (21). In fact, carotenoids are a group of pigments that are produced exclusively by plants, algae, and certain bacteria. There are over 600 types of carotenoids that have been identified, but only around 50 of them are commonly found in the human diet. Among

TABLE 2 Multivariate linear analysis of the association between total carotenoid and serum Klotho level.

Total Carotenoid	Model I β (95%CI)	P	Model II β (95%CI)	P	Model III β (95%CI)	P
Continuous	10.87 (1.53,20.21)	0.02	9.60 (0.90,18.30)	0.03	8.40 (0.48, 16.31)	0.04
Quartiles						
Quartile 1	Reference		Reference		Reference	
Quartile 2	29.09 (1.63,56.55)	0.04	27.5 (0.28,54.71)	0.05	23.82 (-4.08, 51.72)	0.09
Quartile 3	24.1 (-0.34,48.55)	0.05	23.55 (-0.75,47.85)	0.06	25.07 (0.70, 49.44)	0.04
Quartile 4	40.85 (8.57,73.12)	0.01	39.12 (6.90,71.35)	0.02	34.64 (0.34, 69.62)	0.04

Model I: non-adjusted model; Model II: adjusted for year and age; Model III: adjusted for year, age, gender, ethnicity, marital status, education level, family income-to-poverty ratio, smoking status, drinking status, estimated glomerular filtration rate, body mass index, total energy intake, physical activity, medication use, systemic immune-inflammation index, serum 25-hydroxyvitamin D, and self-reported chronic diseases.

TABLE 3 Multivariate linear analysis of associations between carotenoid subgroups and serum Klotho level.

Carotenoid subgroups (Continuous)	Model I β(95%CI)	P	Model II β(95%CI)	P	Model III β(95%CI)	P
α-arotene	13.77 (7.18,20.35)	<0.01	12.57 (5.67,19.47)	<0.01	10.80 (4.49, 17.11)	0.01
β-Carotene	15.58 (9.95,21.21)	<0.01	14.34 (8.59,20.08)	<0.01	11.58 (6.44, 16.71)	<0.01
β-Cryptoxanthin	8.50 (-0.92,17.93)	0.08	9.66 (-0.40,19.73)	0.06	7.06 (-2.16, 16.29)	0.13
Lycopene	-4.56 (-13.18,4.05)	0.29	-4.93 (-13.17, 3.31)	0.24	-2.92 (-10.69, 4.86)	0.45
Lutein with zeaxanthin	15.44 (7.70,23.19)	<0.01	14.1 (6.63,21.58)	<0.01	10.33 (2.02, 18.64)	0.02

Model I: non-adjusted model; Model II: adjusted for year and age; Model III: adjusted for year, age, gender, ethnicity, marital status, education level, family income-to-poverty ratio, smoking status, drinking status, estimated glomerular filtration rate, body mass index, total energy intake, physical activity, medication use, systemic immune-inflammation index, serum 25-hydroxyvitamin D, and self-reported chronic diseases.

these, β-carotene, α-carotene, β-cryptoxanthin, and lycopene are the most extensively researched carotenoids due to their high prevalence in the diet and potential health benefits. In more detail, α-Carotene is a yellow-orange pigment that is present in similar food sources as β-carotene, including spinach, broccoli, pumpkin, mango, apricots, and citrus fruits such as oranges and tangerines. As for the β-Carotene, it is a red-orange pigment found in fruits and vegetables, particularly those with deep green, yellow, or orange hues. It is most abundantly found in sweet potatoes, carrots, pumpkin, spinach, kale, collard greens, and cabbage. Moreover, the β-Cryptoxanthin is an orange-red carotenoid that is found in numerous fruits and vegetables, particularly in deep green leafy vegetables and citrus fruits. Some of the richest sources of this caronete include kale, parsley, spinach, persimmons, winter squash, papaya, and tangerines. Finally, lycopene is a red pigment primarily found in tomatoes, watermelon, pink grapefruit, guava, and apricots, with tomatoes and tomato products being the richest dietary sources of this carotenoid. A meta-analysis, based on Liu et al., demonstrated that zeaxanthin and lutein therapy could reduce the risk of cataracts (34). Evidence suggests that individuals, who frequently eat carotenoid-rich foods, are less likely to suffer from age-related macular degeneration than those who rarely or never consume carotenoids (35). Numerous studies have showed that intakes of carotenoid subgroups, such as zeaxanthin and lutein, can improve visual performance, for example, photo stress recovery, contrast sensitivity, and glare tolerance (36, 37). Furthermore, Carotenoids may be able to reduce oxidative stress, conferring advantages of the ocular health and performance. Besides, carotenoid concentration is associated with better cognitive performance in both healthy and impaired individuals (38, 39). It was further demonstrated that a one-year supplementation of lutein, zeaxanthin, and meso-zeaxanthin had a beneficial effect on memory (40). Finally, epidemiological research has demonstrated that consuming a diet high in carotenoids could help reduce the likelihood of developing osteoporosis and heighten the bone mineral density (41, 42).

Long-term inflammation has a considerable impact on the decrease of Klotho in the serum (43). It has been observed that in middle-aged and older individuals, the plasma level of S-Klotho is inversely proportional to a pro-inflammatory dietary pattern as assessed by the Dietary Inflammatory Index (DII) (44). As for lower concentrations of S-Klotho, they have been associated with a variety of age-related medical conditions, including cancer, high blood pressure, and kidney disease (5, 45, 46). The aging process is often accompanied by a reduction in plasma S-Klotho levels, as evidenced by multiple studies (47–49). It is possible to reduce the inflammation by consuming a diet rich in anti-inflammatory or antioxidant components, such as fiber, nuts, fruits, and vegetables. We speculated that carotenoids might increase S-Klotho plasma levels by reducing oxidative stress and inflammation, particularly for α-carotene, β-carotene, lutein, and zeaxanthin. Subgroup analysis indicated that the beneficial effects of α-carotene, β-carotene, and lutein with zeaxanthin were more significant in male individuals who were older, had a normal weight, and exercised moderate physical activity. It had been suggested that the observed associations stratified by these factors might be attributed to a variety of potential causes. For example, research had indicated that there were sex-based differences in Klotho expression and function (50, 51), which could explain the greater association observed in males. Additionally, individuals with normal body weight tended to have better metabolic health and lower inflammation (52), which could play a role in the relationship. Furthermore, moderate physical activity had been linked to a range of health benefits, including improved insulin sensitivity, reduced inflammation, and enhanced antioxidant defense systems and immune function (53), which could also be contributing to the observed association. These findings required further prospective studies to validate and explain this relationship and their respective underlying mechanisms.

Using the large and nationally representative NHANES database, our findings reveal, for the first time, the presence of a positive correlation of the total carotenoid consumption – especially that of α-carotene, β-carotene, and lutein with zeaxanthin – with the higher level of S-Klotho in aging adults. The large sample size and various sensitivity analyses bolster the reliability of our results, which is our advantage. However, it is important to note that, due to

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TABLE 4 Stratified analysis of associations between carotenoid subgroups and serum Klotho level.

Carotenoid subgroups (continuous)	Model III β(95%CI)	P-value
Stratified by Gender		
Female		
α-Carotene	8.55(-15.54, 32.63)	0.48
β-Carotene	13.22(-3.83, 30.27)	0.13
β-Cryptoxanthin	4.58(-2.99, 12.16)	0.23
Lycopene	4.55(-8.25, 17.34)	0.48
Lutein with zeaxanthin	1.91(-12.85, 16.67)	0.80
Male		
α-Carotene	10.99(7.71, 14.27)	<0.01
β-Carotene	11.09(7.02, 15.16)	<0.01
β-Cryptoxanthin	22.25(-11.25, 55.76)	0.19
Lycopene	-9.09(-19.12, 0.94)	0.07
Lutein with zeaxanthin	14.42(7.14, 21.69)	<0.01
Stratified by body mass index		
Normal weight		
α-Carotene	8.81(3.90, 13.71)	<0.01
β-Carotene	11.5(8.12, 14.88)	<0.01
β-Cryptoxanthin	-3.59(-28.91, 21.73)	0.78
Lycopene	-9.8(-29.36, 9.76)	0.32
Lutein with zeaxanthin	13.34(6.36, 20.32)	<0.01
Overweight		
α-Carotene	-1.95(-24.05, 20.14)	0.86
β-Carotene	-6.73(-26.98, 13.52)	0.51
β-Cryptoxanthin	3.81(-2.30, 9.92)	0.22
Lycopene	7(-7.50, 21.50)	0.34
Lutein with zeaxanthin	-12.52(-33.08, 8.05)	0.23
Obesity		
α-Carotene	40.6(-11.84, 93.05)	0.13
β-Carotene	32.64(-1.35, 66.63)	0.06
β-Cryptoxanthin	33.31(6.25, 60.38)	0.02
Lycopene	-9.65(-24.89, 5.58)	0.21
Lutein with zeaxanthin	24.53(-2.84, 51.91)	0.08
Stratified by physical activity		
With moderate physical activity		
α-Carotene	9.64(3.54, 15.74)	<0.01
β-Carotene	10.71(5.20, 16.21)	<0.01
β-Cryptoxanthin	5.52(-1.89, 12.92)	0.14
Lycopene	-2.61(-12.83, 7.62)	0.61
Lutein with zeaxanthin	13.11(5.27, 20.95)	<0.01
Without moderate physical activity		
α-Carotene	28.95(-15.81, 73.70)	0.20
β-Carotene	19.68(-3.67, 43.02)	0.10
β-Cryptoxanthin	21.21(-21.48, 63.90)	0.32
Lycopene	-5.87(-23.10, 11.37)	0.50
Lutein with zeaxanthin	-1.46(-24.53, 21.61)	0.90

Model III adjusted for year, age, gender, ethnicity, marital status, education level, family income-to-poverty ratio, smoking status, drinking status, estimated glomerular filtration rate, body mass index, total energy intake, physical activity, medication use, systemic immune-inflammation index, serum 25-hydroxyvitamin D, and self-reported chronic diseases. The strata variable was not considered when performing the stratification analysis.

the cross-sectional design of our study using the NHANES database, it is not possible to establish a causal relationship between the carotenoid intake and S-Klotho levels. Further prospective studies will be necessary to investigate this relationship and its underlying mechanisms. Additionally, we adopted two 24-hour dietary recalls to collect dietary data due to the lack of a validated Food Frequency Questionnaire (FFQ) in NHANES 2007-2016. The use of 24-hour dietary recalls for determining carotenoid intake necessarily incurred the risk of recall bias. Since the duration of supplementation with carotenoids was not available in the NHANES database, it may potentially impact the association between the carotenoid intake and the S-Klotho plasma levels. In addition, further studies should encompass more information regarding medications or other supplements that may alter the levels of carotenoids. An additional limitation was the absence of assessment of potential interactions between different variables in the stratified analyses. Despite taking into account certain potential confounding factors, there may be other unknown elements, such as antioxidants and oxidative stress indicators, that could affect our results. Further studies are required to assess the levels of antioxidants, oxidative stress markers, and inflammation response in order to uncover the underlying mechanism. Finally, careful consideration must be taken when attempting to generalize the findings of this study, which was conducted on a Western population, to other populations.

In conclusion, our findings showed a positive link between higher consumption of total carotenoids (especially α-carotene, β-carotene, and lutein with zeaxanthin) and increased levels of S-Klotho for older populations. These findings might provide a unique view on the part of dietary phytochemicals in age-related diseases and offer a comprehensive analysis into a potential intervention that could affect the aging process and thus encourage healthy longevity. Further research is needed to validate these findings and uncover the underlying mechanisms.

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 studies involving humans were approved by National Center for Health Statistics Ethics Review Board. 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. The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

XH: Conceptualization, Investigation, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing. XY: Data curation, Formal Analysis, Methodology, Project administration, Validation, Writing – original draft. XC: Data curation, Formal Analysis, Methodology, Project administration, Validation, Visualization, Writing – original draft. XLC: Investigation, Supervision, Writing – review & 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1283722/full#supplementary-material

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Complex relationship between gut microbiota and thyroid dysfunction: a bidirectional two-sample Mendelian randomization study

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Background: Many studies have reported the link between gut microbiota and thyroid dysfunction. However, the causal effect of gut microbiota on thyroid dysfunction and the changes in gut microbiota after the onset of thyroid dysfunction are not clear.

Methods: A two-sample Mendelian randomization (MR) study was used to explore the complex relationship between gut microbiota and thyroid dysfunction. Data on 211 bacterial taxa were obtained from the MiBioGen consortium, and data on thyroid dysfunction, including hypothyroidism, thyroid-stimulating hormone alteration, thyroxine deficiency, and thyroid peroxidase antibodies positivity, were derived from several databases. Inverse variance weighting (IVW), weighted median, MR-Egger, weighted mode, and simple mode were applied to assess the causal effects of gut microbiota on thyroid dysfunction. Comprehensive sensitivity analyses were followed to validate the robustness of the results. Finally, a reverse MR study was conducted to explore the alteration of gut microbiota after hypothyroidism onset.

Results: Our bidirectional two-sample MR study revealed that the genera *Intestinimonas*, *Eubacterium brachy group*, *Ruminiclostridium5*, and *Ruminococcaceae UCG004* were the risk factors for decreased thyroid function, whereas the genera *Bifidobacterium* and *Lachnospiraceae UCG008* and phyla Actinobacteria and Verrucomicrobia were protective. The abundance of eight bacterial taxa varied after the onset of hypothyroidism. Sensitivity analysis showed that no heterogeneity or pleiotropy existed in the results of this study.

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Conclusion: This novel MR study systematically demonstrated the complex relationship between gut microbiota and thyroid dysfunction, which supports the selection of more targeted probiotics to maintain thyroid–gut axis homeostasis and thus to prevent, control, and reverse the development of thyroid dysfunction.

KEYWORDS

Mendelian randomization, thyroid dysfunction, hypothyroidism, gut microbiota, thyroid-gut axis

Introduction

Thyroid dysfunction stands as one of the prevailing endocrine diseases, and hypothyroidism is one of its main types, affecting approximately 5% of the general population (1). Thyroid hormones exert their influence selectively on multiple organs and tissues. Hypothyroidism, a representation of thyroid dysfunction, exhibits strong associations with cardiovascular diseases (2), diabetes (3), and thromboembolism (4), among other conditions. Recent epidemiological studies globally have unveiled a significant correlation between hypothyroidism and elevated mortality rates (5). Hypothyroidism can arise due to factors such as iodine deficiency, medications, radiation therapy, immune system abnormalities, and pregnancy. Once established, reversing hypothyroidism poses significant challenges. Among these triggers, autoimmune hypothyroidism proves especially problematic, given its enduring nature. Hence, exploring autoimmune hypothyroidism demands comprehensive analysis from diverse angles. It necessitates in-depth research to uncover treatments beyond lifelong thyroxine administration. These treatments should not only impede the disease's progression but also enhance patients' quality of life significantly.

The human gut microbiota boasts unparalleled diversity and complexity among all human organs, with its composition intricately linked to ethnicity, dietary habits, and geographic location (6). Variations in gut microbiota among healthy individuals from diverse backgrounds pose a challenge in establishing a definitive healthy baseline. This microbiota actively participates in the fundamental physiological functions and diseases within the human body, including nutrient production, metabolic balance, immune response, brain behavior, and inflammatory reactions (7). Moreover, it plays a pivotal role in several endocrine diseases, notably diabetes (8) and polycystic ovary syndrome (9). The evolving recognition of the interconnection between gut microbiota and thyroid function is denoted as the thyroid–gut axis (10, 11). Dysbiosis in the gut microbiota has been evidenced to disrupt the absorption of iodine and to impact the synthesis and release of thyroid hormones (12). Researchers have explored this relationship, achieving compelling results by modulating gut microbiota in mouse models and clinical cases of thyroid dysfunction (13–16). For instance, hyperthyroidism correlates with increased Actinobacteria and decreased Bacteroidetes, whereas specific strains of *Bifidobacterium* and *Lactobacillus* were found to interact with human autoantibodies, disrupting thyroid function (14, 17). Clinical study has also linked severe hypothyroidism to higher instances of small intestinal bacterial overgrowth, contributing to gastrointestinal symptoms (18).

Although these studies have indicated associations between gut microbiota and thyroid dysfunction, causality remains elusive in traditional observational studies due to confounding and reverse causation, which can lead to biased conclusions. A bidirectional two-sample Mendelian randomization (MR) study presents an innovative method for studying the causal effects between environmental exposures and diseases, or between two diseases, akin to a randomized controlled trial (RCT) (19). MR employs single-nucleotide polymorphisms (SNPs) in genes as instrumental variables (IVs), effectively circumventing confounding factors, and provides statistically significant causal effects by combining SNPs from genome-wide association studies (GWASs) of exposure and outcome (20, 21). It allows for better modeling of random assignment, thereby reducing bias in observational studies and better inferring causality. Although MR has been applied broadly to investigate the gut microbiota's role in diseases like cancers and autoimmune disorders, its application to thyroid dysfunction remains unexplored (22–24). Therefore, we conducted this bidirectional two-sample MR study utilizing thyroid function–related GWAS data from the international consortia, including the MiBioGen consortium, FinnGen consortium, MRC IEU OpenGWAS project, and ThyroidOmics. In this study, the application of two-sample MR involved the utilization of two distinct, independent European samples for analysis. This approach was employed to mitigate the potential biases associated with a single sample, thereby enhancing the reliability and generalizability of our findings. Furthermore, we used the bidirectional MR that simultaneously considered two different causal directions between the gut microbiota and autoimmune hypothyroidism, thus providing for a more nuanced understanding of the bidirectional causal relationship between them. This comprehensive MR analysis, to our knowledge, marks the first exploration of the association between gut microbiota and thyroid function. These results can determine the bidirectional causal effect between the gut microbiota and thyroid dysfunction and thus guide the regulation of the thyroid–gut axis.

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Methods

Study design

The study's flowchart is depicted in Figure 1. To investigate the interplay between gut microbiota and thyroid dysfunction, we selected gut microbiota as the exposure and thyroid function–related factors as outcomes. MR study adhered to three key assumptions: (1) IVs selected from datasets were linked to exposure; (2) they were unrelated to unidentified exposure confounders; and (3) they influenced outcomes exclusively through exposure pathways (25). SNPs served as valid IVs in the MR study to evaluate the bidirectional causal relationship between exposure and outcome.

Data sources

All data utilized in this study were sourced from diverse GWASs. Regarding thyroid function, the GWASs focused on hypothyroidism, thyroid-stimulating hormone (TSH), thyroxine deficiency, and thyroid peroxidase antibodies (TPOAb) positivity except for gut microbiota data. For the gut microbiota analysis, we extracted the relevant genetic IVs from a comprehensive GWAS dataset provided by the MiBioGen consortium. This dataset was composed of 18,340 participants, primarily of European descent (n = 13,266), and comprised a total of 5,717,754 SNPs after imputation (21). The MiBioGen GWAS amalgamated outcomes from 16S ribosomal RNA gene sequencing effectively eliminated potential batch effects. In total, the dataset contained information on 211 taxa, including 131 genera, 35 families, 20 orders, 16 classes, and nine phyla. As for the thyroid function investigation, the genetic IVs were acquired from the FinnGen consortium (26), MRC IEU OpenGWAS project (27), and ThyroidOmics consortium (28), respectively. The details of each database are summarized in Table 1.

Instrumental variables selection

To infer an accurate and realistic bidirectional causal effect between gut microbiota and hypothyroidism risk, we performed a rigorous quality control to select the best IVs. First, we screened SNPs associated with each bacterial taxon using a mild p-value of 1×10^{-5}. Then, linkage disequilibrium analysis was used to select independent IVs for each bacterial taxon to prevent biased causal estimates. We selected the most suitable parameters for clustering ($R^2 < 0.01$ and clustering distance = 500 kb) to evaluate the linkage disequilibrium among the included SNPs. The population was framed as European. Missing SNPs from bacterial taxa in thyroid dysfunction–related datasets were replaced with proxy SNPs ($R^2 > 0.8$). Palindromic SNPs were excluded to prevent coding distortions. The F-statistic was calculated to detect the presence of weak IV instrument bias. When the F-statistic is greater than 10, it indicates that there is no weak instrument bias.

MR analysis

Five widely used MR methods were employed to detect the bidirectional causal relationships between exposure and outcome, encompassing inverse variance weighting (IVW), weighted median, MR-Egger, weighted mode, and simple mode (29–32). IVW method estimates the causal effect of exposure on the outcome by integrating ratio estimates for each SNP, and it was chosen as the primary method because it can provide a robust and unbiased causal effect when no polymorphism or heterogeneity is found (33). The weighted median method correctly estimates causality when up to 50% of the IVs are invalid (30). MR-Egger method is based on the assumption that instrument strengths are independent of direct effects and thereby allows calibration of pleiotropy and calculation of causal inferences, even if all genetic variants are polymorphic (34). If this assumption is violated, then the weighted model method would have greater power to detect causal effects and produce less bias than the MR-Egger method (34). Finally, the simple model method is an unweighted model of the experienced density function for causal estimation (35). A positive causal effect was affirmed if the IVW results were significant ($p < 0.05$) and beta values from other methods concurred in direction. Wald radio method was used to estimate the causal effect of exposure on the outcome when there was only one SNP, which was the simplest calculation method (36). Then, we further visualized the results of the five MR methods. The

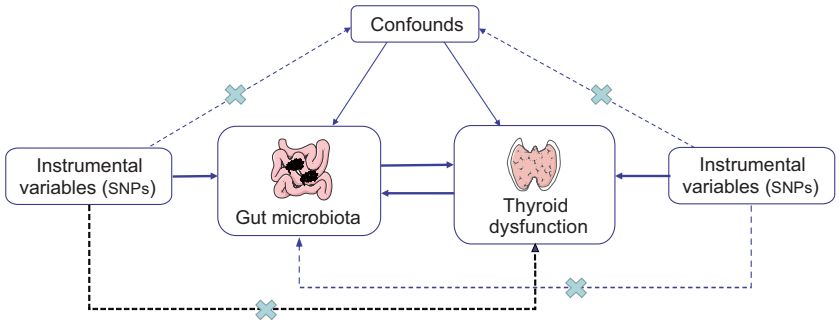

FIGURE 1
Schematic representation of the bidirectional two-sample MR study. The arrows show three key assumptions for instrumental variables commonly required in MR studies, and the crosses represent possible ways that the assumptions could be violated. SNP, single-nucleotide polymorphism.

TABLE 1 Data sources used in the bidirectional two-sample MR study.

Data	Trait	Year	Consortium	Ancestry	Sample size	Number of SNPs
Gut microbiomes	Gut microbiota abundance	2021	MiBioGen consortium	European	13,266	5,717,754
Hypothyroidism	Hypothyroidism, strict autoimmune	2021	FinnGen consortium	European	198,472	16,380,353
TSH alteration	Thyroid-stimulating hormone	2018	MRC IEU OpenGWAS project	European	3,301	10,534,735
Thyroxine deficiency	Medication code: levothyroxine sodium	2018	MRC IEU OpenGWAS project	European	462,933	9,851,867
TPOAb positivity	Thyroid peroxidase antibodies' positivity	2014	ThyroidOmics consortium	European	18,297	10,485,757

bidirectional causal effect was expressed as an odds ratio (OR) calculated from MR analysis. Notably, to obtain more IVs, we did not reach the traditionally strict significance threshold ($p < 5 \times 10^{-8}$) for exposure to gut microbiota. Thus, a false discovery rate (FDR) was corrected for multiple comparisons by the Benjamin–Hochberg method to limit the possibility of false positives, and the threshold was set at 0.05. The bidirectional causal effect was considered significant when $p < 0.05$ and FDR < 0.05 (37).

Sensitivity analyses

Several basic sensitivity analyses were used to validate the results. Cochran's Q statistic was employed to assess heterogeneity among IVs. The heterogeneity should be noted if it exists between different IVs ($p < 0.05$) (38). Horizontal pleiotropy indicates that IVs are associated with the outcome through pathways other than causal effects, potentially leading to false positive results ($p < 0.05$). MR pleiotropy residual sum and outlier (MR-PRESSO) analysis was also used to validate the potential pleiotropy of the direct effect between the selected IVs and the outcome. Subsequently, we applied the leave-one-out method to exclude each SNP from the IVs, evaluatingwhether individual SNPs significantly influenced causal effects using the IVW method. All the above analyses were done using two-sample MR and MR-PRESSOR R packages (35, 39).

Reverse MR analysis

To investigate alterations of gut microbiota following the onset of hypothyroidism, we conducted a reverse MR analysis, treating hypothyroidism as the exposure and gut microbiota as the outcome. The threshold of significance level for IVs was adjusted to be more accurate ($p < 5 \times 10^{-8}$). The procedure for the reverse MR analysis was the same as the MR analysis described above.

Results

Causal effects of gut microbiota on thyroid dysfunction

A total of 14,405 SNPs associated with the gut microbiota were meticulously curated following a rigorous screening process. Through robust linkage disequilibrium clustering and coordination, each bacterial taxon was linked to a varied range of IVs, spanning from 3 to 22. Notably, the F-statistic for all SNPs surpassed 10, indicating the absence of weak instrumental bias in the dataset.

After FDR correction, a meticulous analysis targeted three phyla and seven genera of bacteria taxa, revealing their causal effects on thyroid dysfunction via MR analysis. Six were linked to hypothyroidism, one to alterations in TSH levels, and three to TPOAb positivity (Figure 2). The IVW estimate pointed toward the genera *Intestinimonas* (OR = 1.120, p = 0.014) and *Ruminiclostridium5* (OR = 1.189, p = 0.011) as the risk factors for hypothyroidism. In contrast, genera *Bifidobacterium* (OR = 0.877, p = 0.011) and *Lachnospiraceae UCG008* (OR = 0.871, p = 0.002) and phyla Actinobacteria (OR = 0.827, p = 0.001) and Verrucomicrobia (OR = 0.876, p = 0.012) emerged as the protective factors against hypothyroidism. Moreover, phylum Bacteroidetes displayed an association with reduced TSH levels (OR = 0.711, p = 0.010). Three bacterial genera exhibited potential causal effects on TPOAb positivity: genus *Anaerotruncus* (OR = 0.291, p = 0.025) reduced TPOAb positivity, whereas genera *Eubacterium brachy group* (OR = 1.744, p = 0.034) and *Ruminococcaceae UCG004* (OR = 2.193, p = 0.033) increased TPOAb positivity, as indicated by the IVW method. Intriguingly, no bacteria demonstrated a significant causal relationship with thyroxine deficiency. Rigorous sensitivity analyses revealed the absence of heterogeneity and pleiotropy in the identified causal effects (Supplementary Table 1). Furthermore, the leave-one-out method demonstrated the absence of outlier SNPs in all selected bacteria taxa. Regrettably, this method could not be performed due to the limited number of SNPs associated with TPOAb as the outcome (Figure 3).

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Exposure	Outcome	Method	nSNP	OR	p-value
genus Bifidobacterium	Hypothyroidism	MR Egger	17	0.819 (0.620-1.082)	0.180
		Weighted median	17	0.820 (0.731-0.921)	0.001
		IVW	17	0.877 (0.793-0.970)	0.011
		Simple mode	17	0.843 (0.687-1.035)	0.122
		Weighted mode	17	0.815 (0.716-0.928)	0.007
genus Intestinimonas	Hypothyroidism	MR Egger	16	1.045 (0.811-1.346)	0.738
		Weighted median	16	1.096 (0.971-1.236)	0.138
		IVW	16	1.120 (1.023-1.226)	0.014
		Simple mode	16	1.059 (0.861-1.301)	0.596
		Weighted mode	16	1.098 (0.891-1.352)	0.395
genus Lachnospiraceae UCG008	Hypothyroidism	MR Egger	11	0.566 (0.370-0.867)	0.028
		Weighted median	11	0.904 (0.805-1.016)	0.090
		IVW	11	0.871 (0.798-0.950)	0.002
		Simple mode	11	0.919 (0.744-1.136)	0.453
		Weighted mode	11	0.926 (0.759-1.130)	0.468
genus Ruminiclostridium5	Hypothyroidism	MR Egger	11	1.053 (0.600-1.847)	0.861
		Weighted median	11	1.262 (1.051-1.515)	0.013
		IVW	11	1.189 (1.040-1.361)	0.011
		Simple mode	11	1.323 (0.948-1.845)	0.131
		Weighted mode	11	1.340 (0.948-1.894)	0.128
phylum Actinobacteria	Hypothyroidism	MR Egger	17	0.560 (0.363-0.865)	0.020
		Weighted median	17	0.748 (0.642-0.871)	0.000
		IVW	17	0.827 (0.738-0.926)	0.001
		Simple mode	17	0.744 (0.570-0.972)	0.045
		Weighted mode	17	0.737 (0.608-0.892)	0.007
phylum Verrucomicrobia	Hypothyroidism	MR Egger	12	0.881 (0.672-1.155)	0.381
		Weighted median	12	0.889 (0.773-1.021)	0.095
		IVW	12	0.876 (0.790-0.971)	0.012
		Simple mode	12	0.943 (0.756-1.176)	0.611
		Weighted mode	12	0.907 (0.747-1.102)	0.347
phylum Bacteroidetes	TSH	MR Egger	12	0.562 (0.326-0.970)	0.065
		Weighted median	12	0.701 (0.486-1.011)	0.057
		IVW	12	0.711 (0.549-0.921)	0.010
		Simple mode	12	0.633 (0.369-1.084)	0.124
		Weighted mode	12	0.662 (0.419-1.047)	0.106
genus Anaerotruncus	TPOAb	IVW	2	0.291 (0.099-0.859)	0.025
genus Eubacterium brachy group	TPOAb	IVW	2	1.744 (1.042-2.918)	0.034
genus Ruminococcaceae UCG004	TPOAb	IVW	2	2.193 (1.066-4.511)	0.033

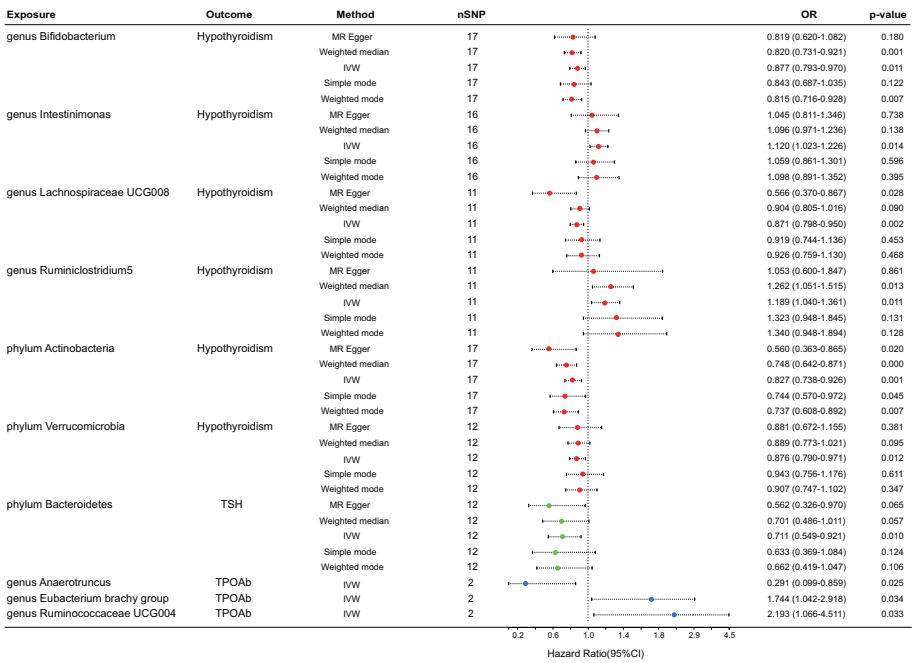

FIGURE 2
Forest plot for the causal effect of gut microbiota on thyroid dysfunction by the five MR methods. The error bar represents the 95% confidence interval of hazard ratio. The red dot represents an outcome of hypothyroidism, the green represents an outcome of TSH alteration, and the blue represents an outcome of TPOAb positivity. nSNP, number of SNPs; OR, odds ratio; MR, Mendelian randomization; IVW, inverse variance weighted.

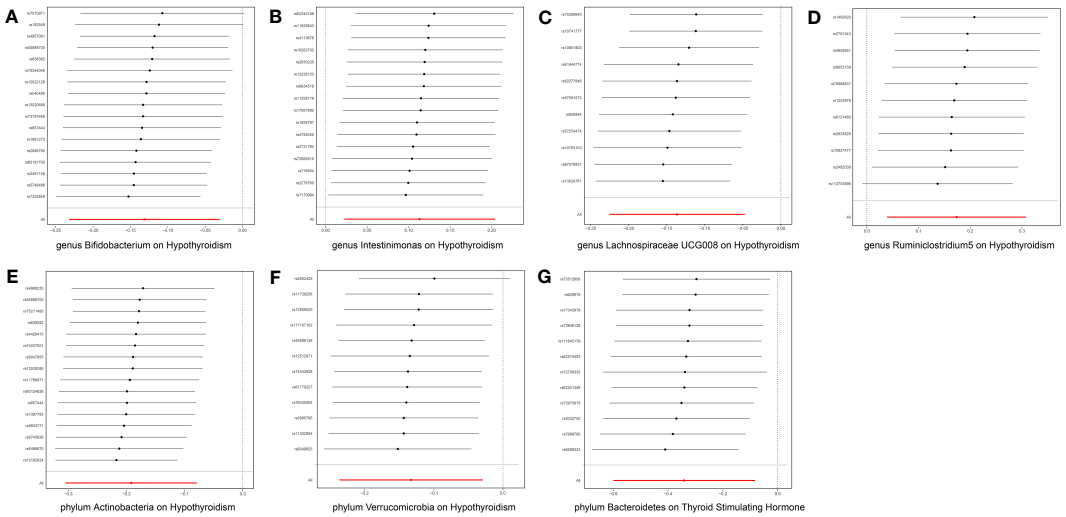

FIGURE 3
Leave-one-out plots for the causal effect of gut microbiota on thyroid dysfunction. The red line is the random effect of the IVW method, and the error bar represents the 95% confidence interval with the IVW method. **(A)** Genus *Bifidobacterium*, **(B)** genus *Intestinimonas*, **(C)** genus *Lachnospiraceae UCG008*, **(D)** genus *Ruminiclostridium5*, **(E)** phylum Actinobacteria, **(F)** phylum Verrucomicrobia, and **(G)** phylum Bacteroidetes.

Causal effects of thyroid dysfunction on the gut microbiota

In the reverse MR analysis, 56 SNPs linked with hypothyroidism were identified. Each bacterial taxon was linked to a minimum of 3 and a maximum of 36 IVs, all exhibiting robustness (F-statistic > 10). Applying the IVW method in the MR analysis, it was observed that class Negativicutes (OR = 1.039, p = 0.048), family Christensenellaceae (OR = 1.065, p = 0.030), genera *Eubacterium ruminantium group* (OR = 1.057, p = 0.042) and *Ruminococcaceae UCG005* (OR = 1.047, p = 0.025), and order Selenomonadales (OR = 1.039, p = 0.048) exhibited upregulation following the onset of

hypothyroidism. Conversely, class Verrucomicrobiae (OR = 0.954, p = 0.029), family Verrucomicrobiaceae (OR = 0.954, p = 0.024), order Verrucomicrobiales (OR = 0.954, p = 0.029), phylum Verrucomicrobia (OR = 0.954, p = 0.024), and genera *Akkermansia* (OR = 0.954, p = 0.029) and *Erysipelotrichaceae UCG003* (OR = 0.906, p = 0.033) were downregulated subsequent to the onset of hypothyroidism (Figure 4). Rigorous sensitivity analyses confirmed the absence of heterogeneity or horizontal pleiotropy in the aforementioned causal effects (Supplementary Table 2). In addition, leave-one-out analysis did not reveal any SNPs driving the causal effect of hypothyroidism on gut microbiota (Figure 5).

Discussion

Thyroid dysfunction has emerged as a global public health concern. Advancements in scientific inquiry have delved deeper into gut microbiota research, particularly in its connection to thyroid function via the thyroid–gut axis, a burgeoning area of interest (1, 40). Hypothyroidism, a prevalent condition stemming from thyroid dysfunction, exhibits multifaceted and poorly understood causes. Beyond external causes such as iodine deficiency, medications, and surgery, autoimmune origins constitute the bulk of primary hypothyroidism, posing significant challenges for explanation and management (41). Gut microbiota serves as the linchpin for stable intestinal lymphoid tissue function, acting as a vital shield in immune homeostasis, enhancing tolerance to autoantigens and non-pathogenic non-autoantigens (11). The interplay between gut microbiota and the host's innate and adaptive immunity potentially influences the susceptibility to autoimmune thyroid disease (42, 43). Moreover, reduced intestinal motility in patients with hypothyroidism might disrupt intestinal substrate utilization and physicochemical conditions, culminating in gut microbiota dysbiosis exacerbating the condition or giving rise to complications (44). To address these intricacies, we conducted a comprehensive bidirectional two-sample MR study utilizing multiple GWAS datasets. This investigation aimed to elucidate the bidirectional causal effects links between gut microbiota and thyroid dysfunction, shedding light on the pathogenesis of autoimmune hypothyroidism and informing strategies for prevention, delay, and reversal of thyroid dysfunction–associated health conditions.

Numerous clinical trials have identified disparities in gut microbiota composition between thyroid dysfunction patients and healthy populations. However, establishing the bidirectional causal relationship between gut microbiota alterations and thyroid dysfunction remained elusive. For instance, a cross-sectional study encompassing 97 cases revealed significantly lower levels of *Alistipes*,

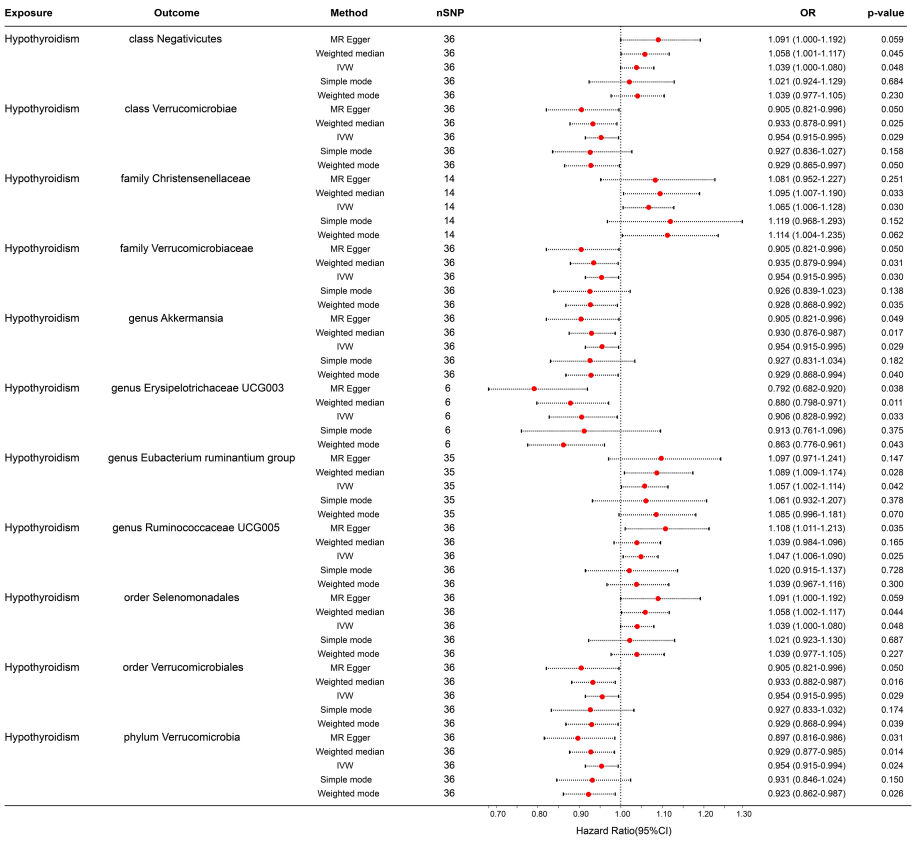

FIGURE 4
Forest plot for the causal effect of hypothyroidism on gut microbiota by the five MR methods. The error bar represents the 95% confidence interval of the hazard ratio. nSNP, number of SNPs; OR, odds ratio; MR, Mendelian randomization; IVW, inverse variance weighted.

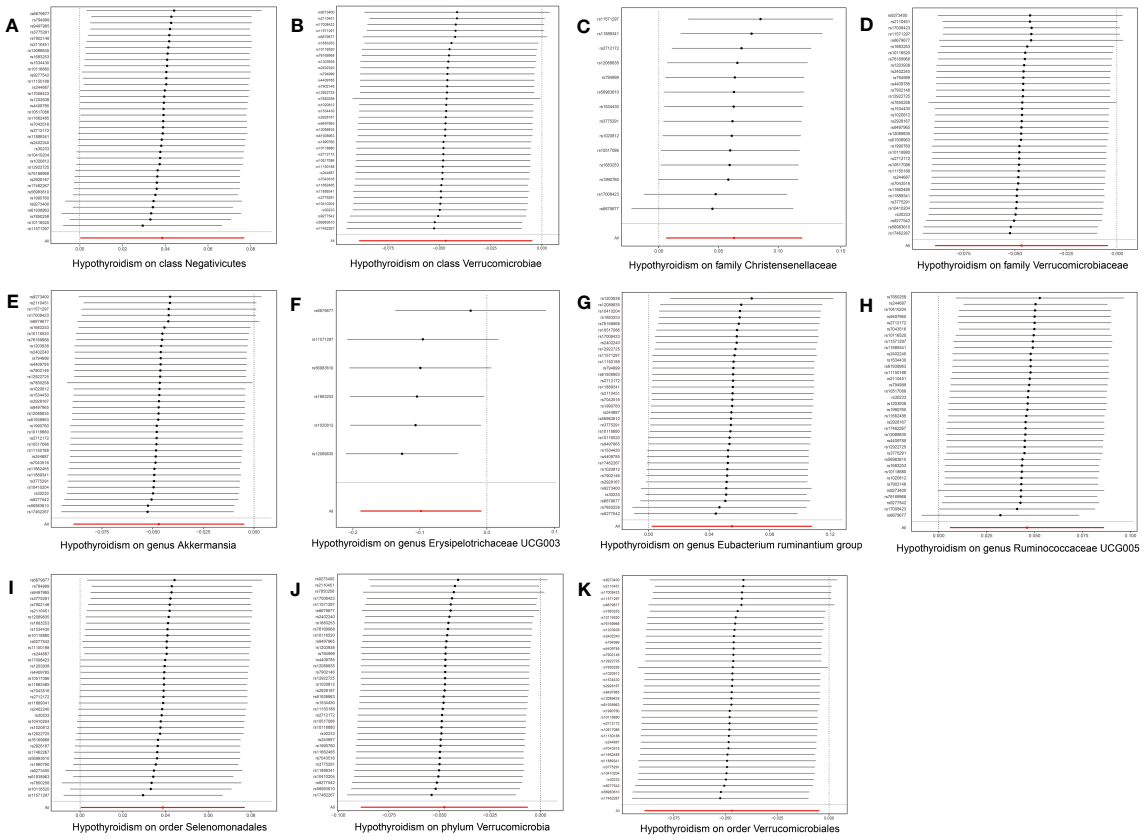

FIGURE 5
Leave-one-out plots for the causal effect of hypothyroidism on gut microbiota. The red line is the random effect of the IVW method and the error bar represents the 95% confidence interval with the IVW method. **(A)** Class Negativicutes, **(B)** class Verrucomicrobiae, **(C)** family Christensenellaceae, **(D)** family Verrucomicrobiaceae, **(E)** genus *Akkermansia*, **(F)** genus *Erysipelotrichaceae UCG003*, **(G)** genus *Eubacterium ruminantium group*, **(H)** genus *Ruminococcaceae UCG005*, **(I)** order Selenomonadales, **(J)** order Verrucomicrobiales, and **(K)** phylum Verrucomicrobia.

Lachnospiraceae, *Intestinimonas*, *Ruminococcus*, and *Subdoligranulum* in hypothyroid patients, whereas *Phascolarctobacterium* and Bacteroidetes were more abundant (45). Another study comparing 29 hypothyroid patients with 11 healthy individuals highlighted a higher prevalence of Actinobacteria and *Enterobacteriaceae* and significantly diminished counts of *Bifidobacteria* and *Ruminococcaceae* in the hypothyroid group (46). Our MR analysis not only validated these findings but also established the bidirectional causal relationship between altered gut microbiota and diminished thyroid function. Notably, the genera *Intestinimonas* and *Ruminiclostridium5* were linked to reduced thyroid function, whereas the genera *Bifidobacterium* and *Lachnospiraceae UCG008* and phylum Actinobacteria mitigated this decline. Furthermore, only after the onset of hypothyroidism did the genera *Eubacterium ruminantium group*, *Ruminococcaceae UCG005*, and *Erysipelotrichaceae UCG003* exhibit changes (47, 48). Previous controlled studies also revealed a significant decrease in Bacteroidetes in hyperthyroid patients with elevated TSH levels, with MR analysis corroborating Bacteroidetes' role in decreasing TSH (49). In an RCT, decreased *Eubacterium* post-probiotic intervention ameliorated complications in patients not treated with radioiodine after thyroidectomy, aligning with our study's identification of Eubacterium as a risk factor for TPOAb positivity (50).

Several mechanisms underpinning gut microbiota's impact on thyroid function via the thyroid–gut axis have been elucidated. The equilibrium between pathogenic and probiotic bacteria is pivotal for maintaining gut barrier function. *Bifidobacterium*, a widely used probiotic, confers several physiological benefits to humans, rendering it a protective genus against hypothyroidism in our study (51). In addition, phylum Actinobacteria and genus *Lachnospiraceae UCG008*, both protective in this study, are implicated in human sugar and protein metabolism. Phylum Actinobacteria participates in the biosynthesis of phenylalanine, tyrosine, and tryptophan, whereas *Lachnospiraceae UCG008* is associated with alanine, aspartate, and glutamate metabolism (52–54). Our results also identified *Ruminiclostridium5* as a risk for promoting hypothyroidism. Although limited information is available on *Ruminiclostridium5*, its increased abundance is linked to systemic inflammation and a negative correlation with secondary and conjugated bile acids (55, 56). Consistent with prior studies, phylum Verrucomicrobia emerged as a protective factor against hypothyroidism in our result. This phylum comprises diverse beneficial bacteria for the gut, whose outer membrane proteins effectively safeguard interactions with other cells (57). Genus *Akkermansia*, a member of the phylum Verrucomicrobia,

plays a significant role in enhancing host metabolic function and immune responses. Intriguingly, its abundance significantly decreases after hypothyroidism onset (58). Despite these insights, in-depth *in vivo* and *in vitro* experiments are imperative to explore the effects and mechanisms of gut microbiota as delineated in our findings.

The bidirectional two-sample MR study offers significant advantages in investigating the bidirectional causal relationship between gut microbiota and hypothyroidism. First, the sample size of clinical trials often lacks the representativeness necessary for generalizability. In contrast, our study utilized gut microbiome data from 13,266 samples. Thyroid function–related data were sourced from four datasets in three databases, encompassing hundreds of thousands of samples, each mutually exclusive. This vast dataset enhances the present results' representativeness and credibility. Moreover, IV analysis grounded in effectively mitigates confounding factors and eliminates the outcome's interference with the exposure's reverse effect. Utilizing the MR-PRESSO approach and a comprehensive set of sensitivity analyses eliminates study pleiotropy and heterogeneity. The non-duplication of GWAS datasets for both exposure and outcome samples significantly reduces and avoids bias. However, it is crucial to acknowledge the study's limitations. Gut microbiota data were derived from the GWAS meta-analysis rather than raw data, precluding subgroup analyses. In addition, the study's inclusion was limited to individuals of European ancestry, restricting the applicability of our findings to broader populations. Furthermore, the lowest taxonomic level for gut bacteria is the genus, precluding an in-depth exploration of gut microbiota's causal effects on hypothyroidism at the species level. Notably, some results, with a limited number of SNPs, necessitate cautious interpretation. It is pivotal to underscore that RCTs remain the gold standard for treatment development and establishing causal relationships in biological contexts. They offer unparalleled control and evidence. Mendelian randomization analysis serves as a potent supplementary tool, especially in scenarios where conducting RCTs proves challenging.

Conclusions

In summary, this study firstly and comprehensively provides evidence to support the effects of various gut microbiota on thyroid dysfunction, including hypothyroidism, TSH, thyroxine, and TPOAb, and further reveals alterations in gut microbiota following hypothyroidism. Genera *Intestinimonas*, *Eubacterium brachy group*, *Ruminiclostridium5*, and *Ruminococcaceae UCG004* are found to be risk factors for decreased thyroid function, whereas genera *Bifidobacterium* and *Lachnospiraceae UCG008* and phyla Actinobacteria and Verrucomicrobia were protective. Eight types of gut microbiota are thought to show altered abundance after the onset of hypothyroidism. This bidirectional two-sample MR study provides fairly strong evidence for the thyroid–gut axis theory to select more targeted probiotics to reverse the disturbed immune system and control the progression of thyroid dysfunction.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories can be found in the article.

Author contributions

XL: Data curation, Methodology, Software, Writing – original draft, Investigation. JL: Methodology, Writing – original draft, Formal Analysis. TZ: Software, Writing – original draft, Data curation. QW: Conceptualization, Validation, Writing – review & editing, Funding acquisition, Methodology, Supervision, Visualization. HZ: Supervision, Writing – review & editing, Project administration.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1267383/full#supplementary-material

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Oxidative balance score: a potential tool for reducing the risk of colorectal cancer and its subsites incidences

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Background: The Oxidative Balance Score (OBS) is commonly used to assess oxidative stress and provides a comprehensive evaluation of dietary and lifestyle-related exposures. However, there is limited research on the association between OBS and colorectal cancer (CRC), its subsites, and complications. The objective of this study was to assess the relationship between OBS and the risk of CRC, its subsites, and common complications in a large prospective cohort study.

Methods: We included data from 175,808 participants in the UK Biobank data sample repository from 2006 to 2010. We evaluated OBS using a scoring system based on 22 dietary and lifestyle factors. Multiple adjustments, including multivariate Cox proportional hazard regression, gender stratification, subgroup analysis, and sensitivity analysis, were performed to fully explore the relationship between OBS and CRC, its subsites, and complications. The mediation analysis was conducted to investigate whether serum albumin, uric acid, and neutrophil levels mediate the relationship between OBS and CRC.

Results: After adjusting for potential confounding factors, a significant negative correlation was found between OBS and the risk of CRC and its subsites (proximal colon cancer, distal colon cancer, and rectal cancer). This correlation was particularly pronounced in male CRC patients. Serum albumin, uric acid, and neutrophil count, which are biomarkers, were found to have a significant mediating effect between OBS and CRC.

Conclusion: Our study suggests that higher exposure to antioxidants assessed through OBS (diet and lifestyle rich in antioxidants) may decrease the occurrence of CRC and its subsites.

KEYWORDS

oxidative balance score, colorectal cancer, UK biobank, mediation analysis, antioxidant

1 Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide, with an increasing incidence rate year by year (1). The prevalence of CRC varies in different regions and is influenced by both unmodifiable factors such as age, gender, and family history, and modifiable factors such as diet and lifestyle. Smoking, alcohol consumption, and high-fat diet are believed to be positively associated with the occurrence of CRC. Conversely, high intake of dietary fiber (fruits, vegetables, whole grains, etc.) and vitamins (vitamin C, D, E) has been found to have a positive protective effect against CRC (2, 3).

Oxidative stress is often considered as a balance between oxidation and antioxidant defense, and the excessive generation of reactive oxygen species (ROS) and the decrease in antioxidant defense capacity are often regarded as initiators and promoters of CRC. Oxidative stress can induce carcinogenesis through mechanisms such as inducing oxidative damage to deoxyribonucleic acid (DNA), protein oxidation, and inflammatory damage to intestinal epithelial cells (4, 5). Antioxidant enzymes and nutrients are believed to play a role in preventing carcinogenesis. However, the impact of individual oxidative balance exposure on carcinogenesis is difficult to measure, which may be closely associated with the combined action of various prooxidants and antioxidants (6). Therefore, in this study, the oxidative balance score (OBS) was used as an evaluation index for combined exposure to antioxidants and prooxidants, providing different assessment criteria for diet and lifestyle. Higher OBS scores represent greater exposure to antioxidants. Previous studies have shown a negative correlation between higher OBS and the occurrence of colorectal cancer (7, 8). However, few studies have explored the correlation between OBS and CRC, as well as its subsites, and the correlation between OBS related to diet and lifestyle and CRC in different genders.

The aim of this study was to investigate the association between OBS and CRC and its subsites (proximal colon cancer, distal colon cancer and rectal cancer) as well as complications (metastasis and abdominal pain) in a large UK Biobank follow-up cohort. Furthermore, we explored whether serum albumin, uric acid, and neutrophil levels mediated the associations mentioned above. Our findings will provide new evidence for the role of oxidative balance in CRC, as well as its subsites, and its related complications. These findings may have implications for early prevention and treatment of CRC in clinical practice.

2 Methods

2.1 Study population

The UK Biobank is a large prospective cohort study that recruited approximately 500,000 participants aged 40-69 from various regions of the UK between 2006 and 2010. Baseline data were collected at this time, followed by four large-scale follow-up visits and additional data collection. The database collected information from various modules, including sociodemographic, dietary, blood and urine specimens, environmental factors, and has been tracking and recording decades of health and medical records. Furthermore, since recruitment, the study has established links with cancer and mortality registries to conduct a tracking investigation on cancer incidence and mortality rates among participants. Approval for accessing patient records for recruitment was obtained from the National Health and Social Care Information Governance Committee for England and Wales, as well as the Community Health Index Advisory Group for Scotland. This biobank has obtained approval from the North West Multi-Centre Research Ethics Committee, and all participants have signed informed consent forms using touchscreen signature capture devices (9).

The dataset included information from a total of 502,619 male and female participants. During the recruitment phase, 311,418 participants with incomplete questionnaire data and were unable to calculate the OBS were excluded. Furthermore, patients with missing data (including race, education level, CRP levels, and Thompson Index (TSI) were excluded. Ultimately, a total of 175,808 participants were included in this study. Further specific details are available in Figure 1.

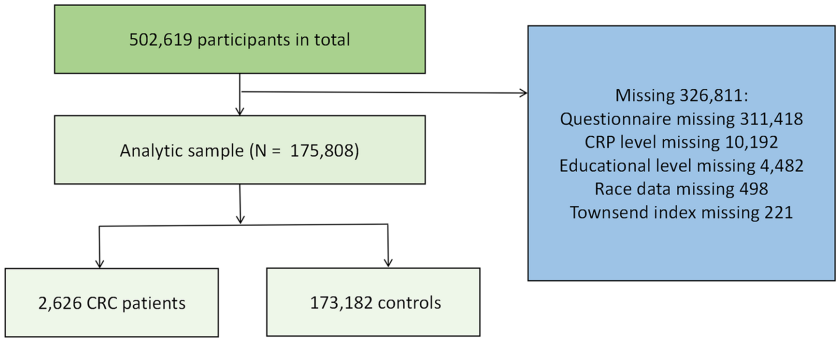

FIGURE 1
The sample sample selection and analysis flowchart.

2.2 Assessment of CRC

Incidence of cancer and mortality cases in the UK Biobank were primarily obtained by linking to routine data from the National Health Service in the UK. Cancer types were coded using the International Classification of Diseases (ICD-10), while histological and morphological information of tumors were coded using ICD-O-3. Participants were followed up from the time of recruitment, and the analysis was initiated from the date when eligible participants were first registered as having cancer. The study exit date, death date, or last follow-up date was December 31, 2014. The endpoint of the analysis was the first diagnosis of CRC (ICD-10 codes C18-C20) or the primary underlying cause of death due to CRC. Proximal colon cancer was defined as tumors occurring in the cecum, appendix, ascending colon, hepatic flexure, transverse colon, or splenic flexure (C18.0-C18.5). Distal colon cancer was defined as tumors occurring in the descending colon (C18.6) or sigmoid colon (C18.7). Rectal cancer included tumors occurring at the rectosigmoid junction (C-19) and rectum (C-20).

2.3 Measurement of OBS

Four categories of data were obtained and collected using a food frequency questionnaire (FFQ), including dietary pro-oxidants (total fat, iron, polyunsaturated fatty acids, saturated fatty acids, meat intake) and lifestyle pro-oxidants (smoking, alcohol consumption, BMI). Dietary antioxidants (calcium, magnesium, vitamin B6, vitamin B12, vitamin C, vitamin D, vitamin E, total folate, carotenoids, dietary fiber, retinol, vegetable intake) and lifestyle antioxidants (tea consumption and physical activity) were also collected. The cumulative OBS score was derived by summing the assigned values (0, 1, 2) based on the predefined tertiles, and participants were further divided into groups based on gender. Participants in the lower tertiles were assigned a value of 0, while those in the higher tertiles were assigned a value of 2. Consequently, a score of 0 represented a low antioxidant level, while a score of 2 indicated a high antioxidant level. Therefore, a higher OBS score indicated a more significant exposure to antioxidants. In smoking, alcohol consumption, and meat intake, non-consumption was defined as 2, and values of 0 or 1 were assigned based on binomial distribution grouping. Meat intake was calculated by summing the intake of beef, lamb, and pork, while vegetable intake was calculated by summing the intake of raw and cooked vegetables. Physical activity was obtained by summing the weekly MET minutes of light, moderate, and vigorous physical activities. For more specific details, please refer to Supplementary Table 1.

2.4 Covariates

In this study, a Cox proportional hazards model was constructed to adjust for potential confounding factors that may influence the association between OBS and colorectal cancer. The adjusted covariates included age, race (European, Asian, African, Chinese, Mixed-race, Others), Townsend deprivation index, education achievement index, BMI, high-sensitivity CRP, dietary energy intake, and NSAIDs medication usage. The education achievement index data were obtained from two domains: "child and adolescent" and "adult skills," reflecting both the mobility and stock of educational disadvantages within a region. Dietary energy intake data were calculated based on recommended data from dietary questionnaire surveys, and NSAIDs medication usage data were obtained from self-reported survey questionnaires.

2.5 Statistical analyses

Continuous variables in this study were presented as mean (± standard deviation). Categorical variables were presented as sample size (percentage). Continuous OBS scores were categorized based on quartiles (Q1: < 25th percentile, Q2: 25th-50th percentile, Q3: 50th-75th percentile, Q4: 75th-100th percentile). In our study, grouping OBS scores into quartiles was aimed at better understanding the distribution of OBS scores among participants and further investigating the relationship between OBS scores and other variables. Wilcoxon rank sum test was used to describe the differences in baseline variable characteristics between quartile groups of continuous OBS scores. Rao-Scott $\chi 2$ test was used to describe the percentage differences of categorical variables. Multiple Cox proportional hazards models were established to explore the correlation between OBS and the occurrence of CRC and its subsites (proximal colon cancer, distal colon cancer and rectal cancer). Model 1: Adjusted for age, race, educational attainment, and Townsend deprivation index; Model 2: In addition to Model 1, adjusted for dietary energy intake; Model 3: In addition to Model 2, adjusted for plasma CRP concentration and NSAIDs medication usage. Furthermore, we conducted subgroup analysis stratified by gender to further investigate the correlation between the two in the three models mentioned above. In this study, we explored the correlation between continuous OBS and the occurrence of complications in CRC (colorectal cancer metastasis, proximal colon cancer metastasis, abdominal pain in proximal colon cancer, abdominal pain in distal colon cancer) using the aforementioned three models. We employed logistic regression models adjusted for the variables in Model 3 to investigate the correlation between continuous variables including serum albumin, uric acid, and neutrophil levels and the occurrence of CRC. In addition, sensitivity analysis was conducted by performing leave-one-out analysis to assess the contribution of each score, and OBS was further divided into diet OBS and lifestyle OBS. The relationship between OBS and CRC occurrence was separately explored in gender subgroups. Mediation analysis was performed to determine whether serum albumin, uric acid, and neutrophil levels mediated the relationship between OBS and CRC. Indirect effect (IE) refers to the influence of serum albumin, uric acid, and neutrophil levels on CRC through OBS. Direct effect (DE) represents the influence of OBS on CRC after controlling for serum albumin, uric acid, and neutrophil levels. Significantly positive IE indicates the presence of a significant mediating effect. All statistical analyses and graphical presentations were performed

using R Project for Statistical Computing (version 4.2.3). All statistical tests were two-tailed, and a p-value < 0.05 was considered statistically significant.

3 Results

3.1 Baseline characteristics

We grouped the participants based on quartiles of OBS and summarized the baseline characteristics of the total population and each group of OBS. The variability in the number of subjects in each group primarily stems from the uneven distribution of OBS scores in the actual data, with different densities across different score ranges. Hence, the unequal distribution of subjects across the four groups. Regarding the representation of quartile ranges in the table, we employed the convention of using closed on the left and open on the right intervals, such as [4,19), indicating that the first quartile range encompasses OBS scores equal to or greater than 4 and less than 19. A total of 175,808 participants were included in this study. The average age of the participants was 55.9 ± 8.0 years. Compared to participants in the lowest quartile of OBS, those in the highest quartile of OBS were more likely to be female, older, of European descent, with lower education levels, plasma CRP levels, NSAIDs medication usage and BMI, higher Townsend deprivation index and dietary energy intake, lower incidence and mortality rate of CRC. For more detailed baseline characteristics, see Table 1. We also compared the general characteristics between males and females. Compared to female participants, male participants were more likely to have lower OBS scores. They were also more likely to be older individuals with colorectal cancer and its related complications (obstruction and abdominal pain). Moreover, male participants had lower TDI scores, Levels of CRP and NSAIDs medication usage, higher level of education, BMI levels, and dietary energy intake. For more details, refer to Supplementary Table 2.

3.2 Association between OBS and CRC and its subsites

As shown in Table 2, in the fully adjusted Model 3, there is a significant negative correlation between continuous OBS and the occurrence of CRC (HR 0.974, 95%CI 0.966-0.982, p<0.001), proximal colon cancer (HR 0.981, 95%CI 0.966-0.996, p=0.012), distal colon cancer (HR 0.966, 95%CI 0.951-0.981, p<0.001) and rectal cancer (HR 0.968, 95%CI 0.953-0.983, p<0.001). Compared to the Q1 of OBS, the risk of CRC decreased by 19.4% in Q2 (HR 0.806, 95%CI 0.714-0.909, p<0.001), 23.0% in Q3 (HR 0.770, 95%CI 0.689-0.861, p<0.001), and 28.7% in Q4 (HR 0.713, 95%CI 0.636-0.799, p<0.001). Compared to the Q1 of OBS, the risk of proximal colon cancer decreased by 19.5% in Q3 (HR 0.805, 95%CI 0.652-0.994, p=0.044) and 21.6% (HR 0.784, 95%CI 0.634-0.968, p=0.024). Compared to the Q1 of OBS, the risk of distal colon cancer decreased by 34.8% in Q2 (HR 0.652, 95%CI 0.520-0.816, p<0.001), 26.8% in Q3 (HR 0.732, 95%CI 0.600-0.893, p=0.002), and 36.5% in Q4 (HR 0.645, 95%CI 0.525-0.792, p<0.001). Compared to the Q1 of OBS, the risk of rectal cancer decreased by 26.2% in Q3 (HR 0.738, 95%CI 0.599-0.910, p=0.004) and 31.9% in Q4 (HR 0.681, 95%CI 0.550-0.841, p<0.001). More details can be obtained in Table 2.

3.3 Association between OBS and CRC and its subsites stratified by sex and race

As shown in Table 3, in male participants, in the fully adjusted Model 3, there is a significant negative correlation between continuous OBS and the occurrence of CRC (HR 0.967, 95%CI 0.956-0.977, p<0.001), proximal colon cancer (HR 0.974, 95%CI 0.954-0.995, p=0.016), distal colon cancer (HR 0.947, 95%CI 0.928-0.966, p<0.001) and rectal cancer (HR 0.978, 95%CI 0.959-0.997, p=0.026). Compared to Q1 of OBS, the risk of CRC decreased by 20.7% (HR 0.793, 95%CI 0.682-0.922, p=0.003) in Q2, 28.2% (HR 0.718, 95%CI 0.622-0.829, p<0.001) in Q3 and 34.1% (HR 0.659, 95%CI 0.568-0.766, p<0.001) in Q4. Compared to Q1 of OBS, the risk of distal colon cancer decreased by 28.2% (HR 0.560, 95%CI 0.422-0.741, p<0.001) in Q2, 38.0% (HR 0.620, 95%CI 0.483-0.796, p<0.001) in Q3 and 49.4% (HR 0.506, 95%CI 0.387-0.663, p<0.001) in Q4. Compared to Q1 of OBS, the risk of rectal cancer decreased by 22.8% in Q3 (HR 0.772, 95%CI 0.597-0.999, p=0.049). In female participants, in the fully adjusted Model 3, there is a significant negative correlation between continuous OBS and the occurrence of rectal cancer (HR 0.970, 95%CI 0.945-0.995, p=0.019). In model 2, compared to the Q1 of OBS, the risk of rectal cancer decreased by 30.9% in Q4 (HR 0.691, 95%CI 0.479-0.996, p=0.048). In model 3, compared to Q1 of OBS, the risk of rectal cancer decreased by 30.8% in Q4 (HR 0.692, 95%CI 0.480-0.997, p=0.048). More specific details can be seen in Table 3.

As shown in Supplementary Table 3, we stratified by age to further assess the correlation between OBS and CRC in different female cohorts above and below the age of 50. In multiple models adjusted for the same covariates as mentioned above, no significant correlation between OBS and CRC was found across different age groups in women. As shown in Supplementary Table 4, we further stratified by race to assess the association between OBS and CRC across different racial groups (European, Mixed, Asian, African, Chinese, and other races). In multiple models adjusted for the aforementioned variables, a significant inverse association between OBS and CRC was found only in the European ancestry population.

3.4 Correlation of OBS with CRC complications

As shown in Table 4, OBS is positively correlated with the risk of CRC metastasis in models 2 and 3. However, OBS is negatively correlated with the risk of proximal CRC metastasis. Furthermore, OBS is negatively correlated with the risk of abdominal pain caused by proximal CRC in models 1, 2, and 3. However, OBS is positively correlated with the risk of abdominal pain caused by distal CRC.

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TABLE 1 Baseline characteristics of participants according to quartiles of oxidative balance score: comparison between the first and fourth quartiles.

	Overall N = 175,808	Quantile OBS groups				p-value
		Q1 [4,19) N = 40,379	Q2 [19,22) N = 35,004	Q3 [22,26) N = 49,209	Q4 [26,41) N = 51,216	
OBS, Mean (SD)	22.5 (5.0)	15.8 (2.1)	20.0 (0.8)	23.5 (1.1)	28.5 (2.2)	<0.001
Colorectal cancer, n (%)	2,626 (1.5%)	671 (1.7%)	502 (1.4%)	715 (1.5%)	738 (1.4%)	0.018
Proximal colon cancer, n (%)	786 (0.4%)	186 (0.5%)	164 (0.5%)	207 (0.4%)	229 (0.4%)	0.700
Distal colon cancer, n (%)	786 (0.4%)	216 (0.5%)	132 (0.4%)	223 (0.5%)	215 (0.4%)	0.008
Rectal cancer, n (%)	757 (0.4%)	196 (0.5%)	154 (0.4%)	202 (0.4%)	205 (0.4%)	0.200
CRC with obstruction, n (%)	275 (0.2%)	63 (0.2%)	60 (0.2%)	64 (0.1%)	88 (0.2%)	0.200
CRC with abdominal pain, n (%)	294 (0.2%)	81 (0.2%)	61 (0.2%)	60 (0.1%)	92 (0.2%)	0.068
Secondary Metastasis of CRC, n (%)	662 (0.4%)	184 (0.5%)	124 (0.4%)	176 (0.4%)	178 (0.3%)	0.084
Death of CRC, n (%)	509 (0.3%)	142 (0.4%)	97 (0.3%)	120 (0.2%)	150 (0.3%)	0.027
Sex, n (%)						<0.001
Female	94,044 (53%)	20,971 (52%)	18,392 (53%)	26,215 (53%)	28,466 (56%)	
Male	81,764 (47%)	19,408 (48%)	16,612 (47%)	22,994 (47%)	22,750 (44%)	
TDI, Mean (SD)	-1.6 (2.9)	-1.3 (3.0)	-1.6 (2.9)	-1.7 (2.8)	-1.8 (2.8)	<0.001
Missing	221	68	39	63	51	
Education, Mean (SD), score	11.7 (13.8)	13.3 (15.1)	11.7 (13.8)	11.2 (13.2)	11.0 (13.0)	<0.001
Missing	4,482	1,108	871	1,199	1,304	
Ethnicity, n (%)						<0.001
European	167,952 (96%)	38,319 (95%)	33,409 (96%)	47,162 (96%)	49,062 (96%)	
Mixed-race	1,054 (0.6%)	299 (0.7%)	197 (0.6%)	282 (0.6%)	276 (0.5%)	
Asian	2,488 (1.4%)	583 (1.4%)	526 (1.5%)	699 (1.4%)	680 (1.3%)	
African	2,082 (1.2%)	706 (1.8%)	410 (1.2%)	467 (1.0%)	499 (1.0%)	
Chinese	495 (0.3%)	90 (0.2%)	106 (0.3%)	142 (0.3%)	157 (0.3%)	
Others	1,239 (0.7%)	268 (0.7%)	253 (0.7%)	333 (0.7%)	385 (0.8%)	
Missing	498	114	103	124	157	
BMI, Mean (SD), kg/m2	26.9 (4.6)	28.3 (4.9)	27.1 (4.6)	26.6 (4.4)	25.8 (4.2)	<0.001
Age, Mean (SD), years	55.9 (8.0)	55.0 (8.0)	55.7 (8.0)	56.0 (7.9)	56.6 (7.9)	<0.001
CRP level, Mean (SD), mg/L	2.3 (4.0)	2.7 (4.3)	2.3 (3.9)	2.2 (4.0)	1.9 (3.7)	<0.001
Missing	10,192	2,377	2,009	2,868	2,938	
Daily energy intake, Mean (SD)	8,862.8 (3,036.6)	7,731.7 (2,481.1)	8,300.7 (2,718.7)	9,135.5 (2,997.0)	9,876.7 (3,285.3)	<0.001
NSAIDs medication usage, n (%)	59,478 (34%)	14,645 (36%)	12,147 (35%)	16,423 (33%)	16,263 (32%)	<0.001
Albumin, Mean (SD), g/L	45.4 (2.6)	45.3 (2.6)	45.4 (2.6)	45.4 (2.6)	45.4 (2.6)	<0.001
Missing	24,201	5,511	4,794	6,689	7,207	
Uric acid, Mean (SD), μmol	306.4 (79.1)	318.6 (82.0)	310.2 (79.7)	304.9 (78.3)	295.5 (75.7)	<0.001
Missing	10,035	2,334	1,988	2,808	2,905	
Neutrophils, Mean (SD), 10^9 cells/Litre	4.1 (1.4)	4.3 (1.4)	4.2 (1.4)	4.1 (1.3)	4.0 (1.3)	<0.001
Missing	6,810	1,608	1,447	1,849	1,906	

OBS, Oxidative Balance Score; TDI, Thomson Deprivation Index; CRC, Colorectal Cancer; SD, standard deviation; BMI, body mass index; CRP, C-reactive protein; Education refered to the age of Highest Level of Education.

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TABLE 2 Association of oxidative balance score with colorectal cancer and subsites.

	Continue	Quantile				P for trend
		Q1	Q2	Q3	Q4	
CRC, HR (95% CI), P-value						
Model 1	0.979(0.971~0.987), <0.001	Ref.	0.807(0.718~0.908), <0.001	0.799(0.718~0.889), <0.001	0.757(0.681~0.842), <0.001	<0.001
Model 2	0.974(0.966~0.982), <0.001	Ref.	0.806(0.715~0.910), <0.001	0.771(0.689~0.863), <0.001	0.715(0.638~0.801), <0.001	<0.001
Model 3	0.974(0.966~0.982), <0.001	Ref.	0.806(0.714~0.909), <0.001	0.770(0.689~0.861), <0.001	0.713(0.636~0.799), <0.001	<0.001
Proximal colon cancer, HR (95% CI), P-value						
Model 1	0.985(0.972~0.999), 0.042	Ref.	0.951(0.769~1.176), 0.641	0.831(0.680~1.016), 0.071	0.823(0.676~1.002), 0.052	0.027
Model 2	0.981(0.967~0.996), 0.015	Ref.	0.953(0.765~1.188), 0.670	0.808(0.654~0.998), 0.048	0.789(0.639~0.975), 0.028	0.012
Model 3	0.981(0.966~0.996), 0.012	Ref.	0.951(0.763~1.185), 0.654	0.805(0.652~0.994), 0.044	0.784(0.634~0.968), 0.024	0.010
Distal colon cancer, HR (95% CI), P-value						
Model 1	0.973(0.959~0.987), <0.001	Ref.	0.663(0.532~0.826), <0.001	0.779(0.644~0.943), 0.010	0.694(0.573~0.841), <0.001	0.002
Model 2	0.966(0.951~0.981), <0.001	Ref.	0.652(0.521~0.816), <0.001	0.732(0.600~0.894), 0.002	0.646(0.526~0.793), <0.001	<0.001
Model 3	0.966(0.951~0.981), <0.001	Ref.	0.652(0.520~0.816), <0.001	0.732(0.600~0.893), 0.002	0.645(0.525~0.792), <0.001	<0.001
Rectal cancer, HR (95% CI), P-value						
Model 1	0.973(0.959~0.987), <0.001	Ref.	0.845(0.682~1.046), 0.122	0.773(0.633~0.943), 0.011	0.726(0.595~0.885), 0.002	0.001
Model 2	0.968(0.953~0.983), <0.001	Ref.	0.843(0.676~1.050), 0.127	0.739(0.600~0.911), 0.005	0.682(0.552~0.843), <0.001	<0.001
Model 3	0.968(0.953~0.983), <0.001	Ref.	0.842(0.676~1.049), 0.126	0.738(0.599~0.910), 0.004	0.681(0.550~0.841), <0.001	<0.001

The hazard ratio value of Q2-Q4 is based on the Q1 group as the reference. Model 1 was adjusted for age, race, educational attainment, and Townsend deprivation index. Model 2, building upon Model 1, adjusted for dietary energy intake; Model 3: In addition to Model 2, adjusted for plasma CRP concentration and NSAIDs medication usage. CRC, Colorectal Cancer; HR, Hazard Ratio.

TABLE 3 Association between oxidative balance score and colorectal cancer and its subsites stratified by sex.

	Continue	Quantile				P for trend
		Q1	Q2	Q3	Q4	
CRC in male, HR (95% CI), P-value						
Model 1	0.968(0.958~0.978), <0.001	Ref.	0.789(0.682~0.913), 0.001	0.726(0.634~0.832), <0.001	0.665(0.579~0.764), <0.001	<0.001
Model 2	0.967(0.956~0.978), <0.001	Ref.	0.794(0.683~0.924), 0.003	0.720(0.624~0.831), <0.001	0.662(0.570~0.769), <0.001	<0.001
Model 3	0.967(0.956~0.977), <0.001	Ref.	0.793(0.682~0.922), 0.003	0.718(0.622~0.829), <0.001	0.659(0.568~0.766), <0.001	<0.001
Proximal colon cancer in male, HR (95% CI), P-value						
Model 1	0.980(0.961~0.999), 0.040	Ref.	1.122(0.851~1.481), 0.414	0.885(0.676~1.159), 0.376	0.802(0.609~1.055), 0.114	0.038
Model 2	0.975(0.955~0.996), 0.020	Ref.	1.119(0.842~1.488), 0.439	0.847(0.638~1.124), 0.249	0.761(0.567~1.022), 0.070	0.020
Model 3	0.974(0.954~0.995), 0.016	Ref.	1.114(0.837~1.481), 0.459	0.841(0.634~1.116), 0.231	0.752(0.560~1.011), 0.059	0.016
Distal colon cancer in male, HR (95% CI), P-value						
Model 1	0.949(0.931~0.967), <0.001	Ref.	0.559(0.425~0.736), <0.001	0.631(0.497~0.800), <0.001	0.512(0.398~0.658), <0.001	<0.001
Model 2	0.947(0.928~0.966), <0.001	Ref.	0.560(0.423~0.741), <0.001	0.621(0.484~0.796), <0.001	0.507(0.387~0.664), <0.001	<0.001
Model 3	0.947(0.928~0.966), <0.001	Ref.	0.560(0.422~0.741), <0.001	0.620(0.483~0.796), <0.001	0.506(0.387~0.663), <0.001	<0.001
Rectal cancer in male, HR (95% CI), P-value						
Model 1	0.976(0.958~0.994), 0.008	Ref.	0.786(0.603~1.024), 0.075	0.759(0.595~0.969), 0.027	0.759(0.595~0.967), 0.026	0.028
Model 2	0.978(0.959~0.998), 0.029	Ref.	0.793(0.602~1.044), 0.099	0.774(0.599~1.002), 0.051	0.790(0.607~1.028), 0.079	0.085
Model 3	0.978(0.959~0.997), 0.026	Ref.	0.791(0.601~1.042), 0.096	0.772(0.597~0.999), 0.049	0.786(0.604~1.023), 0.074	0.079

(Continued)

TABLE 3 Continued

	Continue	Quantile				P for trend
		Q1	Q2	Q3	Q4	
CRC in female, HR (95% CI), P-value						
Model 1	1.000(0.988~1.012), 0.994	Ref.	0.863(0.709~1.051), 0.143	0.965(0.810~1.150), 0.688	0.981(0.828~1.163), 0.824	0.798
Model 2	0.995(0.983~1.008), 0.481	Ref.	0.877(0.716~1.073), 0.202	0.951(0.792~1.143), 0.594	0.940(0.784~1.128), 0.507	0.755
Model 3	0.995(0.983~1.008), 0.477	Ref.	0.876(0.716~1.073), 0.202	0.951(0.792~1.142), 0.593	0.940(0.783~1.128), 0.505	0.752
Proximal colon cancer in female, HR (95% CI), P-value						
Model 1	0.995(0.974~1.015), 0.605	Ref.	0.761(0.545~1.062), 0.108	0.780(0.577~1.052), 0.104	0.861(0.648~1.144), 0.301	0.436
Model 2	0.994(0.972~1.017), 0.602	Ref.	0.772(0.543~1.096), 0.148	0.795(0.578~1.092), 0.157	0.869(0.639~1.183), 0.373	0.521
Model 3	0.994(0.972~1.016), 0.592	Ref.	0.771(0.543~1.096), 0.147	0.794(0.578~1.092), 0.156	0.868(0.637~1.181), 0.366	0.513
Distal colon cancer in female, HR (95% CI), P-value						
Model 1	1.014(0.992~1.037), 0.215	Ref.	0.961(0.658~1.403), 0.837	1.212(0.871~1.687), 0.255	1.230(0.891~1.699), 0.209	0.105
Model 2	1.007(0.983~1.031), 0.585	Ref.	0.941(0.640~1.382), 0.755	1.126(0.799~1.587), 0.498	1.151(0.819~1.619), 0.418	0.276
Model 3	1.006(0.983~1.031), 0.597	Ref.	0.940(0.640~1.381), 0.752	1.125(0.798~1.585), 0.502	1.149(0.817~1.615), 0.426	0.282
Rectal cancer in female, HR (95% CI), P-value						
Model 1	0.976(0.952~1.000), 0.048	Ref.	0.996(0.692~1.435), 0.985	0.840(0.594~1.189), 0.325	0.753(0.533~1.064), 0.107	0.064
Model 2	0.970(0.945~0.995), 0.019	Ref.	1.010(0.698~1.460), 0.959	0.796(0.556~1.140), 0.212	0.691(0.479~0.996), 0.048	0.022
Model 3	0.970(0.945~0.995), 0.019	Ref.	1.010(0.698~1.460), 0.958	0.796(0.556~1.140), 0.213	0.692(0.480~0.997), 0.048	0.022

The hazard ratio value of Q2-Q4 is based on the Q1 group as the reference. Model 1 was adjusted for age, race, educational attainment, and Townsend deprivation index. Model 2, building upon Model 1, adjusted for dietary energy intake; Model 3: In addition to Model 2, adjusted for plasma CRP concentration and NSAIDs medication usage. CRC, Colorectal Cancer; HR, Hazard Ratio. CI, confidence interval.

3.5 Association between serum albumin, uric acid, neutrophils and CRC

As illustrated in Table 5, we classified the continuous levels of serum albumin, uric acid, and neutrophils into quartiles to explore the correlation between these three biomarkers and CRC. Compared to the Q1 group with respect to serum albumin concentration, the risks of CRC occurrence in the Q2, Q3, and Q4 groups decreased by 12.8% (OR 0.872 [0.784-0.971], p=0.012), 11.7% (OR 0.883, 95% CI [0.792-0.983], p=0.024), and 12.3% (OR 0.877, 95% CI [0.786-0.979], p=0.020). The continuous concentration of serum uric acid showed a significant positive correlation with CRC occurrence, with the risks of CRC in the Q3 and Q4 groups increasing by 22.0% (OR 1.220, 95% CI [1.095-1.360], p<0.001) and 37.9% (OR 1.379, 95% CI [1.239-1.535], p<0.001). The continuous levels of serum neutrophils showed a significant positive correlation with CRC occurrence, and the risk of CRC in the Q4 group was increased by 16.5% (OR 1.165, 95% CI [1.045-1.300], p=0.006). More details can be seen in Table 5.

3.6 Sensitivity analysis

Table 6 presents the correlation between lifestyle OBS and dietary OBS with CRC occurrence in the overall participants and different gender subgroups. In the overall participants, both dietary OBS and lifestyle OBS were significantly and consistently negatively correlated with CRC occurrence. In the gender-stratified subgroup

TABLE 4 Correlation of oxidative balance score with colorectal cancer complications.

Complications	Model 1		Model 2		Model 3	
	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
Secondary Metastasis in CRC	1.043(1.000~1.089)	0.050	1.050(1.002~1.100)	0.041	1.052(1.004~1.102)	0.035
Secondary Metastasis in Proximal CRC	0.975(0.948~1.003)	0.079	0.967(0.938~0.996)	0.025	0.967(0.938~0.996)	0.025
Abdominal pain in Proximal CRC	0.962(0.932~0.994)	0.019	0.955(0.922~0.988)	0.008	0.954(0.922~0.988)	0.008
Abdominal pain in distal CRC	1.043(1.000~1.089)	0.050	1.050(1.002~1.100)	0.041	1.052(1.004~1.102)	0.035

Model 1 was adjusted for age, race, educational attainment, and Townsend deprivation index. Model 2, building upon Model 1, adjusted for dietary energy intake; Model 3: In addition to Model 2, adjusted for plasma CRP concentration and NSAIDs medication usage. CRC, Colorectal Cancer; HR, Hazard Ratio; CI, Confidence Interval.

TABLE 5 Association between serum albumin, uric acid, neutrophils and colorectal cancer.

Mediators	OR 95%CI, P-value				
	Continue	Q1	Q2	Q3	Q4
Albumin	1.000(0.999~1.001), 0.206	Ref	0.872(0.784~0.971), 0.012	0.883(0.792~0.983), 0.024	0.877(0.786~0.979), 0.02
Uric acid	1.001(0.999~1.001), <0.001	Ref	1.003(0.896~1.122), 0.957	1.220(1.095~1.360), <0.001	1.379(1.239~1.535), <0.001
Neutrophils	1.001(0.999~1.001), 0.033	Ref	1.068(0.960~1.187), 0.227	1.045(0.938~1.163), 0.423	1.165(1.045~1.300), 0.006

OR, Odds Ratio; CI, Confidence Interval.

analysis, both dietary OBS and lifestyle OBS were significantly negatively correlated with male CRC occurrence. Additionally, we conducted a leave-one-out analysis, in which the contribution of each OBS component score was removed for reanalysis. The results indicated a significant negative correlation between OBS score and CRC occurrence in both the overall participants and male participants. However, no significant correlation was found between OBS score and CRC occurrence in female participants. More details can be seen in Table 7.

3.7 Mediation analysis

We conducted mediation analysis to further explore whether serum albumin, uric acid, and neutrophil levels mediate the relationship between OBS and CRC. In this model, OBS was considered as the independent variable, CRC as the dependent variable, and serum albumin, uric acid, and neutrophils as the mediators. As shown in Figure 2, serum albumin level mediated the association between OBS and CRC, explaining a total of 0.23% of the variance with a significant mediating effect (IE = -1.10E-06). As shown in Figure 3, serum uric acid level mediated the association between OBS and CRC, explaining a total of 19.20% of the variance with a significant mediating effect (IE = -7.30E-05). As shown in Figure 4, serum neutrophil level mediated the association between OBS and CRC, explaining a total of 2.20% of the variance with a significant mediating effect (IE = -4.52E-04).

TABLE 6 Stratified study on the contribution of lifestyle and dietary intake to oxidative balance score.

	Lifestyle OBS	Nutrients intake OBS
CRC, HR (95% CI), P-value		
Model 1	0.935(0.919~0.952)<0.001	0.990(0.981~0.999)0.021
Model 2	0.941(0.925~0.958)<0.001	0.982(0.972~0.991)<0.001
Model 3	0.940(0.924~0.957)<0.001	0.982(0.972~0.991)<0.001
CRC in female, HR (95% CI), P-value		
Model 1	0.971(0.944~0.997)0.032	1.007(0.994~1.021)0.279
Model 2	0.976(0.948~1.004)0.089	1.001(0.987~1.016)0.887
Model 3	0.976(0.948~1.004)0.088	1.001(0.987~1.016)0.891
CRC in male, HR (95% CI), P-value		
Model 1	0.924(0.903~0.944)<0.001	0.978(0.967~0.989)<0.001
Model 2	0.929(0.908~0.951)<0.001	0.976(0.964~0.989)<0.001
Model 3	0.927(0.906~0.949)<0.001	0.976(0.964~0.989)<0.001

Model 1 was adjusted for age, race, educational attainment, and Townsend deprivation index. Model 2, building upon Model 1, adjusted for dietary energy intake; Model 3: In addition to Model 2, adjusted for plasma CRP concentration and NSAIDs medication usage. CRC, Colorectal Cancer; HR, Hazard Ratio; CI, Confidence Interval.

4 Discussion

In this large prospective cohort study of the UK Biobank, we found a negative association between higher OBS and the development of CRC, including proximal colon cancer, distal colon cancer, and rectal cancer. In gender-stratified subgroup analysis, a higher dietary OBS and lifestyle OBS were significantly associated with a reduced risk of CRC in male participants, whereas this association was not significant in female participants. Furthermore, there was a positive correlation between the OBS and the risk of CRC metastasis and abdominal pain caused by distal colorectal cancer. On the other hand, there was a negative correlation between the dietary balance score and the risk of CRC metastasis and abdominal pain caused by proximal colorectal cancer. Additionally, we observed a significant correlation between decreased serum albumin levels, increased uric acid levels, increased neutrophil levels, and the occurrence of CRC. Furthermore, we discovered significant mediating effects of serum albumin, uric acid, and neutrophil levels on the relationship between OBS and the risk of CRC, explaining 0.23%, 19.20%, and 2.20% of the correlation, respectively. These findings provide novel insights into the potential biological mechanisms underlying the relationship between OBS indicators and CRC.

Previous studies have found a negative correlation between OBS and the occurrence of sporadic colorectal adenomas and colorectal cancer (10–14). The above studies were mostly conducted in the United States and the Middle East. Due to differences in diet, environment, and other factors, these findings cannot be generalized to the entire population. Our study constitutes the inaugural large-scale cohort study conducted utilizing the UK Biobank dataset, wherein data from 175,808 subjects were analyzed. It is currently one of the largest and most comprehensive biobanks in the world, making the conclusions drawn from this study more accurate and persuasive. According to research by the World Health Organization (WHO), controlling diet and lifestyle can reduce the incidence and mortality rates of cancer, including physical exercise, avoiding smoking, alcohol consumption, and adopting

TABLE 7 Leave-One-Out analysis of the oxidative balance score impact on colorectal cancer patients.

	CRC		CRC in female		CRC in male	
	HR(95%CI)	p	HR(95%CI)	p	HR(95%CI)	p
OBS without Carotene	0.971(0.962~0.980)	<0.001	0.995(0.981~1.009)	0.459	0.963(0.952~0.975)	<0.001
OBS without Dietary fiber	0.972(0.963~0.981)	<0.001	0.993(0.980~1.007)	0.349	0.965(0.954~0.977)	<0.001
OBS without Vitamin B6	0.971(0.963~0.980)	<0.001	0.995(0.982~1.009)	0.515	0.962(0.951~0.974)	<0.001
OBS without Total folate	0.971(0.962~0.980)	<0.001	0.995(0.981~1.009)	0.457	0.962(0.951~0.974)	<0.001
OBS without Vitamin B12	0.973(0.965~0.982)	<0.001	0.996(0.983~1.009)	0.561	0.965(0.954~0.976)	<0.001
OBS without Vitamin C	0.971(0.962~0.980)	<0.001	0.994(0.980~1.008)	0.389	0.963(0.952~0.975)	<0.001
OBS without Vitamin E	0.972(0.964~0.981)	<0.001	0.994(0.980~1.007)	0.353	0.964(0.953~0.976)	<0.001
OBS without Calcium	0.974(0.965~0.982)	<0.001	0.996(0.983~1.010)	0.567	0.965(0.954~0.977)	<0.001
OBS without Magnesium	0.973(0.965~0.982)	<0.001	0.995(0.981~1.009)	0.456	0.966(0.955~0.977)	<0.001
OBS without Total fat	0.972(0.964~0.981)	<0.001	0.995(0.982~1.008)	0.416	0.967(0.956~0.978)	<0.001
OBS without Iron	0.975(0.967~0.983)	<0.001	0.995(0.983~1.008)	0.453	0.969(0.959~0.979)	<0.001
OBS without Tea	0.972(0.964~0.980)	<0.001	0.995(0.982~1.009)	0.493	0.964(0.953~0.975)	<0.001
OBS without polyunsaturated fatty acids	0.974(0.965~0.982)	<0.001	0.996(0.983~1.009)	0.567	0.967(0.956~0.978)	<0.001
OBS without saturated fatty acids	0.973(0.965~0.981)	<0.001	0.994(0.982~1.008)	0.410	0.968(0.957~0.978)	<0.001
OBS without vegetable	0.971(0.963~0.979)	<0.001	0.994(0.981~1.008)	0.406	0.964(0.953~0.975)	<0.001
OBS without Retinol	0.975(0.966~0.983)	<0.001	0.995(0.982~1.008)	0.455	0.967(0.956~0.977)	<0.001
OBS without Vitamin D	0.972(0.964~0.980)	<0.001	0.995(0.982~1.008)	0.457	0.963(0.952~0.974)	<0.001
OBS without Meat	0.974(0.966~0.982)	<0.001	0.995(0.982~1.008)	0.472	0.967(0.956~0.978)	<0.001
OBS without Alcohol	0.974(0.965~0.982)	<0.001	0.994(0.981~1.008)	0.398	0.966(0.956~0.978)	<0.001
OBS without Body mass index	0.976(0.968~0.984)	<0.001	0.997(0.984~1.010)	0.659	0.969(0.958~0.980)	<0.001
OBS without Physical activity	0.977(0.969~0.985)	<0.001	0.998(0.986~1.010)	0.685	0.970(0.959~0.980)	<0.001
OBS without Smoking	0.978(0.969~0.986)	<0.001	0.998(0.985~1.012)	0.811	0.970(0.959~0.981)	<0.001

Adjusted for age, race, educational attainment, Townsend deprivation index, dietary energy intake, CRP concentration, dietary energy intake, and NSAIDs medication usage. CRC, Colorectal Cancer; HR, Hazard Ratio; CI, Confidence Interval.

an antioxidant-rich diet (15). Moreover, studies have found that a high intake of whole grains, fruits, vegetables, seafood, nuts, dairy products, and a low intake of red and processed meat can significantly reduce the risk of CRC (2, 3).

The mechanisms by which OBS reduces the risk of CRC can be considered from several aspects: First, OBS aims to assess the balance between oxidative stress and the antioxidant system in the body. Excessive free radicals can exert oxidative stress on the human body, leading to DNA damage, gene mutations, and other issues, thereby increasing the risk of developing cancer (7). When the body's antioxidant system is effective, it can eliminate these free radicals and prevent damage to DNA, thereby reducing the risk of cancer. Therefore, a higher OBS may reflect better antioxidant capacity, thus reducing the risk of CRC. In addition, oxidative stress can inhibit cell proliferation and promote cell apoptosis through the regulation of pathways such as the nuclear factor kappa B (NF-κB) pathway and mitogen-activated protein kinase (MAPK) pathway (16). In the case of higher OBS, it may promote apoptosis in damaged cells, preventing their development into cancer cells and thus reducing the risk of CRC. The proximal colon has a higher pH and relatively less microbial diversity. The increase in antioxidants and the reduction in oxidative stress may reduce the risk of proximal colon cancer. The distal colon and rectum are closer to the anus, where carcinogens tend to accumulate, thus an elevated OBS may reduce local oxidative stress and lessen the damage to the mucosa of the distal colon and rectum. Simultaneously, improving the health of the gut microbiome could reduce the risk of distal colorectal and rectal cancers (17, 18).

Previous studies have shown a close association between oxidative reactions and inflammatory responses. Maintaining a proper oxidative balance may help control inflammatory responses, thereby preventing inflammation-induced CRC. Our study identified certain circulating biomarkers associated with the risk of CRC, including serum albumin, uric acid, and neutrophils. Albumin is an essential carrier protein for maintaining colloid osmotic pressure. Nutritional deficiencies can reduce the synthesis of albumin in the liver, impairing its levels and functions in the body (19). Hence, low serum albumin levels may indicate compromised nutritional status in the body, often associated with malnutrition or chronic diseases. Low serum albumin levels may

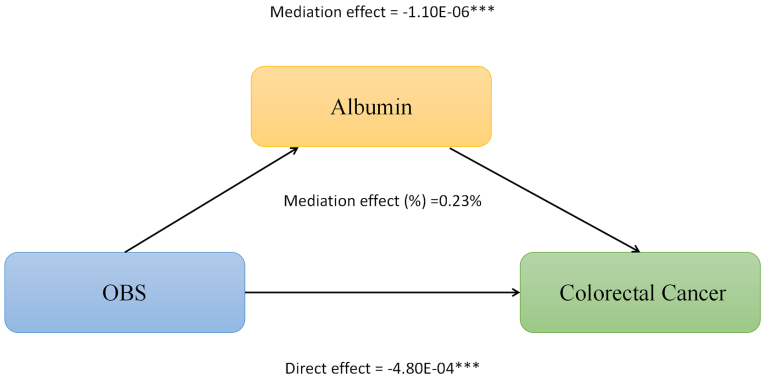

FIGURE 2
Serum albumin partially mediates the relationship between Oxidative Balance Score (OBS) and colorectal cancer (CRC). ***P<0.001.

lead to decreased immune function, thereby accelerating the growth and spread of cancer cells. Serum uric acid is associated with systemic inflammation and closely linked to defects in the insulin signaling pathway and pancreatic cell dysfunction (20, 21). The increased incidence of CRC due to high uric acid levels may be attributed to the interactions of inflammation, insulin resistance, impaired insulin secretion, and other mechanisms. Elevated levels of neutrophils and activated neutrophils may release inflammatory factors, proteases, and reactive oxygen species, leading to systemic inflammation. Moreover, overactivated neutrophils can also secrete factors that promote angiogenesis and tumor cell migration, directly promoting tumor formation and expansion (22–24).

In this study, dietary OBS and lifestyle OBS were significantly negatively correlated with the occurrence of CRC in males, but not in females. This result may be related to several factors: Firstly, males and females differ in physiological and hormonal levels. Considering the potential protective effect of estrogen on CRC risk (25), we further stratified by age to explore the difference in the impact of OBS on CRC between women aged above and below 50 years. Despite exhaustive stratified analyses, we did not observe a clear association between OBS and CRC risk in women groups aged above and below 50 years, suggesting that the OBS estimate does not take into account the possible estrogen-induced effects on antioxidant capacity. Although our data did not directly prove the interaction between estrogen levels and OBS, existing studies have shown that, estrogen has been shown to resist oxidative stress, inhibit inflammatory responses, prevent DNA damage, promote a more favorable gut microbiota, thereby reducing the risk of CRC (25–27). Therefore, the protective effect of estrogen may make females more resilient to oxidative stress compared to males, even if the OBS score is slightly higher, it may not significantly reduce the risk of CRC in females. Future studies should further investigate how estrogen modulates OBS and its impact on CRC risk. Besides, researches should be extended to larger and more diverse populations to validate these preliminary findings and explore other potential biomarkers. Additionally, there are differences in dietary habits and lifestyles between males and females (28, 29). In the comparison of general characteristics between men and women in this study, males exhibited significantly lower OBS scores than females. Studies also have shown that compared to males, females are more likely to choose a healthy diet in their daily lives, including vegetables, fruits, nuts, etc., which can be considered as powerful sources of antioxidants (30). On the other hand, males may be more exposed to environmental factors that induce oxidative stress, such as excessive alcohol consumption, while females have relatively less

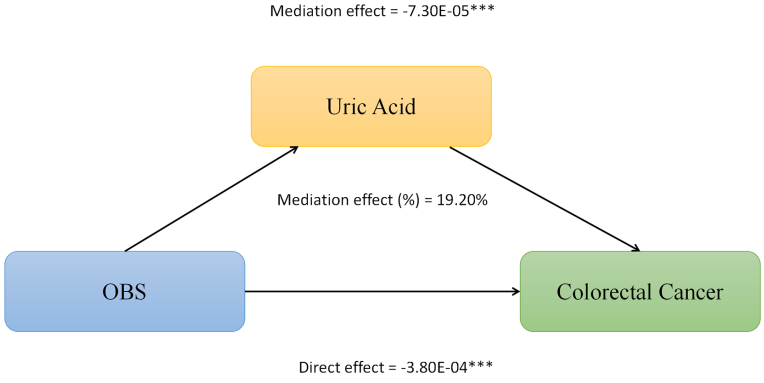

FIGURE 3
Serum uric acid partially mediates the relationship between Oxidative Balance Score (OBS) and colorectal cancer (CRC). ***P<0.001.

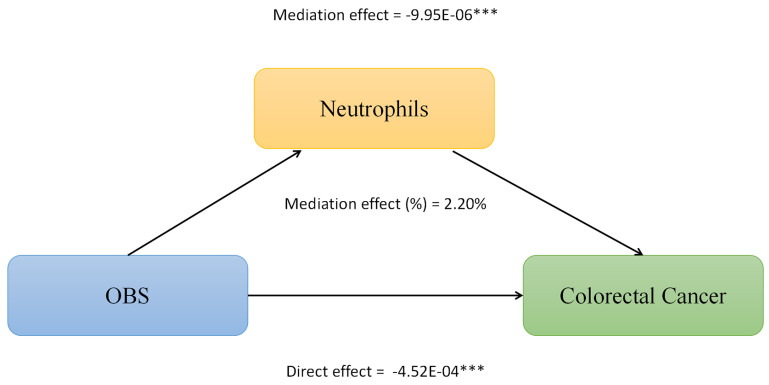

FIGURE 4
Neutrophils levels partially mediates the relationship between Oxidative Balance Score (OBS) and colorectal cancer (CRC). ***P<0.001.

exposure (31, 32). Therefore, the healthy diet and lifestyle are more likely to reduce the incidence of CRC in males.

Our study also underscores the importance of considering racial and ethnic diversity in the relationship between dietary and lifestyle-related oxidative balance and CRC risk. In the European ancestry population, the significant inverse association between OBS and CRC risk may suggest that genetic or environmental factors interact differently with oxidative balance compared to other racial groups. Previous research has demonstrated that dietary patterns significantly affect oxidative stress and inflammation, with notable differences across ethnicities that may influence the efficacy of OBS in reducing CRC risk (33). Furthermore, genetic variations among races may alter the biological response to oxidative stress (34). Future studies should aim to confirm these findings in a larger and more diverse population sample and further explore the potential mechanisms by which racial differences affect the impact of OBS on CRC risk.

Our study found that an elevated OBS reduces the risk of secondary metastasis and abdominal pain caused by proximal colon cancer, yet it elevates the risk of secondary metastasis and abdominal pain induced by distal colon cancer. This may be due to the fact that differences in genetic and molecular characteristics of proximal colon cancer result in antioxidants having a more positive effect, such as reducing DNA damage caused by oxidative stress, thereby lowering the risk of metastasis and symptoms induced by the tumor (35). Some antioxidants may promote the proliferation and metastasis of existing tumor cells at high doses, especially when the tumor cells have adapted to a high oxidative stress environment (36). The balance between antioxidants and pro-oxidants may affect different sites of colon cancer differently, possibly related to the biological characteristics of the tumor, the mechanism of action of antioxidants in different tissues, and individual genetic differences (37). Therefore, future studies will need more in-depth, large-scale prospective clinical trials and biological researches to further explore the complex interactions between biomarkers such as the OBS score and tumor cells and tissue.

Our study has several limitations. First, the design of a cross-sectional study limits causal inferences between OBS and CRC, and further prospective cohort studies are needed to investigate the causal relationship between OBS and CRC. Second, our study collected various components of OBS using self-reported FFQ questionnaires, which may result in partial recall bias. Furthermore, our study lacks exploration of epigenetic and environmental factors that may interact with OBS indicators. Future research should include more studies on genetics and metabolomics to further investigate the antioxidant mechanisms underlying the relationship between OBS and CRC occurrence.

Despite these limitations, our study still has several notable strengths. Firstly, this study provides robust evidence for the utility of OBS indicators in the risk assessment of CRC and its subsites in a large population cohort in the UK. These findings may guide clinical decisions related to CRC and further research is needed to validate the predictive performance of dietary and lifestyle OBS in different cohorts and environments. Moreover, the participant data in this study were obtained from the large-scale UK Biobank database, a long-term, population-based prospective study, which has a massive sample size that effectively minimizes sampling bias. Furthermore, our study highlights the potential role of oxidative stress in CRC and its subsites. OBS, as a composite measure based on oxidative balance, can more accurately reflect the oxidative and antioxidant status in the body compared to individual biomarkers. Our findings provide several guiding directions for future research. Firstly, exploring the impact of genetic and metabolic factors, demographics, lifestyle, and other factors on the association between OBS and CRC and its subsites. This will help to further develop and evaluate strategies for CRC screening and prevention based on OBS. Furthermore, employing more comprehensive and specific methods such as mediation analysis and machine learning to further characterize the dose-response relationship between OBS and CRC would be beneficial for clinicians to explore optimizing antioxidant status through diet, supplements, and lifestyle for improved prognosis of CRC patients.

5 Conclusion

In summary, this large prospective cohort study demonstrates a significant inverse association between OBS and the risk of CRC and

its subsites, with a stronger association observed in the male CRC population. Serum albumin, uric acid, and neutrophils partially mediate the association between OBS and CRC. Our study results emphasize that antioxidant-rich diet and lifestyle may serve as targets for the prevention and treatment of CRC. Further research is needed to elucidate the complex interactions between diet, lifestyle, relevant biomarkers, and the occurrence of CRC.

Data availability statement

The dataset from the UK Biobank can be accessed by researchers through the application process at http://www.ukbiobank.ac.uk/regis. The Application ID for this dataset is 84347.

Ethics statement

The studies involving humans were approved by The North West Multi-center Research Ethics Committee, Manchester, U.K. (REC reference for UK Biobank 11/NW/0382). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

YC: Data curation, Formal analysis, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. FL: Data curation, Project administration, Software, Visualization, Writing – original draft. ZhiW: Investigation, Validation, Writing – original draft. QZ: Investigation, Validation, Writing – original draft. ZDW: Investigation, Validation, Writing – original draft. XH: Investigation, Validation, Writing – original draft. ZhaW: Investigation, Validation, Writing – original draft. CY: Investigation, Validation, Writing – original draft. YL: Investigation, Validation, Writing – original draft. SC: Investigation, Validation, Writing – original draft. HL: Investigation, Validation, Writing – original draft. SH: Investigation, Validation, Writing – original draft. YQL: Resources, Supervision, Writing – review & editing. TT: Resources, Supervision, Writing – review & 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1397512/full#supplementary-material

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Antioxidant insights: investigating the protective role of oxidative balance in inflammatory bowel disease

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Background: Limited studies have investigated the relationship between systemic oxidative stress and inflammatory bowel disease (IBD). The purpose of this study was to explore the relationship between oxidative balance score (OBS) and IBD.

Methods: We included 175,808 participants from the UK Biobank database from 2006 to 2010. OBS scores were calculated based on 22 lifestyle and dietary factors. Multiple variable Cox proportional regression models, as well as gender stratification and subgroup analysis, were utilized to investigate the relationship between OBS and IBD.

Results: There is a significant negative correlation between OBS and the occurrence of IBD, ulcerative colitis (UC), and Crohn's disease (CD). Additionally, OBS is significantly negatively correlated with intestinal obstruction in CD patients. Gender stratified analysis suggest a significant correlation between OBS and CD in female patients, particularly pronounced in those under 60 years old. Sensitivity analysis indicates a significant negative correlation between lifestyle-related OBS and diet-related OBS with the occurrence of CD in females, diet-related OBS is negatively correlated with CD in males.

Conclusion: OBS showed a significant negative correlation with IBD, especially in female CD patients. This study underscores the importance of antioxidant diet and lifestyle, which may provide a greater advantage for female CD patients.

KEYWORDS

inflammatory bowel disease, oxidative balance score, UK biobank, Crohn disease, ulcerative colitis

1 Introduction

Inflammatory bowel disease (IBD) is a group of lifelong autoimmune diseases, including Crohn's disease (CD) and ulcerative colitis (UC). By 2019, there were 4.9 million cases of IBD worldwide (1). IBD affects the most productive period of a person's adulthood, not only imposing a significant economic burden, but also impacting the psychological and social well-being of patients (2).

In recent years, researchers in the field of IBD have shown increasing interest in the role of oxidative stress. Early studies have demonstrated that patients with active IBD exhibit significantly elevated levels of oxidative stress, which is closely associated with the severity of intestinal inflammation. Oxidative stress not only increases the production of inflammatory mediators but also damages the intestinal barrier function. This impairment facilitates the penetration of pathogens and toxins, exacerbating the pathological process of IBD (3, 4). Numerous studies have demonstrated that elevated levels of reactive oxygen species (ROS) or decreased levels of antioxidants can promote the development of IBD (5, 6). In IBD patients, the intestinal mucosa is typically infiltrated by a large number of neutrophils, macrophages, and lymphocytes, and these inflammatory cells produce ROS and reactive nitrogen species (RONS) that can attack the cell membrane lipids, proteins, and DNA of intestinal mucosal cells, thereby exacerbating the progression of IBD (7–9).

RONS are produced as a result of self-inflammatory responses, as well as from exogenous sources such as diet, lifestyle, and environmental toxins (10). Due to the complexity of the human body and the multiple mechanisms involved in the development of autoimmune diseases, the impact of a single factor on the oxidative/antioxidant system in IBD is limited. Therefore, we have introduced a comprehensive index, the Oxidative Balance Score (OBS). The OBS is used to assess the overall exposure to pro-oxidants and antioxidants in an individual (11). A higher OBS indicates a more significant cumulative effect of antioxidant exposure. Recent studies have found an association between OBS and various diseases, such as non-alcoholic fatty liver (12) and metabolic syndrome (13). However, there is currently no research exploring the relationship between OBS and the occurrence of IBD and its related complications.

Considering the potential role of oxidative stress in IBD, the aim of this study is to investigate the association between OBS and the risk of developing IBD and its complications in a large follow-up study utilizing the UK BioBank, including patients with IBD, UC, and CD. Our findings will provide new evidence for the role of oxidative balance in IBD and its related complications. The study aims to deepen the understanding of the molecular mechanisms mediated by ROS and to further explore potential treatment strategies for IBD driven by oxidative stress.

2 Methods

2.1 Study population

The UK BioBank database is the result of a large prospective cohort study that recruited more than 500,000 male and female participants aged 37 to 69 from various locations in the UK between 2006 and 2010 (14). The baseline data for the study were collected at this time, followed by four large-scale follow-ups and additional data collection. The database includes participants' genetic information and blood samples, as well as data on lifestyle and environmental exposures, and tracks their health and medical records for several decades after. The National Health and Social Care Information Governance Board in England and Wales, as well as the Community Health Index Advisory Group in Scotland, approved the access to patient records for recruitment purposes. The UK BioBank obtained ethical approval from the Northwest Multi-center Research Ethics Committee. All participants signed informed consent using touchscreen signature devices. We excluded all the participants with incomplete questionnaire data (n=291,434) during recruitment, and further excluded participants with missing data on CRP levels (n=33,930), dietary habits (n=881), education level (n=12,736), and other covariates. In the end, a total of 175,808 participants were included in this study. Multiple methods were employed for handling missing data, such as mean imputation, multiple imputation, and exclusion. The exclusion criteria were established to ensure the quality and reliability of the study sample and to mitigate potential selection bias. More details are seen in Figure 1.

2.2 Inflammatory bowel disease

All diseases, medication prescriptions, and participant deaths were recorded in the database for utilization and management. Participants were followed up from recruitment until the diagnosis of crohn's disease or ulcerative colitis, withdrawal from the study, date of death, or the last follow-up date, whichever came first. Assessment of inflammatory bowel disease was determined based on the International Classification of Diseases, 10th Revision (ICD-10) codes for primary hospital admission diagnoses (CD events were defined as "K50" and UC events were defined as "K51").

2.3 Oxidative balance score

The calculation plan for OBS was based on prior empirical information (13, 15). This study included data on 5 lifestyle factors (smoking, alcohol consumption, tea consumption, body mass index, and physical activity) and 17 nutrient intake variables (total fat, iron, calcium, magnesium, vitamin B6, vitamin B12, vitamin C, vitamin D, vitamin E, total folate, carotenoids, dietary fiber, retinol, meat intake, vegetable intake, polyunsaturated fatty acids, saturated fatty acids), comprising 14 antioxidants and 8 prooxidants. The total OBS score was the sum of values (0, 1, 2) assigned based on given tertile groupings, with a value of 0 for lower tertiles and a value of 2 for higher tertiles, for both antioxidants and prooxidants. Ultimately, a score of 0 represented low levels of antioxidants, while a score of 2 indicated high levels. Thus, a higher OBS score indicated a more significant exposure to antioxidants. Smoking, alcohol consumption, and meat intake are handled differently to avoid issues of zero inflation in the scores. The scores for these variables are defined as 2 for never intake, and

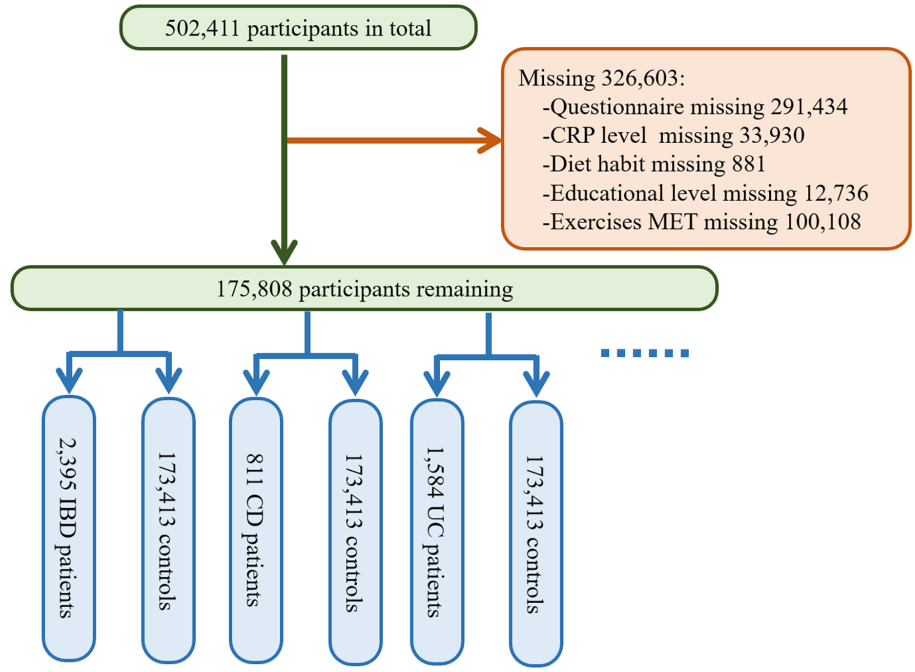

FIGURE 1
Sample Selection and Analysis Flowchart.

assigned values of 0 or 1 based on binarization according to the median. Vegetable intake was calculated by summing the intake of raw vegetables and cooked vegetables. Meat intake was calculated by summing the intake of beef, lamb, and pork. Physical activity was calculated by summing the weekly MET (Metabolic Equivalent Task) minutes of light, moderate, and vigorous physical activity. Due to the differential oxidative stress between genders, values were assigned separately for males and females based on prior empirical evidence (15). The data on these lifestyle factors and nutrient intake were obtained through a baseline completed Food frequency questionnaire (FFQ) by the participants. The questionnaire inquired about the intake frequency of fresh fruits, dried fruits, raw vegetables, cooked vegetables, oily fish, non-oily fish, processed meat, poultry, beef, lamb, pork, cheese, bread, cereal, tea, and drinking water. These intake frequencies were subsequently used to estimate the intake frequencies of essential nutrients such as energy, carbohydrates, and fats. MET data were derived based on the International Physical Activity Questionnaire (IPAQ) guidelines. For more specific details, please refer to Table 1.

2.4 Covariate assessment

In the current study, the following variables were considered as covariates: age, race (European ancestry, Asian ancestry excluding Chinese, African ancestry, Chinese ancestry, other ethnicities, mixed ancestry), educational performance, and Townsend deprivation index. Ethnic background may influence individual genetics, environmental exposures, and lifestyle choices, thereby impacting the occurrence of IBD. Educational level typically reflects an individual's cognitive abilities and social resources, which may be related to the oxidative balance score and the risk of IBD onset investigated in our study. Additionally, to account for individual differences in overall intake and spontaneous oxidative stress, we also included dietary energy intake and high-sensitivity CRP as covariates. Age, race and education level information were assessed through self-reported questionnaires. The acquisition of educational attainment indicators was divided into two subdomains: one related to children and adolescents, and one related to adult skills. These two subdomains aimed to reflect the "flow" and "stock" of educational disadvantage within a region, respectively. Dietary energy intake was calculated using data obtained from the aforementioned dietary questionnaire. The serum high-sensitivity CRP data were obtained through highly sensitive immunoassay testing conducted by skilled researchers using the Beckman Coulter AU5800 system.

2.5 Statistical analysis

Continuous variables were expressed as mean (± standard deviation) in baseline descriptions, and differences were described using the Krusal-Wallis test. Categorical variables were described as sample counts (percentages), and statistical differences were described using Pearson's Chi-squared test. In this study, we categorized the continuous OBS scores based on quartiles (Q1: <25th percentile, Q2: 25-50th percentile, Q3: 50-75th percentile, Q4: 75-100th percentile). We used Cox proportional hazards model to explore the association between OBS and the occurrence of IBD, including its two subtypes (UC and CD). The baseline of this study

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TABLE 1 Components of the oxidative balance score.

OBS components	Unit	Property	Category	Female			Male		
				0	1	2	0	1	2
Total fat	g/day	P	Nutrient Intake	>82.15	56.19~82.15	<56.19	>94.27	64.54~94.27	<64.54
Iron	mg/day	P	Nutrient Intake	>14.59	10.77~14.59	<10.77	>16.26	12~16.26	<12
polyunsaturated fatty acids	g/day	P	Nutrient Intake	>15.5	8.89~15.5	<8.89	>17.59	10.08~17.59	<10.08
saturated fatty acids	g/day	P	Nutrient Intake	>31.32	20.66~31.32	<20.66	>36.36	23.91~36.36	<23.91
Smoking	package years	P	Lifestyle	>17	>0 & <17	=0	>21.5	>0 &<21.5	=0
Alcohol	g/day	P	Lifestyle	>19.52	>0 & <19.52	=0	>31.32	>0 &<31.32	=0
Meat	times/week	P	Lifestyle	>3	>0 & <3	=0	>3	>0 &<3	=0
BMI	kg/m2	P	Lifestyle	>28.30	24.34~28.30	<24.34	>29.00	25.79~29.00	<25.79
Carotene	ug/day	A	Nutrient Intake	<1370.22	1370.22~4147.08	>4147.08	<1014.02	1014.02~3374.59	>3374.59
Dietary fiber	g/day	A	Nutrient Intake	<12.78	12.78~18.49	>18.49	<12.99	12.99~19.13	>19.13
Vitamin B6	mg/day	A	Nutrient Intake	<1.72	1.72~2.37	>2.37	<1.87	1.87~2.58	>2.58
Total folate	ug/day	A	Nutrient Intake	<233.04	233.04~326.78	>326.78	<253.96	253.96~354.33	>354.33
Vitamin B12	mg/day	A	Nutrient Intake	<3.49	3.49~6.48	>6.48	<3.77	3.77~6.76	>6.76
Vitamin C	mg/day	A	Nutrient Intake	<91.45	91.45~178.59	>178.59	<82.29	82.29~168.05	>168.05
Vitamin E	mg/day	A	Nutrient Intake	<6.67	6.67~10.46	>10.46	<6.42	6.42~10.37	>10.37
Calcium	mg/day	A	Nutrient Intake	<749.84	749.84~1049.64	>1049.64	<803.12	803.12~1122.7	>1122.7
Magnesium	mg/day	A	Nutrient Intake	<280.59	280.59~365.89	>365.89	<308.35	308.35~404.4	>404.4
Retinol	ug/day	A	Nutrient Intake	<198.99	198.99~359.63	>359.63	<222.51	222.51~407.21	>407.21
Vitamin D	ug/day	A	Nutrient Intake	<1.03	1.03~2.45	>2.45	<1.2	1.2~2.76	>2.76
Physical activity	MET minutes/week	A	Lifestyle	<1062	1062~2685.17	>2685.17	<1108.5	1108.5~2897.17	>2897.17
Tea	cups/day	A	Lifestyle	<2	2~4	>4	<2	2~4	>4
vegetable	tablespoons/day	A	Lifestyle	<4	4~6	>6	<3	3~5	>5

OBS, Oxidative Balance Score; A, antioxidant; P, pro-oxidant; MET, metabolic equivalent of task; BMI, body mass index.

was set at the time when participants completed the dietary questionnaire, with the censoring date being June 1, 2022, and the date of questionnaire obtained from field number "105010". The follow-up period was calculated by subtracting the date of the questionnaire from either the date of onset or the censor date, varying for each participant. The distribution of follow-up times for all participants is shown in Supplementary Figure 1. In this study, three models were used to investigate the association between OBS and IBD, UC, and CD. Model 1 was adjusted for age, race, educational attainment indicators, and the Townsend Deprivation Index. These basic variables help control for confounding effects of fundamental socioeconomic and demographic characteristics. Model 2 further adjusted for dietary energy intake. This adjustment considers the direct impact that dietary factors might have on the development of OBS and IBD, allowing for a more accurate assessment of the pure relationship between OBS and IBD by eliminating the interference of dietary habits. Model 3 additionally adjusted for serum CRP level based on Model 2. As an inflammatory marker, adjusting for CRP helps evaluate the role of chronic inflammation in the relationship between OBS and IBD, thereby providing insights into how inflammation-mediated physiological mechanisms might influence the association between these two conditions. Furthermore, we conducted subgroup analyses based on gender and age groups to further investigate the impact of OBS on IBD, UC, and CD in different gender. Additionally, using the aforementioned three models, we further investigated the correlation between OBS scores and complications associated with IBD (abscess, obstruction, perforation, fistula, megacolon, Clostridium difficile infection, sepsis). Subgroup analyses of OBS and IBD-related complications were conducted among different gender. Finally, sensitivity analyses were performed by reanalyzing the data based on different types of OBS contributions and using the leave-one-out method to examine the impact of removing a specific score contribution.

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2.6 Data acquisition and statistical software

The UK Biobank data were obtained through our application with ID 84347. Data were queried and downloaded using the web tool at https://biobank.ndph.ox.ac.uk/ukb/. Fields of interest were queried in the "Search" section. Specific data downloading and decompression software were downloaded from the "Download" section. Subsequently, fields were downloaded based on their respective field ID. The data were then imported into R (version 4.2.3) for data cleaning and analysis.

3 Results

3.1 Baseline characteristics

The baseline characteristics of the participants stratified into quartiles of OBS are presented in Table 2. Females accounted for a higher proportion (53%), and the mean ages of the subjects was 55.9 ± 8.0 years. Compared with participants in the lowest quartile of OBS, those in the highest quartile were more likely to be (1) females, (2) of European descent, (3) older, (4) have higher dietary energy intake, and (5) have lower Townsend Deprivation Index, BMI, educational attainment indicators, and CRP levels. They also had lower rates of emergency hospitalization and mortality. There is no statistically significant difference in the rate of IBD-related surgeries among OBS groups. A total of 2,395 IBD patients were included in this study. Of these, there were 811 CD patients and 1,584 UC patients.

3.2 Association between OBS and inflammatory bowel disease and its subtypes

According to the Cox proportional hazards model, OBS was found to be associated with the occurrence of IBD. In model 1, for every one-point increase in the continuous OBS score, the incidence of IBD decreases by 1.0% (HR 0.990, 95%CI 0.982-0.998, p=0.016). Compared to the lowest quartile of OBS (Q1 group), the risk of developing IBD was reduced by 15.0% in the highest quartile (Q4 group) (HR 0.850, 95%CI 0.758-0.952, p=0.005). In model 2, for every one-point increase in the continuous OBS score, the incidence of IBD decreases by 1.5% (HR 0.985, 95%CI 0.977-0.993, p<0.001). Compared to the Q1 group, the risk of developing IBD was reduced by 13.0% in the Q3 group (HR

TABLE 2 Baseline characteristics of participants according to quartiles of oxidative balance score.

	Overall N = 175,808	Quantile OBS groups				
		Q1 [4,19) N = 40,379	Q2 [19,22) N = 35,004	Q3 [22,26) N = 49,209	Q4 [26,41) N = 51,216	p-value
OBS score, Mean (SD)	22.5 (5.0)	15.8 (2.1)	20.0 (0.8)	23.5 (1.1)	28.5 (2.2)	<0.001
IBD, n (%)	2,395 (1.4%)	602 (1.5%)	491 (1.4%)	659 (1.3%)	643 (1.3%)	0.019
CD, n (%)	811 (0.5%)	214 (0.5%)	164 (0.5%)	247 (0.5%)	186 (0.4%)	<0.001
UC, n (%)	1,584 (0.9%)	388 (1.0%)	327 (0.9%)	412 (0.8%)	457 (0.9%)	0.2
Gender, n (%)						<0.001
Female	94,044 (53%)	20,971 (52%)	18,392 (53%)	26,215 (53%)	28,466 (56%)	
Male	81,764 (47%)	19,408 (48%)	16,612 (47%)	22,994 (47%)	22,750 (44%)	
TDI score, Mean (SD)	-1.6 (2.9)	-1.3 (3.0)	-1.6 (2.9)	-1.7 (2.8)	-1.8 (2.8)	<0.001
missing	221	68	39	63	51	
Education score, Mean (SD)	11.7 (13.8)	13.3 (15.1)	11.7 (13.8)	11.2 (13.2)	11.0 (13.0)	<0.001
missing	4,482	1,108	871	1,199	1,304	
Ethnicity, n (%)						<0.001
European	167,952 (96%)	38,319 (95%)	33,409 (96%)	47,162 (96%)	49,062 (96%)	
Mixed-race	1,054 (0.6%)	299 (0.7%)	197 (0.6%)	282 (0.6%)	276 (0.5%)	
Asian	2,488 (1.4%)	583 (1.4%)	526 (1.5%)	699 (1.4%)	680 (1.3%)	
African	2,082 (1.2%)	706 (1.8%)	410 (1.2%)	467 (1.0%)	499 (1.0%)	
Chinese	495 (0.3%)	90 (0.2%)	106 (0.3%)	142 (0.3%)	157 (0.3%)	
Others	1,239 (0.7%)	268 (0.7%)	253 (0.7%)	333 (0.7%)	385 (0.8%)	
Missing	498	114	103	124	157	

(Continued)

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TABLE 2 Continued

	Overall N = 175,808	Quantile OBS groups				
		Q1 [4,19) N = 40,379	Q2 [19,22) N = 35,004	Q3 [22,26) N = 49,209	Q4 [26,41) N = 51,216	p-value
BMI, Mean (SD), Kg/m^2	26.9 (4.6)	28.3 (4.9)	27.1 (4.6)	26.6 (4.4)	25.8 (4.2)	<0.001
Age, Mean (SD)	55.9 (8.0)	55.0 (8.0)	55.7 (8.0)	56.0 (7.9)	56.6 (7.9)	<0.001
CRP level, Mean (SD), mg/L	2.3 (4.0)	2.7 (4.3)	2.3 (3.9)	2.2 (4.0)	1.9 (3.7)	<0.001
missing	10,192	2,377	2,009	2,868	2,938	
Daily energy intake, Mean (SD), KJ	8,862.8 (3,036.6)	7,731.7 (2,481.1)	8,300.7 (2,718.7)	9,135.5 (2,997.0)	9,876.7 (3,285.3)	<0.001
Emergency admission, n (%)	83,369 (47%)	20,236 (50%)	16,449 (47%)	22,933 (47%)	23,751 (46%)	<0.001
IBD-related operation, n (%)	376 (0.2%)	100 (0.2%)	68 (0.2%)	99 (0.2%)	109 (0.2%)	0.4
Death, n (%)	8,670 (4.9%)	2,337 (5.8%)	1,670 (4.8%)	2,283 (4.6%)	2,380 (4.6%)	<0.001

OBS, Oxidative Balance Score; TDI, Thomson Deprivation Index;CD, Crohn's disease; UC, Ulcerative colitis; IBD, inflammatory bowel disease; SD, standard deviation; BMI, body mass index; CRP, C-reactive protein; Education refered to the age of Highest Level of Education

0.870, 95%CI 0.776-0.975, p=0.017) and 21.1% in the Q4 group (HR 0.789, 95%CI 0.702-0.888, p<0.001). In model 3, for every one-point increase in the continuous OBS score, the incidence of IBD decreases by 1.2% (HR 0.988, 95%CI 0.980-0.997, p=0.009). Compared to the Q1 group, the risk of developing IBD was reduced by 17.4% in the Q4 group (HR 0.826, 95%CI 0.732-0.933, p=0.002).

OBS is associated with the occurrence of UC. In model 1, no significant correlation was found between continuous and four-category OBS and UC (All P-values > 0.05). However, in model 2, compared to the Q1 group, the risk of developing UC was reduced by 17.3% in the Q3 group (HR 0.827, 95%CI 0.716-0.954, p=0.009) and 14.6% in the Q4 group (HR 0.850, 95%CI 0.740-0.986, p=0.031). In model 3, compared to the Q1 group, the risk of developing UC was reduced by 17.1% in the Q3 group (HR 0.829, 95%CI 0.715-0.960, p=0.012) and 14.1% in the Q4 group (HR 0.859, 95%CI 0.741-0.995, p=0.043).

OBS is associated with the occurrence of CD. In model 1, for every one-point increase in the continuous OBS score, the incidence of CD decreases by 2.3% (HR 0.977, 95%CI 0.963-0.990, p=0.001). Compared to the Q1 group, the risk of developing CD was reduced by 29.8% in the Q4 group (HR 0.702, 95%CI 0.574-0.859, p=0.001). In model 2, for every one-point increase in the continuous OBS score, the incidence of CD decreases by 2.7% (HR 0.973, 95%CI 0.959-0.987, p<0.001). Compared to the Q1 group, the risk of developing CD was reduced by 33.3% in the Q4 group (HR 0.667, 95%CI 0.541-0.821, p<0.001). In model 3, for every one-point increase in the continuous OBS score, the incidence of CD decreases by 2.9% (HR 0.981, 95%CI 0.966-0.996, p=0.011). Compared to the Q1 group, the risk of developing CD was reduced by 24.8% in the Q4 group (HR 0.752, 95%CI 0.606-0.932, p=0.009). More specific details can be seen in Figure 2.

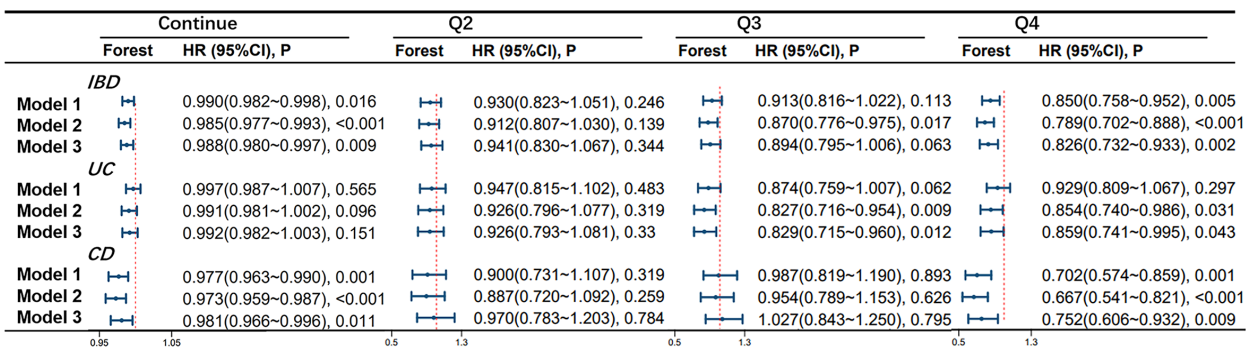

FIGURE 2
Association of Oxidative Balance Score with Inflammatory Bowel Disease and Subtypes. The hazard ratio value of Q2-Q4 is based on the Q1 group as the reference. Model 1 was adjusted for age, race, educational performance indicators, and Townsend deprivation index. Model 2, building upon Model 1, was further adjusted for dietary energy intake. Model 3, extending the adjustments made in Model 2, additionally accounted for plasma CRP concentration.IBD, inflammatory bowel disease; UC, ulcerative colitis; CD, Crohn's disease; HR, hazard ratio.

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3.3 Association between OBS and UC and CD stratified by gender

OBS is significantly associated with the occurrence of UC in the female population. In model 1, compared to the Q1 group, the risk of developing UC was reduced by 26.3% in the Q2 group (HR 0.737, 95%CI 0.587-0.925, p=0.009), 19.9% in the Q3 group (HR 0.801, 95% CI 0.654-0.981, p=0.032), and 19.4% in the Q4 group (HR 0.806, 95% CI 0.661-0.983, p=0.034) in the female population. In model 2, compared to the Q1 group, the risk of developing UC was reduced by 27.4% in the Q2 group (HR 0.726, 95%CI 0.578-0.912, p=0.006), 23.1% in the Q3 group (HR 0.769, 95%CI 0.626-0.944, p=0.012), and 24.2% in the Q4 group (HR 0.758, 95%CI 0.618-0.931, p=0.008) in the female population. In model 3, compared to the Q1 group, the risk of developing UC was reduced by 25.3% in the Q2 group (HR 0.747, 95%CI 0.592-0.944, p=0.015), 21.4% in the Q3 group (HR 0.786, 95%CI 0.636-0.971, p=0.025), and 22.5% in the Q4 group (HR 0.775, 95%CI 0.627-0.958, p=0.019) in the female population.

There is a significant correlation between OBS and CD occurrence in the female population. In model 1, for every one-point increase in the continuous OBS score, the incidence of CD decreases by 3.4% (HR 0.966, 95%CI 0.948-0.985, p<0.001). Compared to the Q1 group, the risk of CD occurrence was reduced by 42.3% in the Q4 group (HR 0.577, 95%CI 0.434-0.767, p<0.001). In model 2, for every one-point increase in the continuous OBS score, the incidence of CD decreases by 3.6% (HR 0.964, 95%CI 0.945-0.983, p<0.001). Compared to the Q1 group, the risk of CD occurrence was reduced by 44.0% in the Q4 group (HR 0.560, 95%CI 0.418-0.751, p<0.001). In model 3, for every one-point increase in the continuous OBS score, the incidence of CD decreases by 2.8% (HR 0.972, 95%CI 0.952-0.992, p=0.006). Compared to the Q1 group, the risk of CD occurrence was reduced by 37.0% in the Q4 group (HR 0.630, 95%CI 0.466-0.851, p=0.003). However, no significant correlation between OBS and CD was observed in the male population (see Figure 3).

3.4 Differences of dietary balance scores in age-stratified female CD patients

In female CD patients, using 60 years as a cutoff point, in the age < 60 group, continuous OBS is significantly negatively correlated with CD occurrence in models 1, 2, and 3, with adjusted HRs of 0.972 (95%CI 0.955-0.988), 0.968 (95%CI 0.951-0.986) and 0.975 (95%CI 0.957-0.993), respectively. However, in the age > 60 group of female patients, no significant correlation was found between continuous OBS and the occurrence of CD. More specific details can be seen in Figure 4.

3.5 Correlation of dietary balance score with intestinal obstruction complications in CD patients

There is a correlation between OBS and intestinal obstruction in CD patients. In model 1, compared to the Q1 group, the risk of obstruction occurrence was reduced by 56.6% in the Q2 group (HR 0.434, 95%CI 0.222-0.849, p=0.015). In model 2, compared to the Q1 group, the risk of obstruction occurrence was reduced by 58.5% in the Q2 group (HR 0.415, 95%CI 0.212-0.814, p=0.010). In model 3, compared to the Q1 group, the risk of obstruction occurrence was reduced by 59.7% in the Q2 group (HR 0.403, 95%CI 0.198-0.822, p=0.012). For more details, please refer to Figure 5.

3.6 Sensitivity analysis

Figure 6 shows the correlation of lifestyle OBS and dietary OBS with IBD, UC, and CD in the overall participants and different gender subgroups. There is a significant and stable negative correlation between dietary OBS and the occurrence of IBD, UC, and CD in patients. In gender-stratified subgroup analysis, both

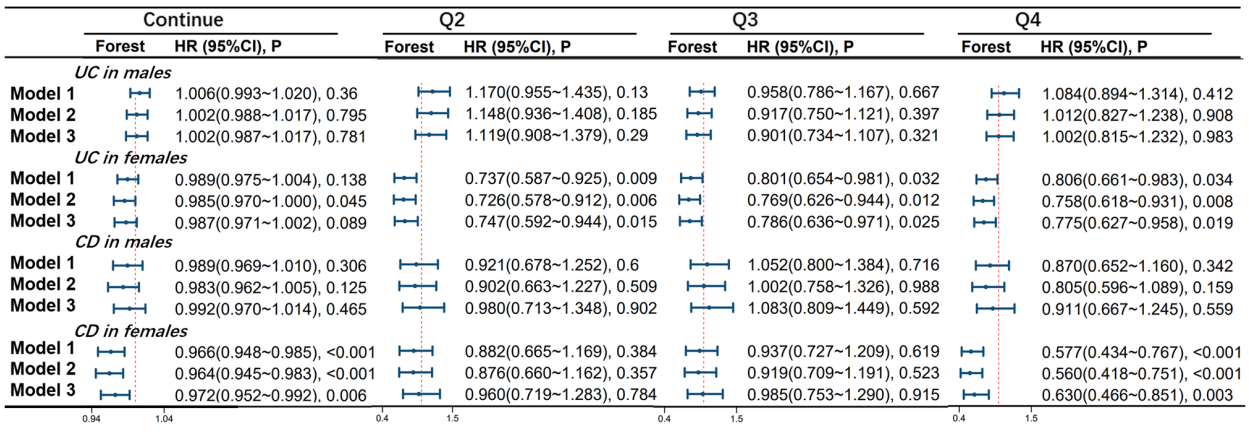

FIGURE 3
Association of Oxidative Balance Score with Stratified Age Groups in UC and CD. The hazard ratio value of Q2-Q4 is based on the Q1 group as the reference. Model 1 was adjusted for age, race, educational performance indicators, and Townsend deprivation index. Model 2, building upon Model 1, was further adjusted for dietary energy intake. Model 3, extending the adjustments made in Model 2, additionally accounted for plasma CRP concentration.IBD, inflammatory bowel disease; UC, ulcerative colitis; CD, Crohn's disease; HR, hazard ratio; CI, confidence interval.

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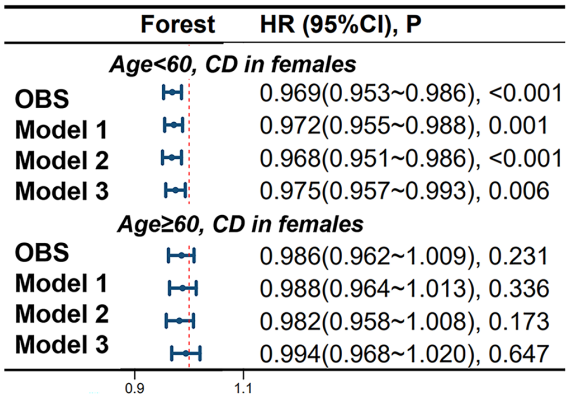

FIGURE 4
Differential Impact of Oxidative Balance Score in Age-Stratified Female CD Patients. Model 1 was adjusted for age, race, educational performance indicators, and Townsend deprivation index. Model 2, building upon Model 1, was further adjusted for dietary energy intake. Model 3, extending the adjustments made in Model 2, additionally accounted for plasma CRP concentration. CD, Crohn's disease; HR, hazard ratio; CI, confidence interval; OBS, Oxidative Balance Score.

diet-related OBS and lifestyle-related OBS are negatively correlated with the occurrence of CD in females. In addition, we also conducted a leave-one-out analysis, which means re-analyzing the data after removing the contribution of a certain component score of OBS, and concluded that there is still a significant negative correlation between OBS and CD occurrence in CD patients and female participants. However, no significant correlation between OBS and CD occurrence was observed in the male population. For more details, please refer to Figure 7.

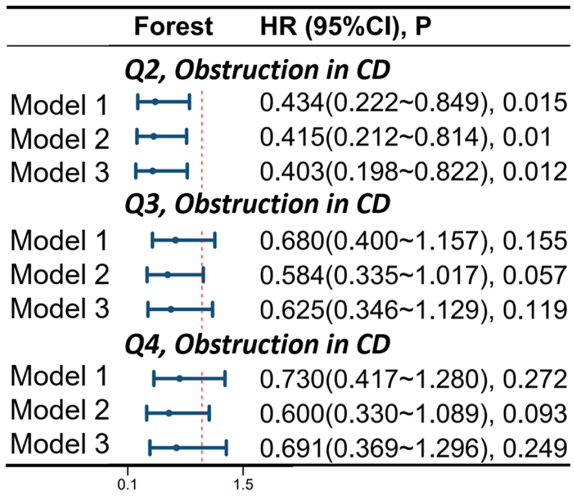

FIGURE 5
Correlation of Oxidative Balance Score with Complications of Intestinal Obstruction in CD Patients. The hazard ratio value of Q2-Q4 is based on the Q1 group as the reference. Model 1 was adjusted for age, race, educational performance indicators, and Townsend deprivation index. Model 2, building upon Model 1, was further adjusted for dietary energy intake. Model 3, extending the adjustments made in Model 2, additionally accounted for plasma CRP concentration. CD, Crohn's disease; HR, hazard ratio; CI, confidence interval.

4 Discussion

In recent years, a growing body of epidemiological evidence has suggested that oxidative stress may play a major role in the pathogenesis of IBD (3). In this large-scale prospective study involving adult participants in the United Kingdom, we observed a negative correlation between overall OBS and dietary OBS with the incidence of IBD, UC, and CD, with a more pronounced effect in female participants. Additionally, we found a negative correlation between higher OBS and the occurrence of intestinal obstruction in CD patients. These findings remained robust even after adjusting for various potential confounders and conducting additional analyses stratified by gender and subgroups.

To the best of our knowledge, this is the first study to investigate the association between OBS and inflammatory bowel disease. While previous studies have shown that substances such as vitamin D and reactive oxygen species can reduce the risk and severity of IBD by alleviating oxidative stress, most of these studies have only used one or a few circulating biomarkers reflecting oxidative stress, such as 8-OHdG and isoprostanes (16, 17). Some studies on chronic diseases have suggested that the combination of multiple factors may have a stronger association with disease risk than considering individual nutrients alone (18, 19). This study, on the other hand, thoroughly assessed the oxidative stress-related exposure by calculating the overall intake of various pro-oxidants and antioxidants using the OBS derived from dietary patterns and lifestyle choices, providing a comprehensive assessment of the overall oxidative balance in the body. Moreover, our study extensively investigated the association between OBS and the risk and severity of inflammatory bowel disease and its complications through long-term follow-up, providing a solid foundation for further optimization of treatment and care strategies for IBD patients. In this study, differences in baseline characteristics that across quartiles of OBS reflect variations in physiological and biochemical statuses among patient groups with different OBS quartiles. By analyzing inter-group differences, OBS may be

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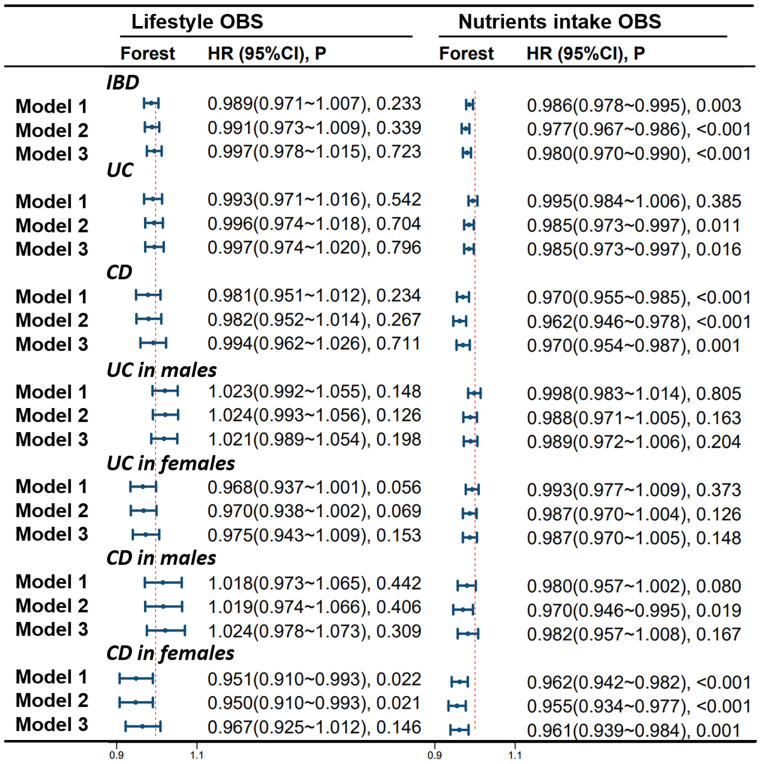

FIGURE 6
Stratified Study on the Contribution of Lifestyle and Dietary Intake to OBS. Model 1 was adjusted for age, race, educational performance indicators, and Townsend deprivation index. Model 2, building upon Model 1, was further adjusted for dietary energy intake. Model 3, extending the adjustments made in Model 2, additionally accounted for plasma CRP concentration. IBD, inflammatory bowel disease; UC, ulcerative colitis; CD, Crohn's disease; HR, hazard ratio; CI, confidence interval; OBS, Oxidative Balance Score.

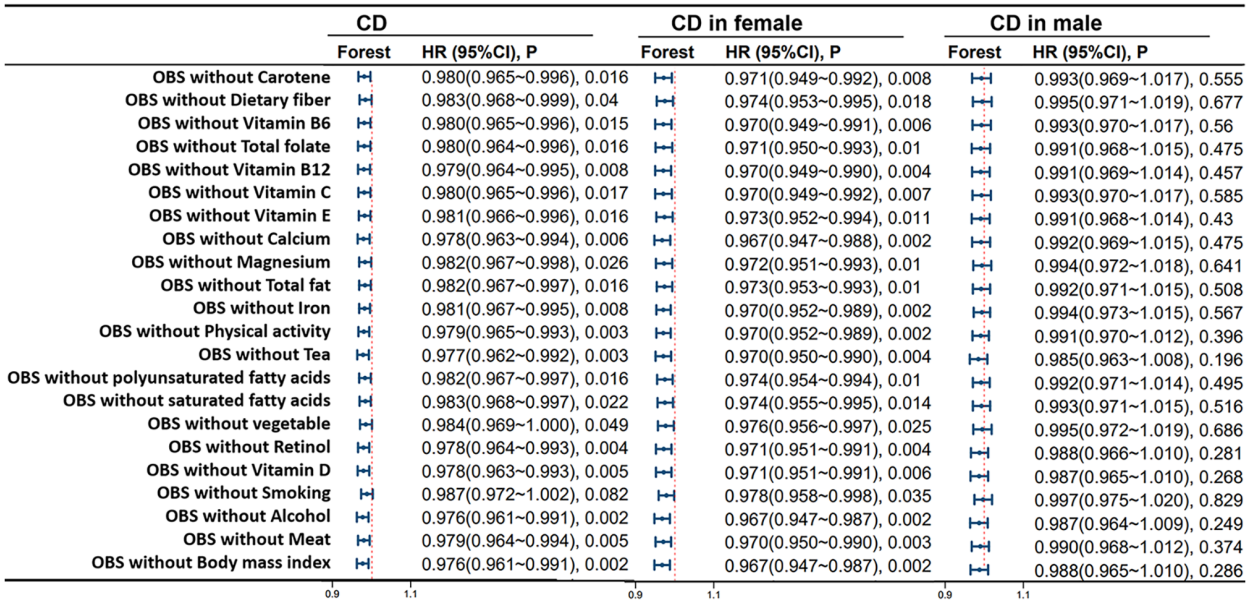

FIGURE 7
Leave-One-Out Analysis for OBS in CD Patients. Model 3 was adjusted for age, race, educational performance indicators, Townsend deprivation index, dietary energy intake and plasma CRP concentration. CD, Crohn's disease; HR, hazard ratio; CI, confidence interval; OBS, Oxidative Balance Score.

closely associated with physiological processes such as inflammatory status and immune function. The group with higher OBS quartiles may exhibit elevated levels of oxidative stress and inflammatory markers, suggesting a potential correlation between oxidative stress and the onset and progression of IBD.

Oxidative stress plays a crucial and multifaceted role in the complex pathogenesis of inflammatory bowel disease. The production of ROS in the gastrointestinal tract may lead to further oxidative stress damage to intestinal cells. The gastrointestinal tract is one of the main sources of ROS in the body, and the intake of substances and intestinal pathogens can promote inflammation, leading to oxidative stress (OS) (20, 21). Intestinal epithelial cells, along with inflammatory cells, participate in the development of inflammation by producing ROS/RNS (reactive nitrogen species) under inflammatory conditions. One of the characteristics of the pathogenesis of IBD is the imbalance between helper T cells and regulatory T cells. The excessive production of Th1-cell-mediated inflammatory cytokines such as IL-12, IL-17, and IL-23 leads to the development of CD (22, 23). These pro-inflammatory cytokines, in combination with other inflammatory cells, promote the production of ROS (20). These ROS, mainly including superoxide and hydroxyl radicals, together with pro-inflammatory cytokines, cause cellular damage and tissue destruction (24). In the intestinal mucosa, ROS can disrupt tight junctions between cells, increase intestinal permeability, ultimately leading to the disruption of the intestinal epithelial barrier and further inflammation (25). Moreover, although low to moderate levels of ROS have a role in resisting pathogen invasion and promoting tissue repair, excessive ROS produced during intense inflammatory responses in IBD can have detrimental effects on the intestinal mucosa. Therefore, based on the above mechanisms, it is evident that ROS are involved in the pathogenesis of IBD. However, antioxidants and compounds can mitigate the multifaceted effects of oxidative stress through various mechanisms. For example, both enzymatic and non-enzymatic antioxidants have a balancing effect on oxidative stress (4). These antioxidants directly form an antioxidant network (26), collectively combating lipid, protein, and DNA damage caused by ROS (9). Furthermore, various antioxidants such as vitamin C, vitamin E, carotenoids, etc., can neutralize ROS, preventing downstream oxidative reactions, aiding in the prevention of damage and shedding of intestinal epithelial cells, and maintaining the integrity of intestinal mucosal epithelium (27). Additionally, substances such as butyrate can reduce oxidative stress in colonic epithelial cells by inhibiting the activity of the NF-kB pathway, alleviating intestinal inflammation and fibrotic reactions, preserving the vitality of intestinal tissues, thereby reducing the risk of intestinal obstruction (28).

Our results also showed a gender dimorphism in the effect of OBS on the development of IBD. In female patients, particularly in the >60 age group, OBS scores significantly reduced the risk of CD incidence. The decline in estrogen levels during menopause may contribute to this outcome. In female patients, higher OBS was associated with a reduced risk of IBD, UC, and CD, with a more pronounced effect in CD patients. Hormonal differences may be responsible for these findings. In a cross-sectional study by Rolston et al. (29), it was reported that female patients with inflammatory bowel disease experienced worsening of symptoms during the menstrual cycle, with a more pronounced effect in CD patients compared to UC patients. The antioxidant effects of estrogen may be mediated through increased expression of antioxidant enzymes (AOEs) (30, 31). Changes in estrogen levels can also affect the expression of inflammatory factors (4). Estrogen can also maintain intestinal epithelial barrier function by affecting epithelial and mucus formation, epithelial permeability, and chloride ion secretion (32). Interestingly, activation of G-protein coupled estrogen receptor (GPER) can reduce mortality in animal models of CD, improve macroscopic and microscopic scores, and decrease CRP levels, which is achieved through the regulation of extracellular signal-regulated kinase (ERK) signaling pathway, involvement in signal transduction and immune response gene expression, and modulation of certain RNA expression (33). In addition, the A-ring phenolic hydroxyl on estrogen is a determining factor in free radical clearance and possesses antioxidant properties independent of activation of estrogen receptors and hormone function (34). Furthermore, sensitivity analysis suggests that both dietary OBS and lifestyle OBS significantly reduce the risk of CD incidence in females, possibly indicating a close association with gender-specific differences in dietary behaviors. Women tend to be more concerned about managing their physique and are prone to engaging in behaviors associated with eating disorders, such as dieting. This may result in inadequate nutritional intake for women, increasing their risk of various types of diseases compared to men (35). Moreover, the absence of a correlation between OBS and IBD among males may not only be attributed to the differences in hormone levels and dietary habits between genders but could also be closely linked to variations in gene expression that influence inflammation and oxidative stress. Research indicates that females are more likely to demonstrate genetic susceptibility to X-chromosome-associated IBD compared to males (36). Our findings provide additional evidence for the gender-specific etiology of IBD and highlight the different effects of OBS on the development of IBD according to gender, thus reinforcing the relevance of OBS in the assessment of IBD susceptibility.

There are several obvious strengths in this study. First, this study provides preliminary evidence that higher antioxidant exposure assessed by OBS is associated with reduced risk of IBD and its complications. Our study is based on OBS, a comprehensive measure of oxidative balance, rather than single biomarkers, to capture the complex relationships between various factors and to investigate the related outcomes comprehensively. Second, the participant data in this study are from a large database, the UK Biobank, which has a large sample size and can effectively avoid sampling bias. Third, this study controlled for many confounding factors, including demographic characteristics, diet quality, dietary energy, blood biomarkers, etc., to maximize the internal validity of the study. Fourth, this study conducted stratified analysis by gender and found that OBS has a more protective effect on female IBD patients, especially those with CD, making the results more targeted. Therefore, these findings have additional public health implications for better managing antioxidant-rich diets and

lifestyles to more effectively prevent CD. However, this study also has several limitations. First, due to limitations of the database, the number of cases for some IBD complications is less than 30, which may introduce bias in the conclusions by not meeting the assumptions of regression analysis. Second, under the assumption that all pro-oxidants and antioxidants are linearly related to oxidative stress, this study overlooks the threshold effects of antioxidants (37, 38). However, it has been demonstrated that certain antioxidants may exhibit pro-oxidative activity at high doses or under certain conditions, thus the association of specific antioxidants may be uncertain. Third, this study was unable to accurately measure and collect the long-term dynamic changes of OBS components, including lifestyle components, and the observational design of the study limits causal inferences to some extent.

Despite these limitations, our study is the first to report a significant association between OBS and IBD in a large, population-based prospective study with long-term follow-up. This provides clinicians with an accurate and feasible risk prediction factor which can be used for assessing individual disease risk, especially for those at risk of IBD, as an intervention measure. This indicator can also be used for the development of screening and preventive strategies. Additionally, in view of the gender differences in the impact of OBS on female IBD patients, clinicians should pay more attention to the treatment and care of female IBD patients. Understanding the influence of estrogen on antioxidant activity can assist doctors in better managing the disease in female IBD patients. Finally, a comprehensive treatment strategy, including controlling pro-oxidants and increasing antioxidant intake, may be helpful in improving treatment outcomes for IBD patients. Clinicians should consider exploring the optimization of antioxidant status through diet, supplements, and lifestyle to improve the prognosis and prevention of IBD, thereby bringing more significant clinical benefits to IBD patients.

5 Conclusion

We found a significant negative correlation between OBS and IBD, particularly in female CD patients. This study emphasizes the crucial role of antioxidant diet and lifestyle, potentially conferring greater advantages for female CD patients.

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 The North West Multi-center Research Ethics Committee, Manchester, U.K. (REC reference for UK Biobank 11/NW/0382). 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

FL: Writing – original draft, Visualization, Project administration, Formal analysis, Data curation. YC: Writing – original draft, Software, Methodology, Investigation, Data curation. ZhaW: Writing – original draft, Validation, Investigation. ZhiW: Writing – original draft, Validation, Investigation. QZ: Writing – original draft, Validation, Investigation. XH: Writing – original draft, Validation, Investigation. ZX: Writing – original draft, Validation, Investigation. CY: Writing – original draft, Investigation. YueL: Writing – original draft, Investigation. SC: Writing – original draft, Investigation. HL: Writing – original draft, Investigation. SH: Writing – original draft, Investigation. YuqL: Writing – review & editing, Supervision, Resources, Conceptualization. TT: Writing – review & editing, Supervision, Resources.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1386142/full#supplementary-material

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Novel perspectives on autophagy-oxidative stress-inflammation axis in the orchestration of adipogenesis

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Adipose tissue, an indispensable organ, fulfils the pivotal role of energy storage and metabolism and is instrumental in maintaining the dynamic equilibrium of energy and health of the organism. Adipocyte hypertrophy and adipocyte hyperplasia (adipogenesis) are the two primary mechanisms of fat deposition. Mature adipocytes are obtained by differentiating mesenchymal stem cells into preadipocytes and redifferentiation. However, the mechanisms orchestrating adipogenesis remain unclear. Autophagy, an alternative cell death pathway that sustains intracellular energy homeostasis through the degradation of cellular components, is implicated in regulating adipogenesis. Furthermore, adipose tissue functions as an endocrine organ, producing various cytokines, and certain inflammatory factors, in turn, modulate autophagy and adipogenesis. Additionally, autophagy influences intracellular redox homeostasis by regulating reactive oxygen species, which play pivotal roles in adipogenesis. There is a growing interest in exploring the involvement of autophagy, inflammation, and oxidative stress in adipogenesis. The present manuscript reviews the impact of autophagy, oxidative stress, and inflammation on the regulation of adipogenesis

and, for the first time, discusses their interactions during adipogenesis. An integrated analysis of the role of autophagy, inflammation and oxidative stress will contribute to elucidating the mechanisms of adipogenesis and expediting the exploration of molecular targets for treating obesity-related metabolic disorders.

KEYWORDS

adipogenesis, autophagy, inflammation, oxidative stress, immune responses

1 Introduction

It is widely acknowledged that adipose tissue performs a dual function, serving as a substantial energy storage organ, regulating the accumulation and release of energy, and as a pivotal endocrine organ contributing to systemic metabolism and preserving the organism's homeostasis (1). Adipose tissue in mammals can be classified into two principal types: white adipose tissue (WAT) and brown adipose tissue (BAT). Moreover, white adipocytes can transdifferentiate into beige adipocytes, which exhibit similar morphological and functional characteristics to brown adipocytes, in response to stimuli such as exercise, cold exposure and other factors. The function of WAT is to store and release energy. Unlike WAT, BAT predominantly engages in thermogenesis, achieving efficient energy consumption via mitochondrial uncoupling. Beige adipose tissue exhibits a hybrid phenotype intermediate between WAT and BAT and can be stimulated to burn energy in response to β-adrenergic activation. Adipocyte hypertrophy (increased fat cell size) and adipocyte hyperplasia (increased fat cell number) constitute the two fundamental mechanisms underpinning the storage capacity of adipose tissue. Adipogenesis, the process whereby precursor adipocytes differentiate into a diverse array of adipocytes, accumulate nutrients, and ultimately mature into adipocytes, is the primary reason for hyperplasia. Adipogenesis is a complex sequence of cellular differentiation processes, including the differentiation of mesenchymal stem cells into mesodermal precursor cells or germinal ganglion precursor cells, the differentiation of mesodermal precursor cells or myogenic ganglion precursor cells into various preadipocytes, and the differentiation of diverse preadipocytes into mature adipocytes (2). Moreover, adipose tissue consists of many types of cells, including endothelial cells, blood cells, fibroblasts, pericytes, precursor cells, adipocytes, macrophages and other immune cells (3, 4). Therefore, many scholars believe that fat is an important immune organ (5).

Adipose tissue is an active metabolic organ that necessitates multiple mechanisms to uphold its function and health. Autophagy represents one of these mechanisms. Autophagy is the process by which vesicles phagocytose intracellular material and fuse it with lysosomes to form autophagic lysosomes that degrade their contents, fulfilling the cell's metabolic needs and renewing certain organelles. Most research has focused on the influence of autophagy on lipid metabolism, while fewer studies have examined the involvement of autophagy in adipogenesis. In recent years, evidence suggests that autophagy is a key regulator of WAT and BAT adipogenesis, and dysregulation of autophagy impairs fat metabolism (6). Many autophagy-related genes, autophagy-related pathways, autophagy-associated transcription factors and regulatory proteins participate in adipogenesis. Therefore, the modulation of autophagy presents a promising therapeutic avenue for treating obesity and its associated complications.

Adipocytes and immune cells within adipose tissue contribute to cytokine secretion, which plays indispensable roles in adipogenesis. These secreted cytokines can influence appetite regulation, energy metabolism, and immunological interactions (7). Immune cells within adipose tissue regulate various cytokines, and certain inflammatory factors, in turn, modulate adipogenesis (8). However, different inflammatory cytokines have different effects on adipogenesis. For example, TNF-α is often thought to function as an inhibitor of adipogenesis (8). However, the expression of IL6 and IL6R is positively correlated with adipogenesis differentiation of MSCs (9). The mechanisms by which inflammatory factors regulate adipogenesis and how dysregulated adipogenesis contributes to inflammation-mediated complications associated with metabolic disorders are currently research hot spots. Moreover, oxidative stress also significantly impacts adipogenesis and adipocyte hypertrophy (10). Elevated peroxide levels within adipocytes can trigger mitochondrial membrane damage, resulting in ATP blockade within the adipocyte and the generation of reactive oxygen species (ROS). These events precipitate morphological and functional alterations in adipocytes (11). Recent findings suggested that in addition to hormonal stimulation, ROS and free radicals might also modulate preadipocyte differentiation (12, 13). This review summarises the most recent investigations concerning the regulatory mechanisms of adipogenesis involving autophagy, oxidative stress, and inflammation. Most of the current studies have focused on the mechanism of single factor regulating adipogenesis, but there is still a need to incorporate multifactorial analyses. For example, autophagy has been playing a protective role by regulating inflammatory responses and ameliorating stress-induced cellular damage, thereby maintaining cellular homeostasis during stress. Therefore, we speculate that the interaction between

oxidative stress, inflammatory factors and autophagy plays an important role in adipogenesis. Here, we discussed the emerging intersections between autophagy, oxidative stress, and inflammation in the context of adipogenesis for the first time. A comprehensive understanding of their roles will facilitate the identification of molecular targets for managing metabolic disorders associated with obesity.

2 The significance of autophagy in adipogenesis

Autophagy is an alternative cell death pathway within cells that involves the breakdown of damaged cellular components through targeted transport to specialized structures called lysosomes. This degradation process produces energy and replenishes metabolic reservoirs within the cell. Autophagy regulates cellular energy metabolism, maintains intracellular environmental balance, and supports metabolic renewal. Eukaryotic cells maintain the homeostasis of energy metabolism through the equilibrium of nutrient synthesis and degradation, encompassing proteins and lipids. The primary role of basal autophagy resides in the degradation of long-lived intracellular proteins and the elimination of damaged or senescent organelles. This intricate process plays a critical function in the preservation of cellular homeostasis. Within adipose tissue, there mainly exist macroautophagy/autophagy, mitophagy and lipophagy. These different forms of autophagy function in collaboration with one another during various physiological states. Their primary function is to govern the quantity, production, structure, and functioning of preadipocytes/adipocytes, thereby influencing adipogenesis. Most research has focused on the influence of autophagy on lipid metabolism, while fewer studies have examined the involvement of autophagy in adipogenesis. This section discusses the recent advancements in understanding the regulation of adipogenesis by autophagy and infers promising targets for manipulating adipogenesis.

2.1 Autophagy-related genes are key factors influencing adipogenesis

2.1.1 ATG5 and ATG7

The discovery of autophagy-related genes (ATG) in yeast in 1990 provided powerful genetic and molecular tools for studying autophagy. More than 35 ATGs have been identified in yeast to date, and the 15 core ATGs required for starvation-induced autophagy are similarly highly conserved in mammals (14). ATGs play essential roles in all stages of autophagy. To understand whether autophagy affects other processes, we must first consider whether ATGs regulate these processes. Studies have shown the involvement of ATG in adipogenesis has been recognized. The initiation of embryonic fibroblast differentiation in mice lacking the ATG5 gene resulted in impaired adipogenesis and a substantial decline in the abundance of white adipocytes (15). This noteworthy finding underscores the involvement of ATG5 in the regular process of adipocyte differentiation, thus implicating a key role in autophagy during adipogenesis. AF4/FMR2 family member 4 (AFF4) is the scaffold protein of the super elongation complex, a class of transcriptional regulators involved in regulating cell development and differentiation (16). Studies have found that it plays an important role in differentiating various cells (17, 18). A recent investigation has demonstrated that the absence of AFF4 in hMSCs and 3T3-L1 preadipocytes inhibits cellular adipogenic differentiation. Over-expression of ATG5 and ATG16L1 rescues the impaired adipogenesis observed in cells where AFF4 is knocked down. Further studies have revealed that AFF4 can directly bind to autophagy-related proteins ATG5 and ATG16L1, thereby impacting adipogenesis (19). Similarly, mice with a deficiency in the ATG7 gene exhibited a remarkable suppression of autophagy, leading to diminished adipocyte differentiation and decreased accumulation of lipid droplets. The mutant mice contained only 20% of the mass of WAT found in WT mice. Crucially, the mutant mice did not appear to have any defects in other organs, and the weight of the lungs, kidneys, liver, heart, and brain did not differ significantly from that of the WT mice. Intriguingly, half of the mutant white adipocytes demonstrated multilocularity. Furthermore, the multilocularity observed in these mutant cells is not attributable to the increase in lipolysis, given that the mutant adipocytes displayed an unaltered basal lipolysis rate and even a reduction in hormone-stimulated lipolysis. These mice were found to be resistant to obesity resulting from a high-fat diet and demonstrated heightened insulin sensitivity (20, 21). This result is likely attributable to either increased energy expenditure, diminished energy utilization and storage efficiency, or both. Notably, the mutant mice exhibit pronounced hyperactivity. In the adipose-specific ATG7-KO models, there is a significant decline in plasma leptin concentrations, which is plausibly a consequence of the substantially decreased WAT mass. Furthermore, deleting the ATG7 gene in adipocytes decreased serum levels of free fatty acids and improved high-fat diet-induced steatosis, liver inflammation, and fibrosis (22). In general, mice models of lipoatrophy exhibit hyperlipidemia and insulin resistance due to lipid storage defects and abnormal lipid deposition in the liver and muscle. Intriguingly, mice with adipose-specific ATG7 knockout exhibit euglycemia and heightened insulin sensitivity. It is postulated that the morphological alterations within the mutant white adipocytes have precipitated fascinating functional modifications. In alignment with an elevated mitochondrial content, these mutant adipocytes display augmented rates of fatty acid β-oxidation. Furthermore, these mutant adipocytes demonstrate diminished rates of hormone-induced lipolysis. These alterations in lipid metabolism within the mutant WAT may have instigated the observed changes in lipid homeostasis within the mutant mice, characterized by reduced plasma levels of free fatty acids (FFA) during feeding and expedited rates of FFA reduction following insulin stimulation. Consequently, these systemic alterations in FFA homeostasis likely contribute to the amplified insulin sensitivity. In addition, ATG7 deficiency also affects brown fat differentiation (23). Recently, a study illustrated that the suppression of FTO (m6A demethylase) expression led to a decrease in the expression of ATG7 and ATG5, thereby hindering the formation of

autophagosomes and impeding autophagy and adipogenesis. The FTO knockout mice exhibited a reduction in ATG5 and ATG7-dependent autophagy and a substantial decrease in white adipose tissue compared to the wild-type mice (24). These findings suggested that the knockdown of both ATG7 and ATG5 genes resulted in a decline in the differentiation of lipogenic cells and the accumulation of lipids.

2.1.2 Other ATGs

The autophagy initiation phase begins with activation of the ATG1 (the homologous gene in mammals is ULK1) complex. There are five ULK1 homologs in the human genome: ULK1, ULK2, ULK3, ULK4 and STK36. As an evolutionarily conserved autophagy regulation-associated serine/threonine protein kinase, ULK1 is responsible for ATG5- and ATG7-independent autophagy (25). The RNAi results revealed that ULK1 is not required for adipogenesis, but ULK2 is indispensable for adipogenesis in 3T3-L1 cells (26). The expression levels of PPARγ and C/EBPα, positive regulators of adipogenesis, were dramatically decreased in cells with knockdown of ULK2, whereas no such decrease was observed in ULK1-knockdown cells. Although ULK1 is dispensable for adipogenesis, inhibition of ULK1 would enhance insulin-responsive glucose uptake and lipid accumulation. On the other hand, ULK1 inhibition leads to increased oxidative stress, thereby potentially exacerbating the development of insulin resistance in adipocytes. ULK1 orchestrates lipid metabolism and facilitates glucose uptake in adipocytes, distinguishing itself from other ATGs. Notably, Y-box binding protein 1 (YBX1), an RNA binding protein, not only can promote ULK1/2-mediated autophagy during white adipogenesis but also can induce brown adipogenesis via PINK1/PRKN-mediated mitophagy (27, 28). With the deepening of research, many biochemical experiments have discovered that more ATGs may be involved in adipogenesis. Chromatin immunoprecipitation (ChIP) results suggested that C/EBPβ binding sites were found on the promoters of five autophagy-related genes-ATG2a, ATG4b, ATG7, ATG9a, and ATG10. However, among these genes, only ATG4b expression was significantly repressed by C/EBPβ siRNA, suggesting that ATG4b is transcriptionally regulated by C/EBPβ (29). Further study confirmed that ATG4b knockdown blocked the terminal differentiation of 3T3-L1 cells. ATG4b expression levels are highly regulated during adipogenesis, and transactivation of ATG4b by C/EBPβ is required to effectively activate autophagy and adipogenesis. Another study has reported that the down-regulation of Beclin-1 (Vps30/ATG6) expression inhibits autophagy in mature adipocytes and adipose tissue of mice following a high-fat diet. The consequences of high-fat diet-induced inhibition of autophagy include the following aspects: accumulation of damaged proteins and organelles, increased oxidative stress, impaired metabolic regulation, enhanced inflammation, development of insulin resistance, increased risk of neurodegenerative diseases, altered immune response, liver dysfunction and cardiovascular disease (30). This study found that in HFD-fed mice, there was an increase in the mRNA expression of several autophagy-related genes (Beclin-1, ATG5, ATG7, and ULK2) compared to chow-fed mice. However, the expression of Gabarapl1 did not significantly change. The protein level of LC3-II, an indicator of autophagy, was also higher in HFD-fed mice, suggesting increased autophagy in obesity. The study also found that berberine suppressed Beclin-1 mRNA expression and reduced LC3-II levels in HFD-fed mice, indicating that berberine could inhibit autophagy. In visceral fat, similar results were observed. This discovery highlights the critical involvement of Beclin-1 as a molecule in regulating autophagy in mature adipocytes (26, 31). But whether it affects adipogenesis remains to be further studied. Moreover, several studies suggest that ATG5、ATG7 and ATG12 participate in BAT whitening, expanding white adipogenesis (32, 33). Within adipogenesis research, it is necessary to focus on the factors that affect the expression and activation of core ATG proteins (Figure 1).

2.2 Autophagy-related pathways in adipogenesis

2.2.1 mTOR is a crucial nod that links autophagy and adipogenesis

For a more comprehensive understanding of the relationship between autophagy and adipogenesis, we must also understand the role of autophagy-related pathways in this process. Several studies have demonstrated the interconnectedness of autophagy and adipogenesis through key signalling nodes, including mTOR, Notch, AMPK, and SIRT. TOR, as a key protein kinase signalling hub, senses changes in intra- and extracellular nutrient content, changes in energy levels, growth factors and other signalling stimuli, and regulates anabolic processes such as cell growth, proliferation, and protein synthesis through activation of its downstream effector proteins. In mammalian cells, mTOR is an important signal regulating autophagy, ribosome biosynthesis and protein synthesis and a key node in regulating signalling pathways related to nutrient metabolism *in vivo*. Activation of mTORC1, for instance, would influence various autophagy-related proteins and genes, such as Beclin-1 and LC3 I/II, thereby regulating autophagy. MTORC1 is susceptible to rapamycin, which is a rapid inhibitor of mTOC1. Treatment of hepatocytes with rapamycin enhanced intracellular autophagic activity and increased lipid oxidation levels and lipolytic activity (34). In C. elegans, rapamycin treatment enhances lysosomal acid lipase activity in cells (35). One recent study revealed that metformin upregulated Beclin-1 and LC3 I/II expression and downregulated mTOR expression, thereby stimulating autophagy induction. Also, metformin can mitigate adipogenesis of fibro-adipogenic progenitors after rotator cuff tears by mediating mTOR/ULK1-regulated autophagy (36). The inhibitory effect of metformin on mTOR may be mediated through AMPK. It has been shown that AMPK can inhibit mTORC1 in at least two parallel pathways: one is by phosphorylating TSC2 to directly inhibit the viability of RHEB and mTORC1, and the other is by directly phosphorylating the RAPTOR on the mTORC1 complex, which results in a structural change in the complex and thus inhibits the viability of the complex (37). It is known to all that the important biological functions of metformin are its attenuation of mitochondrial respiratory chain

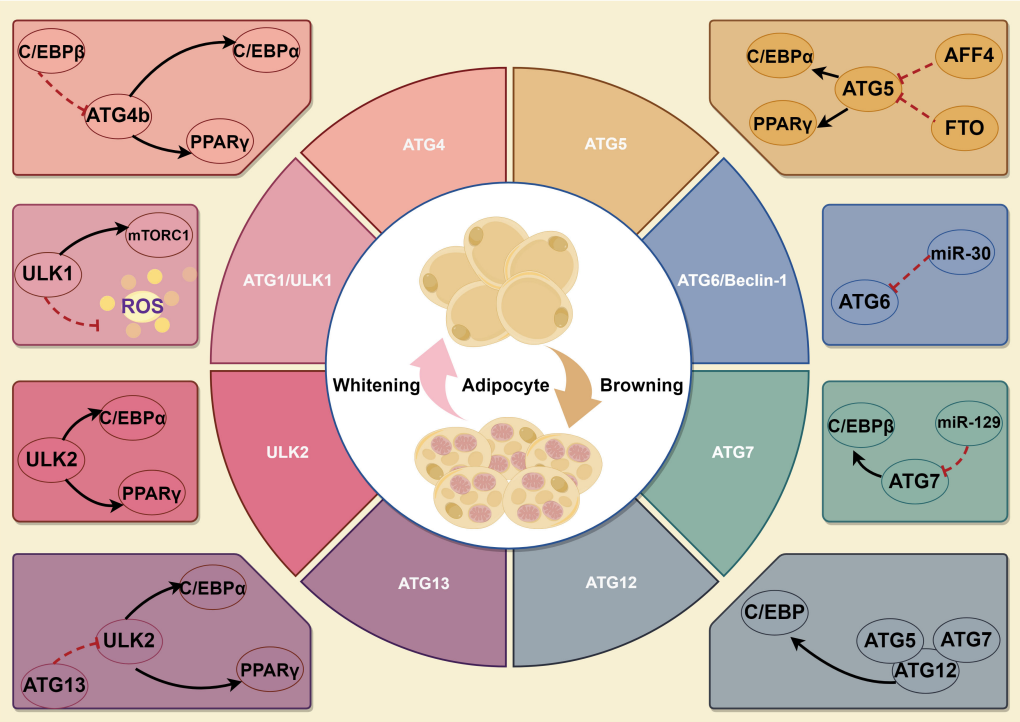

FIGURE 1
A schematic illustration depicting the role of ATGs in adipogenesis. Firstly, ULK1 is not required for adipogenesis, but ULK2 is indispensable for adipogenesis. Though ULK1 is dispensable for adipogenesis, it still affects autophagy, lipid metabolism and glucose uptake in adipocytes. Of which, YBX1 not only can promote ULK1/2-mediated autophagy during white adipogenesis but also induce brown adipogenesis via PINK1/PRKN-mediated mitophagy. Secondly, ATG4b can regulate PPARγ and C/EBPα, key positive adipogenesis regulators. Thirdly, ATG5 and ATG7 promote white adipogenesis and are involved in BAT whitening. In addition, ATG6 regulates autophagy in mature adipocytes, but whether it can affect adipogenesis remains unknown.

complex I activity and its enhancement of phosphorylation of AMPK (38). Under hyperglycemic conditions, enhanced autophagy is crucial in potentiating osteogenic differentiation in adipose-derived stem cells and ultimately mediates adipocyte differentiation (39). Alternatively, type I collagen has been shown to reduce intracellular lipid accumulation and adipocyte differentiation by inactivating autophagy through the YAP pathway. In the presence of collagen-coating conditions, there is an increase in lysosome-mTOR co-localization, accompanied by elevated downstream p-S6K protein expression and subsequent autophagy suppression (40).

2.2.2 Notch signal in adipogenesis

The Notch signalling pathway, encompassing Notch, ligand Delta/Serrate/LAG-2 and CBF1/Su (H)/Lag-1 (CSL, a DNA-binding protein), is a critical component of cellular communication, orchestrating many cell differentiation processes (41). Notch and its ligands are single transmembrane proteins and play indispensable roles in adipogenic differentiation. The expression of PPARγ and C/EBPα was impeded upon exposure to the Notch ligand jagged1 or overexpression of the Notch target gene Hes-1 in 3T3-L1 cells. Intriguingly, these cells' adipogenic differentiation potential was attenuated following Hes-1 knockdown via siRNA (42). A finding has elucidated that the inhibition of Notch signalling promotes autophagy-mediated adipogenic differentiation of MSCs through the PTEN-PI3K/AKT/mTOR pathway (43). In addition to its role in adipogenic differentiation, Notch signalling has been implicated in suppressing osteogenic differentiation via the inhibition of Wnt/β-catenin signalling (44). However, other studies have suggested that Notch signalling may promote osteogenic differentiation through interaction with BMP2 signalling (45). Consequently, the Notch signalling pathway modulates both adipogenesis and osteogenesis of MSCs through direct gene targeting or interaction with other signalling pathways. Interestingly, inhibiting autophagy through the inhibitor DAPT reduces Notch signalling activity, consequently impairing osteogenic differentiation in Dop ASCs. Nonetheless, the impaired osteogenic differentiation of Dop ASC can be restored by administering Torin1, a selective inhibitor of mTORC1/2 and autophagy agonist, which activates the Notch signalling pathway via the upregulation of autophagy (46).

2.2.3 AMPK is a promising target for equilibrating autophagy and adipogenesis

AMPK, as a regulatory centre of cellular energy metabolism, can regulate individual metabolic emergencies by directly acting on metabolism-related proteins or indirectly affecting gene expression. Under stress, activated AMPK can directly regulate various downstream metabolism-related enzymes, such as mTOR, acetyl-CoA carboxylase, fatty acid synthase, glycerol phosphate

acyltransferase, and PPARγ coactivator 1α (PGC-1α), to regulate different energy production/consumption pathways, thus maintaining the balance of cellular energy metabolism and homeostasis. AMPK is localized to the lysosome during autophagy, and AMPK regulates ULK1 complex activity by antagonizing mTORC1 and PI3KC3/VPS34 complex activity (47). Moreover, the mitochondria-localized AMPK is also involved in the regulation of mitophagy (48). The regulation of lipid metabolism is the primordial role of AMPK, whereby its activation manifests in a concomitant reduction in lipid storage (49). AMPK exerts a suppressive influence on the *de novo* biosynthesis of cholesterol, triglycerides, and fatty acids. It both inhibits and phosphorylates substrates intricately associated with the biosynthesis of fatty acids. AMPK impairs cholesterol biosynthesis by phosphorylating and suppressing the enzymatic activity of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (50). Furthermore, AMPK elicits the augmentation of mitochondrial genesis and β-oxidation utilizing the orchestration of PGC-1α activity (51). The loss of PGC-1α functionality precipitates a diminished manifestation of mitochondrial and thermogenic genes within white adipose tissue. AMPK has been demonstrated to exert an inhibitory effect on adipogenesis by impeding the initial stages of mitotic clonal expansion, concomitant with diminished expression of early and late adipogenic factors, including fatty acid synthase, sterol regulatory element-binding protein-1c, and adipocyte protein 2 (52). The suppression of adipogenesis through the utilization of small-molecule activators (RSVA314 and RSVA405) targeting AMPK, with concomitant inhibition of the MCE phase, accompanied by decreased expression of C/EBPβ, suppression of C/EBPα, PPARγ, and the subsequent downregulation of late adipogenic factors, including SREBP-1c, FAS, and FABP4, was observed (53). Similarly, the activation of AMPK by A769662 led to a decrease in lipid droplets and the activation of PPARγ, C/EBPα, as well as early adipogenic transcription factors such as C/EBPβ and C/EBPδ (54). A study suggests that the effect of AMPK on adipogenesis is autophagy-dependent. Pre-inhibiting or pre-promoting autophagy with siATG7 or rapamycin would block AMPK. The study showed inhibiting or improving autophagy would promote or inhibit the role of AMPK prohibition in adipose-derived stem cell adipogenesis (55). Moreover, the selection of AMPK-mediated signalling cascades also involved BAT activation. These pathways are categorized into three distinct processes: the development of brown adipocytes, mitochondrial health, and browning. Firstly, the AMPK/mTOR axis is the core signal involved in autophagy and protein synthesis in the developmental stage and glucose uptake in adult brown adipocytes. Secondly, AMPK can activate PRDM16, an important DNA-binding transcription factor that indirectly induces adipogenesis and lipolysis, via α-ketoglutarate. Thirdly, AMPK triggers the activation of SIRT1 via deacetylation, culminating in the activation of the peroxisome PGC1α and subsequent augmentation of mtDNA content, mitochondrial dimensions, and abundance, ultimately resulting in diminished adipogenesis. Finally, AMPK affects the abundance of UCP1 expression, which is a key factor in WAT browning (56). These compelling evidences indicate that AMPK plays a pivotal role in developmental and functional processes of BAT. It also has been showed that AMPK has the capacity to elicit BAT activation through a diverse array of signaling pathways. Meanwhile AMPK-mediated autophagy and mitophagy play important roles in this process. Clarifying the roles of autophagy related signaling pathways in adipogenesis is helpful to explore molecular targets for the treatment of obesity-related metabolic disorders. Next we will discuss the specific nodes where these factors or pathways act on adipogenesis.

2.3 Autophagy-associated transcription factors and regulatory proteins regulate adipogenesis

Additional investigations have revealed that other autophagy-associated proteins, regulatory factors, and transcription factors exert direct or indirect influences on the process of adipogenesis. Some adipogenic factors regulate adipogenesis by targeting autophagy-associated genes (such as Beclin-1, LC3B, and p62) and autophagy transcription factors (including FOXO1, TFEB, and XBP1) (57). In bone marrow-derived mesenchymal stem cells (BMSCs) expressing Scara3, p62 was suppressed while the expression of LC3B and FOXO1 was upregulated, thereby promoting autophagy (58). Oil Red O staining results demonstrated that overexpression of Scara3 impeded lipid droplet formation. However, this effect was disrupted by silencing FOXO1b expression. Therefore, Scara3 potentially affects lipid differentiation through the FOXO1-autophagy signalling axis. Recently, it has been determined that microRNA (miRNA) and long non-coding RNA (lncRNA) play pivotal roles in autophagy and adipogenesis. For example, miRNA-9 targets PNPLA3, reducing lipid droplet accumulation and triglyceride content while promoting AMPK pathway phosphorylation, ultimately inhibiting adipogenesis (59). Bioinformatic analysis has predicted that the lncRNA, lncIMF4, is involved in various biological processes, including cell proliferation, differentiation, and autophagy. Knockdown of the lncIMF4 gene in pigs has resulted in a significant upregulation of the autophagy-associated gene p62, downregulation of LC3, and a substantial increase in intracellular lipid droplets. These findings suggest that the knockdown of lncIMF4 attenuates lipolysis by suppressing autophagy, ultimately promoting intramuscular lipogenesis (60). Furthermore, it has been observed that the Vangl2 protein directly binds to lysosome-associated membrane protein and targets lysosomes for degradation. This process inhibits adipogenesis by scavenging lipogenic transcription factors TLE3 and ZNF423 (61). Deficiency of the transcription factor Nrf2 attenuates autophagic flux and inhibits the fusion of autophagosomes and lysosomes *in vivo* and *in vitro*. Subsequently, this decrease in autophagy leads to hepatic lipid accumulation. Additionally, adipogenesis can be stimulated by enhancing the activity of the transcription factor SREBP-1c (62). Collectively, the studies mentioned above indicate that autophagy-associated transcription factors and regulatory proteins possess the potential to regulate or interact with factors implicated in adipogenesis and related pathways (Table 1). Thus, the modulation of autophagy is a promising strategy for preventing and controlling obesity through influencing adipogenesis.

TABLE 1 Autophagy-associated factors regulate adipogenesis.

Autophagy-related gene	Effect on adipogenesis	References
ATG5/ATG7	FTO promotes the expression of ATG5/ATG7 and induces autophagosome, affecting adipogenesis	(24)
ATG5/ATG16L1	AFF4 directly binds to ATG5 and ATG16L1 and regulates autophagy during adipogenesis	(19)
Rubicon	Loss of RUBCN mediates the upregulation of autophagy, which further causes a reduction in NCOA1/2 level, inhibiting adipogenesis.	(63)
Pink1/Parkin	Loss of YBX1 decreases the Pink1/Parkin level, alleviating mitophagy and inhibiting the thermogenic program.	(28)
Type I collagen	Type I collagen inactivates autophagy by up-regulating YAP-mediates mTOR activity.	(40)
Scara3	Scara3 controls the cell fate by promoting Foxo1 expression and autophagy flux.	(58)
Nrf2	Nrf2 deficiency attenuates autophagic flux and inhibits the fusion of autophagosomes and lysosomes.	(62)
PNPLA3	PNPLA3 plays an essential role in lipophagy in hepatocytes	(59)
lncIMF4	knockdown lncIMF4 facilitates intramuscular adipogenesis through attenuating autophagy to repress the lipolysis	(60)
FUNDC1	Ablation of FUNDC1 results in defective mitophagy and impaired mitochondrial QC	(64)
TP53INP2	Autophagy-related protein TP53INP2 activates autophagy during the early stage of differentiation in bovine adipocytes and positively regulates adipocyte differentiation by affecting autophagy.	(65)

2.4 Autophagy regulates key adipogenesis regulators PPARγ and C/EBPα

On the basis of summarizing promising autophagy-related genes and pathways affecting adipogenesis, we still hope to identify the specific targets of autophagy regulation of adipogenesis. Therefore, we focus on some adipogenesis regulators. PPARγ plays a pivotal role in regulating adipocyte differentiation, while TP53INP2, an autophagy protein, stimulates autophagy and promotes PPARγ expression. The activation of PPARγ effectively compensates for the decline in lipid droplets caused by TP53INP2 knockdown, thus governing the adipocyte differentiation process (65). Fasting induces a notable decrease in Rubicon, a negative regulator of autophagy, in adipose tissue, which is accompanied by an increased level of autophagy. Adipose-specific Rubicon-knockout mice exhibit systemic fat loss (63). Implied in the autophagy-induced reduction of adipogenic gene expression is the degradation of coactivators of PPARG/PPARγ, specifically NCOA1/SRC-1 and NCOA2/TIF2. The degradation of these substrates during fasting leads to a decline in mRNA levels of adipogenic genes in adipocytes. Furthermore, the knockout of Rubicon in adipocytes results in adipose atrophy in the liver, owing to the downregulation of adipogenic gene expression. The activation of PPARγ can restore the expression of these genes. Additionally, autophagy facilitates the degradation of SRC-1 and TIF2, coactivators of PPARγ. This degradation relies on their interaction with GABARAP family proteins and is significantly reduced in Rubicon-deficient or senescent adipocytes (66). Mechanistically, the excessive autophagy instigated by Rubicon inhibition within adipocytes precipitates an LIR/GIM-dependent diminution of SRC-1 and TIF2, functioning as coactivators of PPARγ. It is postulated that SRC-1 and TIF2 within adipocytes are translocated to the cytosol and degraded via autophagy in response to external energy demand. These reductions instigate a decline in PPARγ activity and adipocyte function, facilitating energy supply for other tissues. Moreover, given that the overexpression of LIR/GIM mutant SRC-1 and TIF2 failed to fully rescue the reduction in adipogenic gene expressions instigated by Rubicon knockdown, it is plausible that the absence of Rubicon fosters the autophagic degradation of other specific constituents implicated in adipogenesis. C/EBPα overexpression upregulates the expression of LC3B, ATG5 and Beclin1 and further induces autophagy. Moreover, C/EBPα affects the expression of P62 and its binding to Beclin1 through acetylation modification (at positions K298, K302 and K326) to induce autophagy (67). In turn, pre-promoting or pre-inhibiting autophagy with rapamycin or 3MA decreased or increased C/EBP-α expression, but the underlying mechanism remains unknown (68). At present, there are few clear targets for autophagy to regulate adipogenesis. Exploring these targets and analyzing their mechanism will be hot topics for the next research.

2.5 Mitophagy has effects on fat metabolism and adipogenesis

While the above discussion mainly focused on the effect of macroautophagy (autophagy) on adipogenesis, this section will specifically discuss the role of selective autophagy in adipogenesis, which is the current research hotspot. Several recent studies suggest that selective autophagy (especially mitophagy) may be a key factor influencing adipogenesis (28, 69, 70). The process of adipogenesis is the result of intracellular energy changes and metabolism. Mitochondria, as a vital biological energy centre, must be paid more attention. Mitophagy is the targeted phagocytosis and destruction of mitochondria by cells and is generally regarded as the main mechanism of mitochondrial quality control. Initiation of mitophagy involves the recruitment of the cytosolic E3 ubiquitin ligase PARK2/Parkin to the injured mitochondrion, facilitated by the protein kinase PINK1. Once they are recruited, PARK2 ubiquitinates mitochondrial substrates, thereby initiating mitophagy. This intricate process assumes a central role in cell types rich in mitochondria, such as brown and beige adipocytes,

where it governs adipocyte differentiation and supports homeostasis within beige adipocytes (71). Interestingly, the mitophagy receptor FUNDC1 dysfunction significantly affects adipose inflammatory metabolism and insulin resistance, compromising adipocyte production and exacerbating diet-induced obesity (64). Mechanistically, the hyperactivation of the MAPK/JNK pathway precipitates insulin resistance, a condition ameliorable through the abrogation of MAPK8/JNK1 in the FUNDC1 knockout model. Dysregulated maintenance of mitochondrial quality control, stemming from impaired mitophagy receptor FUNDC1, establishes a connection with fat metabolism disorders through the mediation of MAPK signalling and the orchestration of inflammatory responses. As a newly identified mitophagy receptor, Bcl-2-like protein 13 (Bcl2l13) has been demonstrated to be critical for adipogenic differentiation (72). Bcl2l13 knockdown significantly impaired adipocyte differentiation. Knockdown of Bcl2l13 triggered cellular reprogramming, augmenting the reliance on glycolysis to fulfil ATP requisites in compromised oxidative phosphorylation. Bcl2l13 depletion in embryonic mesenchymal stem cells induced increased mitophagy. Furthermore, Bcl2l13 also served to alleviate apoptosis during the process of adipogenesis. As one of the best-studied selective autophagy receptors, p62 takes part in different types of selective autophagy, including aggrephagy, pexophagy, and mitophagy. The p62 is an adapter molecule, engaging directly with ubiquitinated molecules on the autophagosome. The inhibition of p62 entirely obstructs the clearance of compromised mitochondria. Hence, activating the PINK1/PARKIN/p62 axis is important in effectively eliminating damaged mitochondria, a process indispensable for maintaining their quality control. P62 has been shown to play an important role in adipose tissue metabolism and adipogenesis. Obesity and insulin resistance have been noted in p62-deficient murine models, with basal lipolytic hydrolysis manifesting a decreasing tendency compared to their wild-type counterparts. Furthermore, p62-knockout mice exhibit an augmented quantity of intracellular lipid droplets, elevated triglyceride synthesis, and an enlargement in adipocyte size (73). Specifically, the main organ affected is the BAT, where p62 controls mitochondrial function directly. The deficiency of p62 in BAT affects mitochondrial structure and function and impairs thermogenesis. The study also shows that p62 controls the activation of p38 *in vivo*, which is crucial for BAT nonshivering thermogenesis and UCP1 function. The loss of p62 in adipocytes impairs the activation of p38 and its downstream pathways, affecting uncoupling *in vivo* and *in vitro*. It suggests that p62 controls mitochondrial function via the p38/Ppargc1a pathway. The study also found that oxidative capacity, measured by Cox activity, was decreased in BAT of adipocyte-specific p62-knockout mice. The study concludes that p62 plays a critical role in regulating transcriptional programs controlling mitochondrial homeostasis, which underlies the phenotype of adipose-specific p62-KO mice. A recent study showed that p62 knockdown enhanced mitophagy (74). Knockdown of p62 in human adipose-derived stem cells (hADSCs) yielded a pronounced decrement in stabilizing capacity, consequently culminating in heightened mitophagy. Conversely, suppressing p62 increased the efficiency in activating alternative mediators, thus instigating mitophagy. In this case, the role of p62 as a mediator for mitophagy becomes dispensable. During the adipogenic differentiation of hADSCs, the knockdown of p62 led to an uneven distribution of autophagic fluxes: a relatively increased mitophagy flux. Consequently, the refreshment of mitochondria was ensured, further propelling the synthesis of lipids and the progression of differentiation. Notably, YBX1, an RNA-binding protein, exhibits robust expression in BAT and is induced by exposure to cold and β-adrenergic agonists in mice. Loss-of-function experiments have illustrated that YBX1 deficiency impairs the differentiation of primary brown adipocytes and their capacity for thermogenesis. Subsequent investigations have revealed that YBX1 exerts a positive regulatory influence on thermogenesis by enhancing mitophagy (28). RNA immunoprecipitation studies have identified direct targeting of PINK1 and PARKIN transcripts by YBX1. Additionally, RNA decay assays have demonstrated that YBX1 deficiency diminishes the mRNA stability of PINK1 and PARKIN, leading to reduced protein expression. Consequently, this impairment compromises mitophagy and represses the thermogenic program. At present, there are few studies on the regulation of adipogenesis by mitophagy. We believe that further research in this area will help us understand the changes in energy metabolism and the mechanism of cell transformation (the differentiation of diverse preadipocytes into mature adipocytes).

3 New advances in the impact of oxidative stress on adipogenesis

3.1 Oxidative stress is an important driver of adipogenesis

In addition to autophagy, oxidative stress is closely related to intracellular material and energy metabolism. So, we're also interested in its role in adipogenesis. Oxidative stress refers to the discrepancy between the generation of ROS in the cell's internal and external environment and the cell's intrinsic antioxidant capacity, resulting in a disturbance of the intracellular redox balance. This imbalance in a tendency to oxidize impairs normal cellular function.

Oxidative stress is strongly associated with adipogenesis, as heightened levels of peroxides within adipocytes cause damage to the mitochondrial membrane, hindering ATP production and generating ROS. In turn, it contributes to morphological and functional alterations in adipocytes. Generally, adipocytes exhibit a higher spontaneous production of intracellular and extracellular ROS than preadipocytes (75). For instance, the expression of MCPIP induces the production of reactive oxygen species/reactive nitrogen species in 3T3-L1 cells and enhances adipogenesis. Conversely, inhibiting the expression of reactive oxygen species hampers the ability of MCPIP to stimulate adipogenesis (76). Furthermore, oxidative stress induced by heme inhibits Sirt1, which disrupts the regulation of PPARγ and C/EBPα. These factors are known to promote adipogenesis and preadipocyte hypertrophy (77). NOX4, a crucial NADPH oxidase, belongs to a

protein class that converts oxygen into reactive oxygen radicals. Catalase-knockout mouse embryonic fibroblasts appeared to differentiate into adipocytes more easily than wild-type cells. Silencing catalase significantly increases H_2O_2 concentration, followed by increased NOX4 expression and decreased AMPK levels. Ultimately, this cascade leads to adipogenesis (78). Suppression of the adipose-specific protein BAMBI results in increased expression of NOX4. The study found that increased white fat deposits in BAMBI knockout mice were due to elevated levels of NOX4. NOX4 is involved in stimulating ROS production in mitochondria and C/EBPβ expression. Another study also shows that administering the antioxidant NAC to BAMBI knockout adipocytes can reverse lipogenic differentiation (79). MSCs are adult stem cells with self-renewal capacity and multidirectional differentiation potential, and the expression level of cystathionine β-synthase in human adipose MSCs is low. This low level is accompanied by increased inflammatory factors and markers of oxidative stress, including elevated intracellular reactive oxygen species and decreased intracellular glutathione levels. They lead to an upregulation of cellular lipogenic genes (80). In glucocorticoid-induced osteoporosis, MSCs display a substantial reduction in the expression of osteogenic genes and an increase in lipogenic gene expression. It is due to GCs elevating the level of oxidative stress in the cells and inducing the expression of SENP3, promoting lipogenic differentiation. Further studies have demonstrated that SENP3 knockdown suppressed the detrimental effects of glucocorticoid-induced osteoporosis on osteogenic differentiation and reduced lipid formation and triglyceride content. These findings suggest that SENP3 knockdown inhibits lipid accumulation and lipogenic differentiation through oxidative stress (81). GPX7, an antioxidant enzyme located on the endoplasmic reticulum, inhibits ROS production and promotes osteogenic differentiation of bone marrow MSCs by stimulating endoplasmic reticulum stress and the mTOR pathway. Knockdown of GPX7 in MSCs leads to an increase in lipogenic capacity (82).

Although the prevailing view is that oxidative stress contributes to adipogenesis, some studies still report that oxidative stress may impede adipogenesis under certain circumstances. Suppression of protein tyrosine phosphatase 1B (PTP1B) enhances mitochondrial dynamics, attenuates oxidative stress, and potentiates the adipogenic differentiation capacity of adipose-derived stem cells (83). In a comparable investigation, the activity of PTP1B was obstructed by MIS-1436, resulting in diminished oxidative stress, decreased expression of endoplasmic reticulum stress-related proteins, and heightened levels of apoptosis-related proteins. The cumulative effect of these mechanisms may have synergistically amplified the adipogenic differentiation potential of cells towards mature adipocytes (84). Prolonged exposure of adipocytes to low levels of H_2O_2 elicits changes in mitochondrial dynamics, reduces the efficiency of cellular respiration, and down-regulates the expressions of PPARγ and C/EBPα, as well as the lipogenic marker Plin1. Ultimately, these events culminate in impaired adipogenesis (12). Moreover, there is compelling evidence that ROS can impede adipogenesis by up-regulating the lipogenic inhibitory factor CHOP-10/GADD153 (85). It suggests that oxidative stress may play a double-edged role in regulating adipogenesis. These opposite results may be due to differences in the intensity and timing of oxidative stress or to a combination of other factors. Therefore, more studies are needed to uncover the special mechanisms involved.

3.2 Oxidative stress regulates energy metabolism in BAT

Although most of the research on adipogenesis has been done in WAT, the growth and differentiation of brown adipocytes have also attracted much attention from researchers. A recent study has uncovered that the intertissued administration of enoxacin remarkably augments the mitochondrial count within adipocytes, reinforcing fatty acid oxidation metabolism and fuel consumption while diminishing adipocyte size and body fat composition (86). Enoxacin works by regulating miRNAs within adipose tissue, such as miRNA-34a-5p. Subsequently, oxidative metabolism is stimulated via the FGF21 signalling pathway, and the expression of thermogenic genes UCP1, Dio2, and Ppargc1a are activated. Ultimately, it promotes energy expenditure and decreases lipid accumulation. In a distinct investigation, it was observed that oxidative stress downregulates the expression of pivotal adipogenesis regulators, including PPARγ, C/EBPβ, and cell death-inducing DFFA-like effector A and FABP4, in mature brown adipocytes (87). As a result, adipogenesis in brown fat is impaired. Simultaneously, oxidative stress leads to a decline in the expression of thermogenic genes such as UCP1 and PGC-1α, thereby reducing thermogenesis and energy expenditure. While the effects of oxidative stress on adipogenesis remain controversial, researchers generally believe that oxidative stress can promote adipogenesis (Table 2). Differences in these findings may be due to different types of adipose tissue. The differential regulation mechanisms of adipogenesis by oxidative stress in WAT and BAT need to be further revealed. A comprehensive understanding of the role played by oxidative stress in adipogenesis would be pivotal in advancing our comprehension of the pathogenesis of obesity and associated disorders and facilitating the development of more productive prevention and treatment strategies.

4 New revelations regarding the impact of inflammatory cytokines on the process of adipogenesis

Adipose tissue consists of many types of cells, including endothelial cells, blood cells, fibroblasts, pericytes, precursor cells, adipocytes, macrophages and other immune cells (3, 4). Consequently, adipose tissue is widely acknowledged as a primary reservoir of immune cells (5). Studies have revealed that immune cells play an intricate role in the secretion of inflammatory factors, which concurrently modulate adipogenesis. Although the impacts of certain inflammatory cytokines on adipogenesis have been reported, ongoing investigations are dedicated to discerning novel functions and roles of these cytokines in this physiological process.

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TABLE 2 Oxidative stress-related factors regulate adipogenesis.

Target	Function	Effect on adipogenesis	References
Nrf2	Oxidative stress promotes Nrf2 recruitment to the sterol regulatory element binding protein one promoter, inducing lipogenesis.	Promoting	(13)
PPARγ	Sustained low levels of oxidative stress reduce the PPARγ level, inhibiting preadipocytes from differentiating to mature adipocytes.	Promoting	(12)
SENP3	SENP3 promotes adipose differentiation during oxidative stress by PPARγ2 DeSUMOylation	Promoting	(81)
MCPIP	MCPIP induces adipocytes to produce ROS/RNS, which in turn regulates key transcription factors for adipogenesis	Promoting	(76)
NOX4	NOX4 induces oxidative stress and reduces AMPK activity, resulting in disturbed energy metabolism and fat deposition	Promoting	(78)
CHOP-10/ GADD153	CHOP-10/GADD153 triggers hypoxia-dependent inhibition of adipocyte differentiation	Inhibiting	(85)
PTP1B	Inhibition of PTP1B reduces oxidative stress, improves mitochondrial biocompetence and kinetics, and enhances the lipogenic differentiation potential of adipocytes.	Inhibiting	(84)
Sirt1	Heme-dependent oxidative stress negatively regulates Sirt1 activity by enhancing the expression of lncRNA and subsequent adipogenesis.	Inhibiting	(77)
BAMBI	BAMBI inhibits NOX4 activity and reduces ROS expression, which in turn affects C/EBPβ activity, reducing lipid synthesis	Inhibiting	(79)
miRNA-34a-5p	MiRNA-34a-5p promotes energy expenditure and reduces lipid accumulation by mediating the FGF21 to induce oxidative metabolism.	Inhibiting	(86)

(Continued)

TABLE 2 Continued

Target	Function	Effect on adipogenesis	References

Recently, several other inflammatory factors have been found to impact adipogenesis.

4.1 TNF-α inhibits adipogenesis

TNF-α has demonstrated formidable suppressive effects on adipogenesis, primarily utilizing TNFR1 activation, leading to subsequent stimulation of the NF-κB, ERK1/2, and JNK signalling pathways. The reinstatement of 3T3-L1 cell differentiation can be achieved by impeding NF-κB and JNK signalling through specific inhibitors. In addition, the Wnt/β-catenin/TCF-dependent cascade and the repression of transcription factors are also involved in TNF-α-mediated inhibition of adipogenesis (8). A recent investigation has revealed that lactic acid bacteria-fermented skimmed milk exerts inhibitory effects on adipogenesis by hindering the activities of the principal transcription factor PPARγ (88). This effect is realized via the upregulation of the proinflammatory cytokine TNF-α in 3T3-L1 cells.

4.2 Interleukin-induced adipogenesis

IL-1β, an interleukin, is predominantly synthesized by THP-1 macrophages in adipose tissue and, to a lesser degree, by adipocytes. It impedes adipocyte production by binding to the IL-1β receptor and activating the NF-κB signalling pathway. IL-6, belonging to the GP130 family of cytokines, predominantly binds to the GP130 receptor to form homodimers or heterodimers, thereby facilitating signal transduction. In most instances, IL-6 is bound to the membrane IL-6Rα receptor, which induces β-receptor dimerization, activates the JAK/STAT signalling pathway, and transmits the signal intracellularly, thereby promoting inflammation (89). A small portion of IL-6 can also bind to soluble IL-6Rα, forming a complex that promotes energy metabolism through transmembrane signalling. Generally, the pro-inflammatory factor IL-6 regulates adipocyte production and counteracts obesity, but its role in insulin sensitivity remains controversial. Reduction of TNF-α and IL-6 downregulates the mRNA expression of PPAR-γ, sterol regulatory element binding protein-1c, and leptin. However, these cytokines lead to upregulation of lipocalin and uncoupling protein-1 (UCP-1) expressions (90). During Brucella infection, there is an upregulation of IL-6 and MMPs-2/9 secretion in preadipocytes and adipocytes, resulting in the downregulation of lipocalin and leptin expression in differentiated

adipocytes (91). Moreover, it has been demonstrated that IL-6R promotes the adipogenic differentiation of MSCs by activating the p38 signalling pathway, as evidenced by both IL-6 receptor knockdown and overexpression experiments (9). IL-11, also a member of the Gp130 cytokine family, primarily targets adipocytes through the IL-11 receptor. It suppresses the mRNA expression of PPARγ and C/EBPα by inhibiting Dkk1 and Dkk2, thus augmenting Wnt signalling and impeding adipogenesis (92).

Macrophages, dendritic cells, cardiomyocytes, and smooth muscle cells predominantly secrete IL-20. The IL-20 receptor comprises a heterodimeric complex composed of IL-20Rα and IL-20Rβ subunits. Activation of IL-20 receptors triggers the activation of various signalling pathways, including STAT3, p38, and JNK, with STAT3 serving as the principal signal. During the adipose differentiation process, IL-20 induces the expression of TNF-α, which subsequently modulates adipose differentiation. In murine *in vitro* experiments, IL-20 has been shown to regulate the differentiation of adipocytes and the polarization of bone marrow-derived macrophages toward M1-type pro-inflammatory cells (93). Furthermore, IL-20 enhances the expression of netrin 1, leptin, and MCP-1 in adipocytes by upregulating TNF-α, MCP1, netrin 1, and un5b expression in macrophages, thereby promoting adipose tissue inflammation and macrophage retention. IL-17, predominantly produced by γδT cells, orchestrates a diverse repertoire of immune responses and primarily controls the initiation of pro-inflammatory reactions (94). In the context of adipocyte differentiation, IL-17 stimulates the production of PGE2 in human bone marrow mesenchymal stem cells (hBM-MSCs), thereby inhibiting the differentiation of preadipocytes. Additionally, IL-17 modulates adipogenesis by influencing the expression of Kruppel-like family members (KLF), including KLF15, KLF2, and KLF3, while also impeding the activity of PPARγ and C/EBPα. Notably, IL-17A markedly suppresses FABP4 and PPARγ and facilitates the recruitment of type 17 lymphocytes that harm adipose tissue function (95).

Additionally, some other interleukins also linked to inflammation are associated with adiposity. IL-38, an anti-inflammatory cytokine belonging to the IL-1 family, elevates the expression of GATA-3 and GLUT4 mRNA while suppressing the secretion of IL-1β, IL-6, and MCP-1 from 3T3-L1 cells (96). In turn, it restrains the differentiation of human adipocytes and the production of inflammatory cytokines by 3T3-L1 cells. In adipocytes, the knockdown of IL-21R diminished the lipogenic capacity of ADSCs without impacting the proliferation and mitochondrial activity of ADSCs. The relationship between IL-21 and the process of lipogenic differentiation warrants further investigation (97). IL-35 could regulate the equilibrium between osteogenic and lipogenic differentiation of progenitor cells through the Wnt/β-catenin-PPARγ signalling pathway. Moreover, IL-35 administration promotes osteogenesis while inhibiting adipogenesis (98). The effect of interleukins on adipogenesis deserves further study.

4.3 Regulation of adipogenesis by interferons

IFN-α is a polymorphic immunomodulatory cytokine produced by monocytes/macrophages, lymphoblasts, and various cell types in response to diverse stimuli. It is extensively studied for its role in treating cancer and viral infections. During adipocyte differentiation, IFN-α hinders adipocyte differentiation at the initial stages of adipogenesis by governing the expression of PPARγ and C/EBPα and regulating the cell cycle through modulation of the JAK/STAT1 signalling pathway (99). IFN-γ is a pro-inflammatory factor that serves various biological functions in immune regulation, tumour resistance, and induction of cell differentiation. In adipocytes, IFN-γ represses the expression of PPARγ, C/EBPβ, and C/EBPα while also triggering apoptosis in adipocytes. Studies have also shown that activated IFN-γ and CD8+ T cells promote lipogenesis in mice's bone marrow mesenchymal stromal cells with aplastic anaemia *in vitro* or *in vivo* (100). Moreover, evidence suggests the interferon signalling pathway plays a regulatory role in lipogenesis (101). In mouse preadipocytes lacking IRF3, a heightened PPARγ and PPARγ-mediated lipogenic gene expression led to enhanced adipogenesis. Furthermore, another study has validated the significance of single nucleotide polymorphisms in the porcine IFN-α-16 and TNFRSF19 genes in promoting intramuscular fat deposition in pigs (102).

During the adipose differentiation and production process, there is a discernible alteration in the transcript levels of diverse miRNAs, and several miRNAs are intricately associated with inflammatory responses. Compare people with significant differences in obesity degree, a total of 25 miRNAs have been identified as targeting three upregulated adipogenesis-associated inflammatory genes, namely interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-α), and interleukin-1 beta (IL-1β). Remarkably, these miRNAs were observed to undergo conserved changes throughout adipogenesis (103). Furthermore, analysis of the relationship between TNF-α and miR-424 in childhood obesity has revealed that miR-424 is regulated by TNF-α and plays a crucial role in adipogenesis, with its mechanism of action being closely related to the Wnt/β signalling pathway (104). Continual exploration of the influence of inflammation on adipogenesis holds the potential to identify novel targets for ameliorating aberrant adipose tissue development. These invaluable findings may pave the way for developing novel therapeutic strategies to address obesity and its associated pathologies.

5 The crosstalk between autophagy, oxidative stress and inflammation plays a key role in regulating adipogenesis

The studies above suggest that several factors, including autophagy, oxidative stress, and inflammation, always influence adipogenesis outcomes. Although our studies predominantly focus on a single aspect of the adipogenesis regulatory mechanism, it remains necessary to incorporate multifactorial analyses. Autophagy consistently exerts a protective influence by regulating the inflammatory response and ameliorating stress-induced cellular damage, thus preserving cellular homeostasis during stresses. There exists a closed correlation between oxidative stress, inflammatory

factors and autophagy. ROS can induce autophagy by directly modulating the activity of multiple upstream autophagy pathways, including AMPK, mTOR, MAPK, and PI3K. Appropriate autophagy can also suppress excessive inflammatory responses. Many pathologically relevant investigations have demonstrated the interplay between autophagy, oxidative stress, and inflammation, ultimately impacting the physiological state of tissues and organs (105–107). Although few studies have examined their roles in adipogenesis simultaneously, the synergistic and antagonistic effects of all three of them in adipogenesis may be of great significance. Here, we discussed the interplay between autophagy, oxidative stress, and inflammation during adipogenesis for the first time.

5.1 The autophagy-oxidative stress axis mediates adipogenesis

Firstly, there is a close link between oxidative stress and autophagy. ROS can initiate autophagy by directly modulating the activity of multiple upstream autophagy pathways, such as AMPK, mTOR, MAPK, and PI3K (108). ROS can also modify autophagy-related proteins, thereby regulating autophagy activity (109). Conversely, autophagy attenuates oxidative stress by eliminating damaged organelles and excessive oxidizing intermediates. Deficiencies in autophagy-related proteins contribute to the accumulation of cellular ROS. Recent studies have revealed both autophagy and oxidative stress are involved in adipogenesis. In the fibroblasts of patients with Graves' orbitopathy, autophagy induced by IL-13 promotes inflammation, ROS production, and fibrosis, thereby impacting adipose differentiation. Conversely, neferine inhibits autophagy-associated inflammation, oxidative stress, and adipogenesis by activating Nrf2 and PI3K/Akt/mTOR signalling pathways (110). The mineralocorticoid receptor (MR) signalling pathway plays a key role in the normal physiological differentiation and maturation of adipocytes. However, excessive MR activation can lead to excessive oxidative stress, the release of pro-inflammatory cytokines, and dysregulation of adipocyte autophagy (111). In adipocytes, the MR antagonist suppresses the transcription of lipogenic and inflammatory cytokines by activating the Akt-FOXO1 pathway and reduces the transcriptional levels of ROS-producing enzymes, consequently promoting the transcription of adipose regulators PPARγ and sgk1, as well as autophagic flux (112). Overexpression of MCPIP in 3T3-L1 preadipocytes increases ROS production through p38 activation, which accordingly impacts autophagy levels and adipogenesis. Induction of precursor adipocytes using a combined approach enhances intracellular autophagy levels, but this effect is mitigated by MCPIP knockdown (76). Autophagy is activated in MSCs and plays a crucial role in their self-renewal and survival. Notably, adipose tissue-derived MSCs exhibit a superior phenotype to bone marrow-derived MSCs, as they possess a higher potential for proliferation and differentiation and a slower senescence rate (113). The hypoxic state of MSCs promotes the maintenance of their stemness, and hypoxia can trigger autophagy in MSCs. Furthermore, autophagy in MSCs is regulated by ROS. Thus, in MSCs, the intracellular hypoxic microenvironment acts as a trigger for autophagy. Autophagy functions to maintain low levels of intracellular ROS. The intricate interplay between autophagy and ROS levels determines the fate of stem cell differentiation into preadipocytes. Conversely, the interplay between autophagy and ROS influences the transcriptional regulation of adipose regulatory factors, ultimately affecting the differentiation of preadipocytes. Recently, a research group established a LEPTIN-deletion pig obesity model (114). LEPTIN deletion in pigs causes type II diabetes and non-alcoholic fatty liver disease. Porcine LEPTIN deficiency inhibits JAK2-STAT3 signalling and enhances oxidative stress. JAK2-STAT3 signalling affected the expressions of lipogenic-related SOCS3, SREBP1c, ACSL3 and ACSL5. Moreover, LEPTIN knockout activates the AMPK signal and enhances mitophagy. Notably, there are no significant changes in the activation of mTOR, MAPK and PI3K-AKT pathways. More surprising is that LEPTIN-deficient rat livers are void of hepatic fibrosis, mitochondrial autophagy and oxidative stress. By comparing these results, they found that the phosphorylation level of AMPK was responsible for these differences. Based on these studies, it can be inferred that Nrf2, p38, mTOR, AMPK and FOXO1 may serve as key nods between autophagy and oxidative stress during adipogenesis. There may exist other pathways linking autophagy and oxidative stress. For instance, β-cypermethrin promoted adipogenesis via oxidative stress-mediated autophagy disturbance, and it caused macrophage polarization by mediating miR-34a (115). Arsenite treatment induces oxidative stress by decreasing UCP1 expression. Meanwhile, arsenite inhibited autophagy necessary for the homeostasis of brown adipose tissue by suppressing Sestrin2 and ULK1 (116). However, the specific signalling regulation has not been further investigated. More research is needed to identify novel targets linking autophagy and oxidative stress in adipogenesis.

5.2 The adipogenesis is regulated by autophagy-inflammation crosstalk

Autophagy represents a vital process for maintaining homeostasis, exerting diverse effects on the immune system. Notably, autophagy has the potential ability to regulate inflammation, which has systemic implications and directly impacts the development of both innate and adaptive immunity, thereby influencing various disease states and cellular physiological conditions (117). On one hand, the regulation of autophagy is influenced by pro-inflammatory cytokines. While on the other hand, it functions to restrain excessive inflammatory responses. Metformin and vitamin D modulate adipogenesis by impeding the formation of autophagosomes and suppressing adipose inflammation, thereby hindering adipogenesis in WAT and facilitating the differentiation of BAT. Nevertheless, this context's mutual regulation between autophagy and inflammation remains elucidated (118). Members of the Angiopoietin-like protein (ANGPTL) family serve as natural inhibitors of lipoprotein lipase and play a crucial role in lipoprotein and triglyceride metabolism in response to nutritional cues. ANGPTL8 has been implicated in NF-

κB-mediated inflammation, autophagy, adipogenesis, intracellular lipolysis, and circadian rhythm regulation. Inflammations can downregulate ANGPTL8 expression in human adipocytes by enhancing the levels of miR-221–3p. A strong association was observed in a murine model between the cycling of ANGPTL8 and lipopolysaccharide-induced acute inflammatory responses i. Further studies have verified that ANGPTL8 negatively regulates NF-κB activity by facilitating the kinase IKKγ autophagic degradation (119). Another bioflavonoid, Kaempferol, has been demonstrated to activate autophagy in osteoblasts through the inhibition of adipogenesis, reduction of inflammation and oxidative stress, as well as promotion of osteoclast autophagy and survival, thereby achieving osteoprotective effects. The mitigation of oxidative stress and inflammatory responses through autophagy represents a critical step in preventing and treating osteoporosis (120). Additionally, while neuregulin4 knockdown does not appear to impact adipogenesis, studies have revealed its detrimental effect on the insulin responsiveness of 3T3-L1 adipocytes. Moreover, neuregulin4 knockdown has modulated the expression of pro-inflammatory cytokines and autophagic degradation (121). Adipocyte-specific depletion of TBK1 mitigates obesity induced by a high-fat diet by augmenting energy expenditure. Subsequent investigations reveal that TBK1 directly impedes AMPK, suppressing respiration and enhancing energy accumulation (122). Conversely, activating AMPK under catabolic circumstances promotes the phosphorylation of TBK1 through its downstream effector, ULK1. More surprisingly, TBK1 knockout also exaggerates adipose tissue inflammation and insulin resistance. TBK1 exerts its anti-inflammatory effects by phosphorylating and provoking the degradation of the IκB kinase NIK, consequently inhibiting NF-κB activity. In addition, TBK1-mediated AMPK activity hurt NF-κB activation. These studies highlight a significant interplay between inflammation, autophagy, and oxidative stress in adipogenesis. However, there remains a dearth of comprehensive investigations analysing the synergistic mechanisms contributing to adipogenesis. Based on this research, it is reasonable to speculate that mTOR, AMPK, FOXO1, and NRF2 signalling pathways may serve as pivotal nodes that interconnect autophagy, oxidative stress, inflammation, and adipogenesis (Figure 2).

6 Summary and perspectives

Adipogenesis is a process influenced by multiple factors. Studies have identified autophagy, oxidative stress, and inflammatory

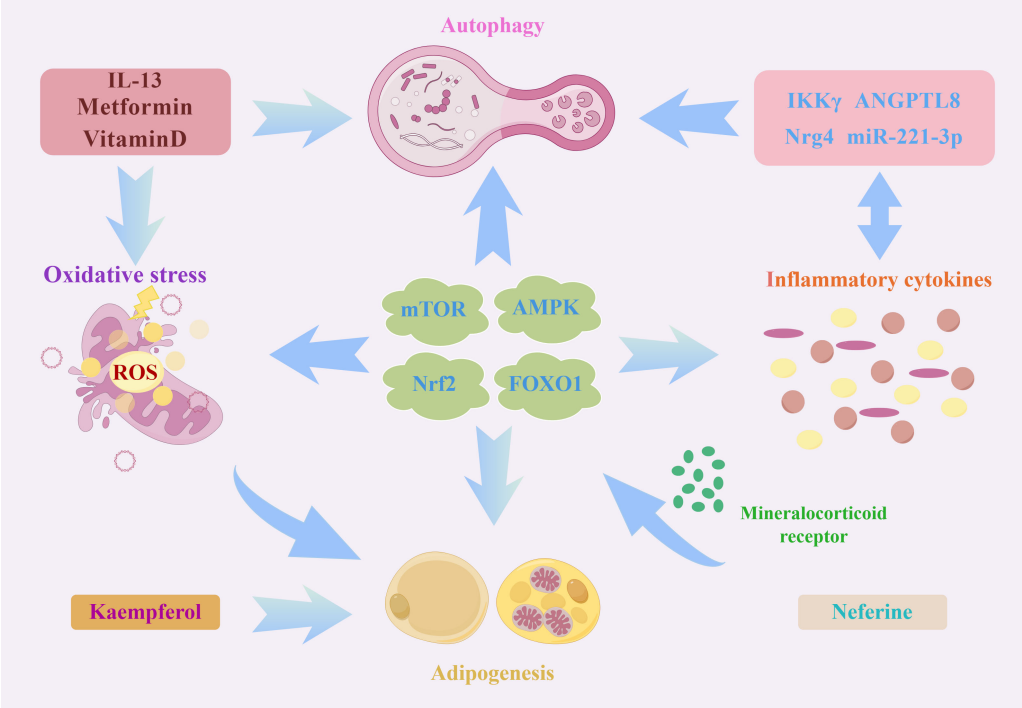

FIGURE 2
A schematic illustration depicting the intricate interplay between autophagy, oxidative stress, and inflammation in the process of adipogenesis. As an important intracellular energy receptor, AMPK is the most critical node linking autophagy, oxidative stress and inflammation. On the one hand, AMPK can affect the expressions of lipogenic genes (including SOCS3, SREBP1c, ACSL3 and ACSL5) through JAK2-STAT3 signalling. On the other hand, AMPK can regulate different autophagic processes through specific phosphorylation regulation of ATGs, such as ULK1 and Beclin-1. In addition, AMPK affects the expression of various oxidation-related proteins such as Nrf2, SOD and UCP2. Moreover, the activated AMPK is also involved in ameliorating inflammation by mediating NF-κB activation. Similarly, Nrf2 and PI3K/Akt/mTOR signalling pathways are also worthy of attention. Several molecules, such as MR and neferine, affect the autophagy-oxidative stress-inflammation axis through these pathways. In addition, some autophagy proteins, ATG7, TP53INP2 and autophagy negative regulator Rubicon, can regulate adipogenesis by impacting PPARγ activation. All these signals and molecules are important factors in the regulation of autophagy and redox equilibrium and participate in the transcription of many lipogenic genes and pro-inflammatory cytokines.

factors as key regulators of adipogenesis, which participate in regulating these pivotal signals. Autophagy protein ATG7, TP53INP2, and autophagy negative regulator Rubicon all impact PPARγ activation (63, 65, 123). Furthermore, cellular autophagic flux is a critical determinant of MSC adipogenic differentiation (124). In turn, these adipogenic factors can also regulate adipogenesis by targeting autophagy-related genes (such as Beclin-1, LC3B, and p62) and autophagy transcription factors (including FOXO1, TFEB, and XBP1) (57). Cytokines TNF-α, IL-4, IL-6, IL-7, and IFN-γ inhibit adipogenesis through diverse mechanisms, including activation of the Wnt/β-catenin/TCF-dependent pathway or inhibition of PPARγ and C/EBPs (8, 125). Furthermore, MCP-1 induces autophagy and endoplasmic reticulum stress during the initial phase of adipogenesis. This is accompanied by increased expression of adipocyte differentiation factors such as C/EBPs and PPARγ (126). Sustained ROS stimulation alters mitochondrial dynamics in adipocytes, decreases cellular respiration efficiency, and downregulates the expression levels of PPARγ, C/EBPα, and the lipogenic marker PLIN1 via Nrf2 or SIRT1 signalling, resulting in impaired adipogenesis (12, 13, 127). Inhibition of autophagy, oxidative stress, and inflammation reduces adipose tissue mass and impacts obesity. Moreover, there is evidence that autophagy inhibitors, ROS scavengers, and pro-inflammatory cytokine antagonists contribute to regulating adipogenesis and enhancing lipid metabolism (128–130). Therefore, combined targeting of autophagy, inflammation, and oxidative stress is a promising therapeutic avenue for obesity and its associated complications.

However, autophagy, inflammation and oxidative stress are double-edged swords in many physiological processes. Many lines of evidence show that autophagy, inflammation, and oxidative stress can either promote adipogenesis or induce dyslipogenesis. Although most research suggests that autophagy and oxidative stress predominantly facilitate adipogenesis, a few contrasting findings persist. One study found that when autophagy initiation-related protein ULK1 was knocked down in 3T3-L1. However, autophagy was significantly inhibited, adipogenesis was not affected, and the expression of transcription factors (C/EBPs and PPARγ) in adipose differentiation was not significantly changed. The study suggests that ATG5-dependent autophagy, rather than ULK1-dependent autophagy, may be critical for adipogenesis (26). Similarly, ROS can impede adipogenesis by heightening the inhibitory factor of lipogenesis, CHOP-10/GADD153 (85). Additionally, the influence of diverse inflammatory factors on adipogenesis exhibits considerable variability (8). Adipogenesis is a very complex process involving numerous events, such as cell fate determination, energy metabolism and physiological homeostasis. Based on the characteristics of adipogenesis and the results of many studies, we believe that the autophagy-oxidative stress-inflammation axis plays a key role during adipogenesis. Autophagy, oxidative stress, and inflammatory response are intricately linked, and their interplay may collectively dictate the course and fate of adipogenesis. Previous studies have usually focused on one of these points, ignoring the fact that adipogenesis is actually the result of multifactorial regulation. An integrated study of the modulation of adipogenesis by multiple factors will be the focus of the next study. Therefore, the identification of signalling pathways or molecules that can comprehensively regulate autophagy, inflammation, and oxidative stress will be important for elucidating the mechanisms of adipogenesis and accelerating the exploration of molecular targets for the treatment of obesity-associated metabolic disorders. Here, we point out that mTOR, AMPK, FOXO1, and Nrf2 may be promising targets that simultaneously affect autophagy, oxidative stress and inflammation during adipogenesis. Continued research into the specific mechanisms by which these nods integrally regulate adipogenesis is a significant work. A thorough exploration into the roles of autophagy, inflammation and redox homeostasis in adipose tissue and their interaction carries profound implications for elucidating the mechanisms of adipogenesis and expediting the exploration of molecular targets for treating obesity-related metabolic disorders.

Author contributions

CHo: Writing – original draft, Writing – review & editing. XL: Writing – original draft, Writing – review & editing. KZ: Writing – original draft, Writing – review & editing. QH: Writing – original draft, Writing – review & editing. BL: Writing – review & editing. HX: Writing – review & editing. BH: Validation, Writing – review & editing. FM: Writing – review & editing. XZ: Writing – review & editing. DT: Writing – review & editing. CHu: Writing – review & editing. CT: Writing – review & editing. LJ: Writing – review & editing. YC: Writing – review & editing. HW: Writing – review & editing. BD: Writing – review & editing. SW: Writing – original draft, Writing – review & 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.

Publisher's note

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The emerging role of oxidative stress in inflammatory bowel disease

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Inflammatory bowel disease (IBD) is a chronic immune-mediated condition that affects the digestive system and includes Crohn's disease (CD) and ulcerative colitis (UC). Although the exact etiology of IBD remains uncertain, dysfunctional immunoregulation of the gut is believed to be the main culprit. Amongst the immunoregulatory factors, reactive oxygen species (ROS) and reactive nitrogen species (RNS), components of the oxidative stress event, are produced at abnormally high levels in IBD. Their destructive effects may contribute to the disease's initiation and propagation, as they damage the gut lining and activate inflammatory signaling pathways, further exacerbating the inflammation. Oxidative stress markers, such as malondialdehyde (MDA), 8-hydroxy-2'-deoxyguanosine (8-OHdG), and serum-free thiols (R-SH), can be measured in the blood and stool of patients with IBD. These markers are elevated in patients with IBD, and their levels correlate with the severity of the disease. Thus, oxidative stress markers can be used not only in IBD diagnosis but also in monitoring the response to treatment. It can also be targeted in IBD treatment through the use of antioxidants, including vitamin C, vitamin E, glutathione, and N-acetylcysteine. In this review, we summarize the role of oxidative stress in the pathophysiology of IBD, its diagnostic targets, and the potential application of antioxidant therapies to manage and treat IBD.

KEYWORDS

inflammatory bowel disease, oxidative stress, antioxidant therapy, oxidative stress markers, IBD treatment

1 Introduction

Inflammatory bowel disease (IBD) is a condition that affects the digestive system and is caused by a chronic immune response. There are two main types of IBD: Crohn's disease (CD) and Ulcerative colitis (UC). The CD is characterized by the discontinuous pattern of the ileum and colon caused by transmural inflammation, while UC occurs only in the colon

and rectum. It solely affects the mucosa (1). There has been a significant rise in the prevalence of IBD in the past few years (2). According to estimates, 3.7 million people in American and European populations have IBD (3). It has been reported that IBD situations in Asia are more severe than in the West (4). Although the exact cause of IBD is still unknown, it is believed to be due to a complex interplay of genetic, environmental, and immunological factors (5). The treatment for IBD is constantly evolving, and researchers continually explore new therapies to improve patient outcomes. Currently, there is no cure for IBD, but several new medications and treatment approaches are being developed, including targeted therapies (6) and personalized medicine (7). Biologics, which target specific molecules involved in the inflammatory process, have shown great promise in the treatment of IBD (8). Additionally, stem cell therapies (9) and fecal microbiota transplantation (FMT) (10)are being studied as potential treatments for IBD. Since IBD is considered a significant global public health problem (11), There is an urgent need to explore its pathogenesis and new effective treatment options (12).

An increasing amount of evidence derived from both clinical investigations and experimental models indicates that oxidative stress signaling contributes to the development of IBD through various functional pathways. The term' oxidative stress', first introduced by Helmut Sies in the late 1980s (13), occurs when the production of oxidants exceeds the antioxidant defenses, leading to potential damage to biological systems (14). Oxidative stress is commonly viewed as detrimental to the body because it has the ability to harm cells, DNA, and proteins. Despite its harmful effects, some degree of oxidative stress is crucial for multiple physiological processes such as cellular signaling and immune response. Oxidative stress can damage the RNA machine involved in transcription and translation in bacteria, a critical function in bacterial survival (15). Similarly, studies indicated that protective mechanisms against oxidative stress within the bacterial cell envelope are essential for the cell's survival (16, 17). In cancer cells, oxidative stress can act as a stimulus for inducing cell death; ROS can trigger the process of apoptosis in cancer cells by causing damage to crucial cellular components like DNA, proteins, and lipids (18). The stimulation of tumorigenesis and proliferation of cancer cells may occur due to low levels of ROS. Conversely, high levels of ROS can induce cell death (19). Therefore, oxidative stress can either be detrimental or advantageous to pathogens and cancer cells, contingent upon the concentration of ROS and the situation in which it manifests.

In IBD, evidence suggests that oxidative stress plays a crucial role in the onset and progression of the disease (20). Chronic intestinal inflammation is known to cause an overproduction of ROS and RNS, which in turn causes oxidative and nitrosative stress, respectively. These two types of stress have been linked to several human disorders, including IBD (21). Oxidative stress causes GI tract mucosal layer degradation and bacterial invasion, which triggers the immune system and leads to IBD (22). These features show that oxidative stress is a potential agent in the pathogenesis of IBD.

Over the past decades, extensive research has been conducted to understand the mechanisms underlying oxidative stress in IBD. Several studies have identified various sources of ROS and RNS in IBD, including neutrophils, macrophages, and inflamed intestinal tissue (23). The oxidative stress mechanisms may include increased ROS production, impaired antioxidant defense, biomolecule damage, mitochondrial dysfunction, epithelial cell damage, and the activation of inflammatory pathways (24–26). Considering these observations, multiple treatments that involve antioxidants, such as dietary modification, organic antioxidants, and drugs, have been suggested to decrease oxidative harm and alleviate inflammation in individuals with IBD. The prospect of oxidative stress in IBD means that antioxidant therapy may be a potential strategy for managing and treating IBD. However, further research is needed to fully understand oxidative stress's role in IBD and determine the most effective antioxidant interventions. This review aims to summarize the mechanisms of oxidative stress, its role in the development of IBD, and the applications of oxidative stress in the diagnosis and therapeutics of IBD.

2 Mechanism of oxidative stress in IBD

While the precise mechanisms responsible for the development of IBD remain unclear, it is widely accepted that multiple factors contribute to its etiology, including overproduction of ROS, damage to biomolecules, mitochondrial dysfunction, recruitment of immune cells, impaired antioxidant defense system, and the activation of inflammatory pathways (Figure 1).

2.1 Overproduction of ROS

One hallmark feature of IBD is the overproduction of ROS, which causes dysregulation of signal transduction, an inflammatory response, and DNA damage, all of which contribute to the progression and deterioration of the disease (27). ROS include superoxide ($O_{2)}$, nitric oxide (NO), hydroxyl radical (-0H), hydroperoxyl radical (O_2H), hydrogen peroxide (H_2O_2), and singlet oxygen (28). ROS are highly reactive molecules that occur naturally as byproducts of cellular metabolism and aerobic respiration. These compounds significantly impact physiological functions such as cell differentiation, cell signaling, cell survival, and the creation of inflammatory factors (29). Proteins, lipids, DNA, and other macromolecules are all susceptible to oxidation by ROS, which can result in chemical changes and harmful outcomes (30). Under physiological conditions, aerobic metabolism produces ROS predominantly in the mitochondria. However, excessive ROS production can disrupt cellular homeostasis, resulting in severe oxidative damage (28). In IBD, an imbalance in the redox system is caused by excessive production of ROS in colonic tissues, shown by oxidative changes to lipids, proteins, or DNA (31). The excessive production of ROS and the resulting disruption in the redox balance can give rise to oxidative stress, characterized by an increased presence of oxidative free radicals and ROS, which is closely linked to chronic inflammation and the development of metabolic diseases (24). Oxidative stress caused by ROS overproduction can contribute to the pathogenesis of IBD by damaging cellular

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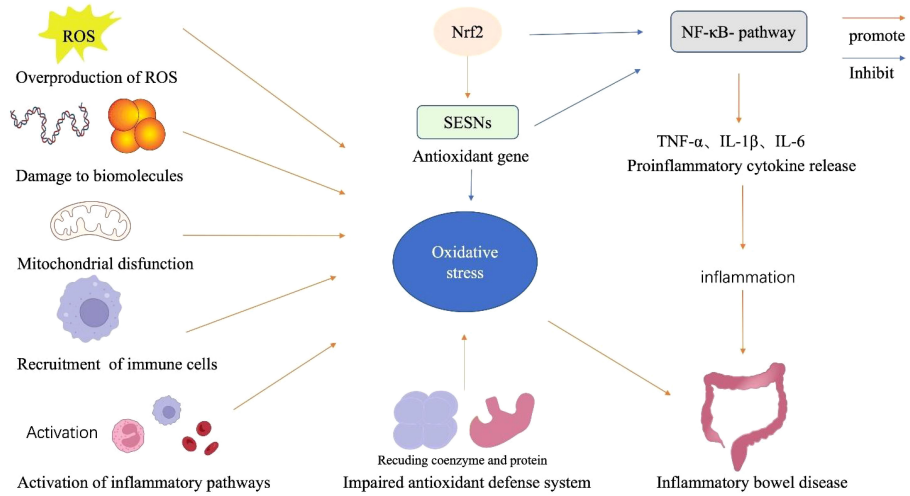

FIGURE 1
Components of the oxidative stress mechanism in IBD. The alteration of mitochondrial dysfunction, overproduction of ROS, damage to biomolecules, immune cell recruitment, and impaired antioxidant system participate in the mechanism of oxidative stress condition in IBD. Oxidative stress also triggers NF-κB activation and enhances inflammatory responses, a vital pathological component of IBD. Additionally, Nrf2 increases a variety of genes, allowing renal cells to act as antioxidants and reducing the production of cytokines and adhesion molecules that promote inflammations.

components, activating proinflammatory signaling pathways, and impairing the intestinal epithelial barrier. For instance, high levels of ROS produced by inflammation are essential for activating macrophages in a way that further promotes inflammation (32). This activation results in the continuing release of proinflammatory mediators like IL-1β, IL-6, TNF-α, and IFN-γ, as well as increased levels of ROS (33). Hence, the interactions caused and perpetuated by the overproduction of ROS within the proinflammatory milieu lead to a self-reinforcing vicious loop that significantly contributes to the pathogenesis of IBD.

2.2 Damage to biomolecules

ROS can damage various cell biomolecules, including lipids, proteins, and DNA. Uncontrolled lipid peroxidation leads to harmful lipid peroxidation products. Lipid peroxidation occurs when free radicals attack and damage cell membrane lipids, particularly PUFAs (34). ROS interact with PUFAs, forming lipid radicals, which react with molecular oxygen to create lipid peroxyl radicals. The hydrophobic tails of unsaturated fatty acids receive a hydroperoxy group during lipid peroxidation. This change may affect the structural properties of biomembranes and lipoproteins by interfering with hydrophobic lipid-lipid and lipid-protein interactions or cause the production of hydroperoxyl radicals and reactive aldehyde derivates, which may result in secondary modifications of other cell components (35). In IBD, ROS, including O2−, H_2O_2, and •OH, can initiate lipid peroxidation by oxidizing PUFAs in the cell membranes of intestinal epithelial cells; this process leads to the formation of lipid peroxides, such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which are highly reactive and cause cellular damage and inflammation (36). The elevated production of lipid peroxidation products in IBD can have several detrimental effects. For instance, 4-hydroxynonenal treatment reduces tight junction protein expression in the colon, boosts bacterial movement from the gut to circulation, and intensifies Toll-like receptor-4 signaling activation (37). Lipid peroxides damage cell membranes, disrupting the intestinal epithelial barrier and increasing permeability, allowing harmful substances and bacteria to enter underlying tissue and triggering an immune response (38). These products also activate inflammatory pathways in IBD, inducing the expression of proinflammatory cytokines, chemokines, and adhesion molecules (39).

Protein oxidation plays a critical role in the development of IBD (40). Proteins are central to cellular structure and function, and their optimal activity relies on proper folding and maintenance of sulfhydryl groups. Oxidative stress disrupts this equilibrium, leading to protein oxidation. It involves the reaction of proteins with ROS or RNS, resulting in the modification of amino acid residues, changes in protein conformation, and altered protein functions. For instance, the two most studied sulfur-containing amino acids in proteins, cysteine, and methionine, can undergo oxidation and induce changes in protein conformation (41–44). One study reported *HP1021* as a redox switch protein identified in *Helicobacter pylori*; the study shows that cysteine residues in *HP1021* are easily oxidized under cellular and laboratory conditions. This oxidation impacts the protein's capacity to attach to DNA, and the oxidative state of the regulatory domain influences this connection with DNA (45). Hence, *HP1021* is a redox switch

protein and could be a target for *H. pylori* control strategies. Besides, protein undergoes carbonylation, another form of protein oxidative modification in which ROS groups bind to specific amino acids, affecting protein stability and activity (46). It can be produced by oxidative cleavage of the Protein's backbone or by the attack of ROS radicals on some specific amino acids in the side chains, such as lysine, arginine, proline, and threonine. Significantly, ROS can modify amino acid residues in proteins, causing structural changes and loss of enzymatic activity (47). This process can also lead to the formation of protein aggregates and disruption of normal cellular functions.

Furthermore, ROS/RNS can directly attack DNA, causing oxidative DNA damage in IBD (41). High amounts of DNA lesions can be sustained over time because cellular repair systems are compromised by prolonged exposure to oxidative stress (42). The oxidative DNA damage is more dangerous to cells because it affects the cell cycle and can lead to mutations and cancer (48). Numerous studies have shown that DNA damage plays a major role in other chronic diseases, such as various cancers, neurodegenerative diseases, inflammation/infections, aging, and cardiovascular disease (49). It has been reported that individuals with IBD exhibit elevated oxidative stress and DNA damage, particularly in lymphocytes, as observed through studies using the comet assay technique (50). The IBD patients' DNA damage in peripheral blood lymphocytes is significantly higher, indicating that basal DNA damage may be related to insufficient antioxidant capacity and excessive ROS/RNS generation, contributing to the IBD disease's pathogenesis.

2.3 Mitochondrial dysfunction

Mitochondria, with their functions in energy production, calcium homeostasis, and membrane excitability, are thought to substantially impact the pathology of IBD, as their dysfunction may initiate and advance the disease. The intestinal tract harbors abundant bacteria along with their metabolic byproducts, immune-activating molecules, molecules associated with cellular damage (DAMPs), foreign substances, and environmental pollutants capable of harming mitochondria (51). A study has reported that mitochondria in the intestinal epithelium exhibit unique protein profiles (52). Notably, these mitochondria show increased expression of ATP-binding cassette transporters, which is likely a response to the specific requirements of the gut environment (53). Additionally, it's important to note that the gut, unlike other body parts, heavily depends on the gut microbiota for energy and the well-being of enterocytes containing mitochondria (54). Interactions with harmful bacteria such as adherent-invasive *E. coli LF82* disrupt the functioning of mitochondria in the cells lining the gut. This disrupts the balance of mitochondrial regulation mechanisms due to their strong connection with the gut's microbial community (55). Animal models that lack genes responsible for protecting the gut's epithelium, such as Mdr1a−/−, Irgm1−/−, and Sod2−/− transgenic mice, exhibit an increased presence of impaired mitochondria in intestinal cells and are more susceptible to experimentally induced colitis (56). Notably, 5% of the IBD genetic factors from human GWAS relate to mitochondrial balance. The leading gene associations are *SLC25A28 (mitoferrin 2), VARS (valine-tRNA ligase), and RNF5 (E3 ubiquitin ligase).* These genes control mitochondrial iron, tRNA transport, and ubiquitination (57–62). In addition, there is a connection between IBD and the *HSPA1-A, -B*, and *-L genes*, responsible for heat-shock protein 70, a key player in the mitochondrial unfolded protein response (63).

Similarly, mitophagy genes such as *PARK7* and *LRKK2* are linked to UC and CD, respectively (64, 65). Single nucleotide variations in the *C13orf31 gene*, resulting in amino acid changes in *p.C284R* and *p.I254V* in a protein of unidentified function, play a role in the development of systemic juvenile idiopathic arthritis and are associated with heightened susceptibility to leprosy and CD (66).This suggests that individuals with IBD may have an inherent vulnerability to mitochondrial dysfunction, particularly influenced by the gut environment. Additionally, intriguing connections with genes like *mitoferrin 2* hint at potentially specific pathogenic issues that remain incompletely understood. Variations in genes related to maintaining mitochondrial balance are strongly linked to the susceptibility to CD and its clinical progression. These genes include *SLC22A5*, which encodes *OCTN2, IRGM* and *UCP2* (67–69). Similarly, a study on the proteome of children with CD found that the function of mitochondria was compromised. This was particularly evident in the mitochondrial proteins responsible for detoxifying H_2S, and this downregulation was associated with a higher disease severity (70). Again, a study documented that examinations of the mitochondria within epithelial cells of CD patients revealed disrupted and irregular mitochondrial structures, suggesting impaired function (71). These changes occur before other early inflammatory events, like modifications to tight junctions that regulate barrier function. Notwithstanding, the precise ways in which mitochondrial dysfunction affects the development of IBD are currently being studied. However, it is speculated that the impact of mitochondrial dysfunction on energy metabolism, calcium control, and membrane excitability can interfere with intestinal homeostasis, weaken immune responses, and cause the chronic inflammation seen in IBD (72).

2.4 Recruitment of immune cells

Within the gastrointestinal (GI) tract, the innate immune system comprises epithelial cells, neutrophils, macrophages, dendritic cells, and natural killer (NK) cells (73). In contrast, the adaptive immune system includes T lymphocytes and B cells. When activated, T lymphocytes and B cells release cytokines and antibodies (74). Under normal conditions, there is a well-regulated equilibrium in the GI mucosa between inflammatory cytokines (such as TNF-α, IL-1, IL-6, IL-8, IL-17, and IL-23) and anti-inflammatory cytokines (like IL-5, IL-10, IL-11, and TGF-β) (75). IBD impacts both innate and adaptive immunity. However, in the case of CD, it's important to note that while adaptive immunity can perpetuate inflammation, it doesn't trigger the initial inflammation (64). The root cause of IBD

pathogenesis involves a disruption in the equilibrium between T helper (Th) cells and regulatory T cells, emphasizing the inability of regulatory T cells to function effectively.CD is characterized by inflammation driven by Th1 cells, resulting in an overproduction of IL-12, IL-17, and IL-23 (65). In contrast, UC is primarily influenced by cytokines like IL-4, IL-5, IL-10, and IL-13 produced by Th2-type T cells. In CD, the microbiota triggers the Th1 response, leading to the release of IFN-γ and TNF-α, ultimately resulting in damage to the mucosal barrier (76). In patients with IBD, the intestinal lining is frequently exposed to numerous environmental stressors, such as microbial antigens and inflammatory cytokines. In response to these triggers, immune cells such as neutrophils, monocytes, macrophages, and T cells are recruited to the inflamed mucosal tissue. This recruitment is orchestrated by chemokines, adhesion molecules, and other signaling molecules released by the inflamed tissue (77).Understanding the complex interplay between immune cell recruitment and oxidative stress in IBD is crucial for developing targeted therapies. Strategies to modulate immune cell infiltration and ROS production could potentially mitigate oxidative stress and limit tissue damage in IBD patients.

2.5 Impaired antioxidant defense system

Antioxidants play a major role in mitigating ROS's effects to maintain the body's redox balance. Antioxidants shield cells from harmful and unstable molecules by employing processes that eliminate them, thus preventing the oxidation of endogenous or non-endogenous molecules. Endogenous substances found within cells can be categorized into enzymatic antioxidants, which include superoxide dismutase (SOD), catalases (CAT), and peroxidases, or non-enzymatic antioxidants, which encompass tocopherol, glutathione, and ascorbic acid (78). Within the group of natural antioxidants, glutathione in its reduced form (GSH) primarily functions to eliminate reactive oxygen intermediates and free radicals generated during metabolic processes (79). GSH serves as a substrate for the antioxidant enzyme GPx and helps remove reactive species. It transforms into its oxidized form, GSSH, and can be converted back to GSH by glutathione reductase. Excessive free radicals can slow down this process, causing GSSH to accumulate in the cell (80). SOD is an antioxidant enzyme responsible for facilitating the conversion of the highly reactive superoxide anion (O_2^-) into less reactive molecules, specifically O2 and H_2O_2 (81). Similarly, CAT and GPx facilitate the conversion of H_2O_2 into water (82). Moreover, several of the antioxidant genes are recognized to have genetic variations, which can lead to differences in enzyme activity and responsiveness (83). Certain genetic variations in antioxidant enzyme genes have been linked to specific diseases, with certain genetic profiles related to a higher vulnerability to oxidative stress (84). Inadequacies in antioxidant enzymes or their compromised metabolism will increase the concentration of reactive oxygen species (ROS) and, in essence, cause oxidative stress. Research has shown that the levels of antioxidant defenses, as assessed through activities of SOD, catalase, and glutathione peroxidase, are naturally minimal within the colon and are primarily limited to the epithelial cells (85).

2.6 Activation of inflammatory pathways

The two main transcription factors that control cellular reactions to oxidative stress and inflammation are nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and nuclear factor-κB (NF-κB) (86). The coordination of the NF-κB signaling pathway and the Nrf2 pathway plays a crucial role in driving the complex process of oxidative stress in the context of IBD.

2.6.1 Nrf2 signaling

Nrf2 is a crucial transcription factor that plays a significant role in preserving mucosal balance by inhibiting the excessive production of ROS in IBD.Nrf2 has a dampening effect on the inflammatory response and the damage to the mucosal lining through its antioxidant actions (87). Numerous studies reveal that Nrf2 benefits cell survival and proliferation in various ways, ranging from redox homeostasis and drug/xenobiotic metabolism to DNA repair (88). Nrf2 controls the expression of several enzymatic antioxidants and results in the modulation of the levels of ROS, such as SOD, CAT, GPx, and heme oxygenase-1 (HO-1), which are vital in maintaining redox balance and cellular homeostasis. Nrf2 is a transcription factor sequestered in the cytoplasm by its inhibitor protein, Keap1, under normal conditions (89). When cells are exposed to oxidative stress, Nrf2 is released from Keap1 and translocates into the nucleus. Once in the nucleus, Nrf2 binds to antioxidant response elements (AREs) in the promoter regions of target genes. This leads to the transcription of genes involved in detoxification, antioxidation, and anti-inflammatory responses (Figure 2) (90). Nrf2 genes encode enzymes like heme oxygenase-1 (HO-1), NAD(P), H quinone dehydrogenase 1 (NQO1), and glutathione S-transferases (GSTs) (91). A recent study shows that upregulation of Nrf2 gene expression in a mice experiment led to increased NQO-1 protein content and activity, as well as elevated HO-1 protein content and activity in the brain, while in the liver, HO-1 activity and mRNA levels, NQO-1 activity, and protein content were augmented (92).This study reveals the tissue-specific regulation of Nrf2 signaling and downstream antioxidant enzymes in the mice, highlighting its adaptive response to varying oxygen concentrations. Similarly, in Nrf2-KO mice, there are higher levels of proinflammatory genes such as *IL-1β, IL-6, IL-8, iNOS*, and *COX-2*, and a decrease in the expression of antioxidant enzymes like hemeoxygenase-1 and GST Mu-1 (93). The activation of IER3 in the mucosa downregulates Nrf2 through the PI3K/Akt pathway, leading to decreased ROS production and apoptosis in a colitis model, which keeps Nrf2 levels low in IBD (94). Conversely, Nrf2 has been documented to reduce NOX activation and inhibit protein kinase C, consequently leading to a decrease in ROS levels and production in Nrf2-KO mice due to elevated antioxidant GSH levels, highlighting its role in mitigating oxidative stress.

2.6.2 NF-κB signaling

NF-κB is a key regulator in many pathogenic processes and is abnormally active in IBD. It is a central regulator of immune and inflammatory responses (95). It controls gene transcription in immune activation, cytokine production, cell survival, and inflammation. The NF-κB pathway is a complex signaling cascade that regulates the expression of various genes involved in immunity, inflammation, and cell survival (96). It comprises a group of transcription factors linked to

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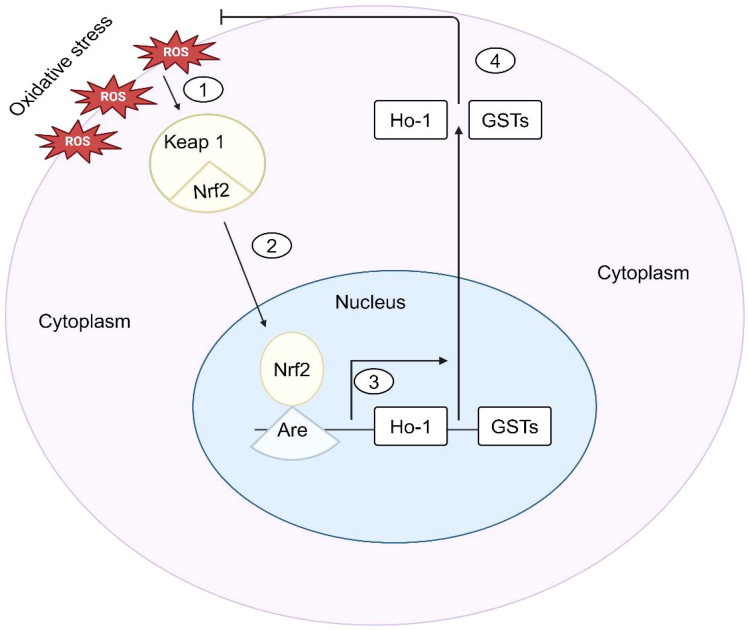

FIGURE 2
Nrf2-Mediated signaling in response to oxidative stress in IBD. (1) Upon sensing oxidative stress, cells phosphorylate Nrf2, which is normally sequestered in the cytoplasm by kelch-like ECH-associated protein 1 (Keap1). (2) The antioxidant response element (ARE) of the antioxidant genes is then bound by Nrf2 when it translocates into the nucleus. (3) HO-1 and GSTs are examples of antioxidant genes whose transcription is stimulated by Nrf2. (4) The antioxidant genes are then expressed, which prevents oxidative stress and keeps cells' redox balance.

IκBs inhibitor proteins and remains dormant in the cytoplasm. ROS can activate the NF-κB pathway by promoting the degradation of IκBs, allowing NF-κB to translocate into the nucleus and initiate gene transcription (Figure 3) (97). Numerous studies have demonstrated the involvement of the NF-κB pathway in the development of IBD. NF-κB is linked to IEC homeostasis and can alter the permeability of the intestinal layer and intensify the chronic intestinal inflammation observed in the mucosa of UC and CD patients (98). Prolonged NF-κB signaling can exacerbate the persistent inflammation seen in UC and CD patients (99). In intestinal epithelial cells, the activation of toll-like receptors (TLRs) and the recognition of TNF-α by these receptors initiate the downstream NF-κB signaling pathway. This signaling pathway is also vulnerable to the effects of oxidative stress.

Additionally, the activation of NF-κB leads to increased expression of key genes, including proinflammatory cytokines like IL-6, IL-8, IL-16, and TNF-α, which contribute to inflammation. NF-κB activation upregulates genes involved in cell survival and proliferation, such as PUMA, leading to epithelial cell apoptosis and contributing to UC development (100).

3 Components of oxidative stress and their contributions to IBD pathogenesis

3.1 ROS in IBD pathogenesis

The superoxide anion (O2•−), which is produced when molecular oxygen gains one electron, is the most prevalent free radical in human tissues (101). Complexes I and III of the mitochondrial electron transport chain, which transforms 1–3 percent of total oxygen into the superoxide anion, is the primary source of O2•−in a cell (102). An additional source of O2•− is an enzymatic reaction that is catalyzed by membrane enzyme complexes known as NADPH oxidases (NOX) and xanthine oxidase (XO) (103). Out of the five isoforms of the NOX family, colon epithelium expresses NOX1 at a high level (104). Numerous studies have shown the vital role of NOX1 in IBD pathogenesis. A previous study shows the overexpression of NOX1 in large and small bowel cancers in humans (105). NOX1 expression correlates with RAS mutational status in colorectal cancer, and immunohistochemical analysis indicates overexpression in specific cancers, emphasizing its relevance as a therapeutic target in colorectal and small intestinal cancer. In IBD, analyses of biopsies from patients with CD showed increased JNK1/2 activation, as well as NOX1 and Lipocalin-2 (LCN-2) expression (106). This indicates that NOX1 might play a key role in mucosal immunity and inflammation by controlling LCN-2 expression. Again, this recent study (107) explores the impact of NOX1 loss-of-function mutations on IBD. TNF-α induces higher ROS production in NOX1-WT colonoids than NOX1-deficient ones, affecting the stem cell niche and cell. It emphasizes ROS modulation for future IBD therapies. Notably, NOX1 is vital in IBD. A recent study highlights NOX1's involvement in peroxynitrite production induced by the microbiota. Certain NOX1 variants, such as *NOX1 p. Asn122His*, are linked to impaired gut barrier function. The research further examines the structural dimension, indicative of a crucial asparagine residue in the NOX1-p22phox complex, vital for

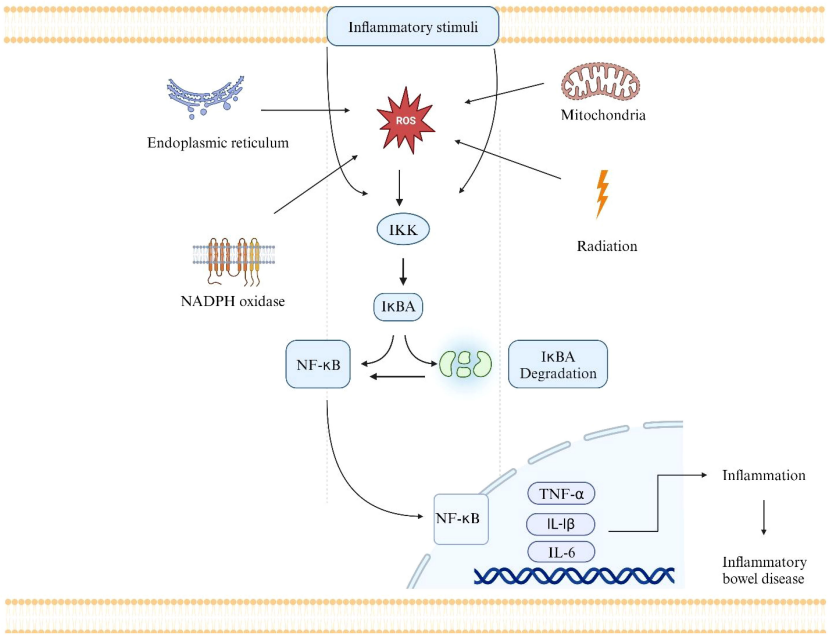

FIGURE 3
The NF-κB pathway as one of the mechanisms by which ROS can contribute to the pathogenesis of IBD. Radiation, mitochondria, NADPH oxidase, and Endoplasmic reticulum are the main sources of ROS.ROS activates the IKK complex. The IKK phosphorylates IkBA, which leads to its degradation.NF-κB is activated and translocated into the nucleus. NF-κB binds to particular DNA sequences and triggers the transcription of many different genes, including those that produce adhesion molecules, pro-inflammatory cytokines, and other inflammatory mediators (e.g., TNF-α, IL-6, and IL-1).The transcription of pro-inflammatory genes leads to the production of inflammatory cytokines and other molecules that contribute to the inflammatory response in the intestinal mucosa. Immune cells like neutrophils and macrophages are drawn to the area of gut tissue inflammation by these inflammatory signals. The influx of immune cells and the ongoing inflammation can result in tissue damage, ulceration, and the chronic inflammation that characterizes IBD.

the electron transfer process in human NADPH oxidases (108). Similarly, NOX1 facilitates the transmembrane electron transfer to two molecular oxygens, forming when activated. It has been proposed that NOX1-induced O2•− at the colon's luminal surface Influence various processes such as bacterial virulence, expression of bacteriostatic proteins, epithelial renewal, restitution, and microbiota composition to control the intestinal innate immune defense and homeostasis (109). NOX1 and NOX4 have been linked to the pathologies associated with the hepatitis C virus as long-lasting, endogenous ROS generators (110). Besides, NOX4 has been specifically linked to oncogenic H-Ras- (H-RasV12-) induced DNA damage and senescence, suggesting a potential role in HCV-related oncogenesis (111).

Moreover, oxidative stress causes an increase in O2•− concentrations, which triggers the Haber-Weiss reaction and excessive production of the harmful hydroxyl radical (OH •). A Fenton reaction catalyzed by Fe^{2+} and H_2O_2 also produces the hydroxyl radical (112). Other transient metals, such as copper, chromium, or cobalt, may contribute to the generation of OH• in place of ferrous metals. These reactions become a significant source of OH• in the presence of oxidative stress conditions or when the concentration of free, unbounded transient ions rises, as in the case of hemodialysis. The OH• depolymerizes GI mucin, inactivates pyruvate dehydrogenase, an essential mitochondrial enzyme, and damages mitochondrial RNA and DNA in the GI tract (113). The perhydroxyl radical (HOO•) is another protonated form of O2•− that starts the peroxidation of fatty acids (114). Lipid peroxidation alters lipoproteins into pro-inflammatory forms, disrupts biomembranes' fluidity, permeability, and integrity, and produces potentially hazardous byproducts (115). Furthermore, it has been demonstrated that lipid peroxidation products have carcinogenic and mutagenic qualities (116). In addition to mitochondria, peroxisomes and plasma membrane NADPH oxidases are other sources of free radicals in cells. These organelles use oxygen to produce H_2O_2. Catalase (CAT) converts peroxisome-derived H_2O_2 to water and oxygen under physiological conditions (117). On the other hand, a damaged peroxisome contributes to oxidative stress by directly releasing H_2O_2 (118). In Fenton and Haber-Weiss reactions, H_2O_2 may be transformed, along with O2•−, into the highly toxic and oxidizing OH• hydrogen peroxide (119). XO is the primary source of O2•− in the GI tract. As a result, the reaction mediated by GPx and/or CAT transforms it to H_2O_2.MPO uses the H_2O_2 neutrophil produced to create hypochlorite ions (OCl −) (120). The O2•− is a highly unstable, highly reactive, and short-lived form of ROS and reacts quickly to become membrane-impermeable. As a result, it acts close to its source, oxidizing nearby biomolecules, while H_2O_2 can freely diffuse across cell membranes and oxidize molecules farther away, such as pathogen membrane lipids. Aquaporin-8 (AQP8) facilitates H_2O_2 diffusion in GI (121). This specific aquaporin isoform is crucial in controlling H_2O_2 membrane permeability and signaling, making it an essential player in redox signaling processes (122). These studies (123–125)

documented the involvement of AQP8 in modulating H_2O_2 transport through the plasma membrane, influencing redox signaling pathways associated with leukemia cell proliferation (126, 127). It's interesting to note that enterocytes exhibit varying baseline levels of ROS. The small intestine, for instance, maintains a lower concentration of ROS, whereas the colon has a higher concentration (128). The variations in ROS generation could affect the amounts of oxidized proteins, lipids, and DNA damage, increasing the colon's vulnerability to GI disorders at these two intestinal sites (129). Circulating XO binds to vascular endothelial cells in pathological states, causing site-specific oxidative damage to intestinal tissue (130). In IBD, a retrospective study documented XO activity concerning adverse effects from thiopurine therapy. The results indicated lower XO activity in patients experiencing adverse effects; the findings imply that monitoring XO activity might be useful in predicting and managing thiopurine-induced toxicities (131). Furthermore, O2•− is produced during a sequence of events known as "the respiratory burst" that activated neutrophils go through (132). Research has demonstrated that NOX enzymes, particularly NOX2, are involved in this process. This is because mice lacking NOX2 exhibit lower levels of oxidative burst and are less vulnerable to experimentally induced UC (133).

3.2 RNS in IBD pathogenesis

RNS comprise the second category of free radicals, produced as a byproduct of nitric oxide synthases(NOS) and expressed in specific intestinal submucosa and mucosal regions. Through a five-electron oxidative reaction, NOS converts arginine to citrulline and produces the nitric oxide radical (NO•) (134). Three main isoforms of NOS are inducible NOS (iNOS), which is present in various cells and tissues; endothelial NOS (eNOS), which is initially identified in vascular endothelial cells; and neuronal NOS (nNOS), which is discovered primarily in neural tissue (135). The iNOS continuously produces NO•, in contrast to the pulsative nature of eNOS. The overproduction of RNS in activated macrophages, leukocytes, and epithelial cells in the intestinal mucosa is caused by iNOS, which is only found in inflammatory tissue (136). Numerous studies have shown the involvement of NOS isoforms in IBD. According to this study (137), it has been shown that in UC, the activation of the iNOS/COX-2/5-LOX loop and increased levels of their end products, such as NO, prostaglandin E2 (PGE2), and leukotriene B 4 (LTB 4), which lead to the overproduction of free radicals and the impairment of the antioxidative system. For instance, this study found that patients with active IBD exhibited elevated mRNA expression of iNOS in intestinal biopsies, indicating increased inflammation. This suggests a specific role of iNOS in the inflammatory response associated with IBD, emphasizing its potential significance in understanding the disease's pathophysiology (138). Similarly, in an earlier study, the up-regulation of iNOS in the intestinal epithelial cells (IECs) has been closely associated with the initiation and maintenance of intestinal inflammation in IBD, which can be potentially used as a non-invasive blood-based biomarker of IBD, as documented (139). The role of iNOS in IBD is further complicated by its relationship with cytokines and pro-inflammatory cytokines, which upregulate iNOS expression (140). Furthermore, nitrotyrosine is produced when tyrosine and NO derived from iNOS react. Research has shown that patients with UC but not collagenous colitis exhibit strong nitrotyrosine-stained epithelium linked to neutrophil infiltration (141). Additionally, iNOS is among the central downstream genes of NF-κB, but, in turn, iNOS can promote and inhibit NF-κB activity (142).

On the contrary, the Enos isoform, which is localized to the microvasculature at the submucosa–mucosa interface, catalyzes the capillary recruitment of absorptive hyperemia. The vasodilatory actions of NO• play a prominent role in this process (143). Again, the nitric oxide radical reduces leukocyte adhesion to endothelial cells and shields epithelial cells from toxicity induced by H_2O_2 (144). Notably, the increased expression of the eNOS gene reduces the expression of adhesion molecules in endothelial cells, mitigates colitis induced by DSS in mice, and is associated with severe cases of UC, as documented (145). This suggests that eNOS could serve as both a potential prognostic marker and a target for therapeutic intervention. Besides, the nNOS isoform also plays a significant role in the pathophysiology of irritable bowel syndrome (IBS) and other gastrointestinal disorders, including IBD. For instance, this study (146) utilized a neonatal maternal separation stress model in mice to simulate irritable bowel syndrome (IBS) and identified neuronal nitric oxide synthase (nNOS) as a novel and reliable biomarker for interstitial cells of Cajal stimulation in IBS. This is further supported by studies (147), which identified deficits in nNOS neurons in various enteric neuropathies, including those associated with IBD.

Moreover, One mechanism through which RNS contributes to IBD pathology is the formation of Peroxynitrite(ONOO-), a highly reactive oxidant formed by the reaction of NO• with O2•− (148). ONOO-is produced by cells that contain NOS enzymes, such as smooth muscle or endothelial cells, as well as by stimulated leukocytes during an inflammatory response. ONOO- can induce damage to cellular structures, including lipids, proteins, and DNA, leading to oxidative stress and further exacerbating inflammation and tissue injury (149). Research elucidates a novel HMGB1-mediated inflammatory pathway in Non-Alcoholic Fatty Liver Disease (NAFLD), revealing a redox signaling mechanism where ONOO-, formed through NADPH oxidase activation, plays a pivotal role in TLR-4 activation and cytokine release (150). The findings highlight the significance of ONOO-as a key mediator in intestinal inflammation in NAFLD. In IBD, the increased production of NO•, coupled with excessO2•−, results in elevated ONOO-levels. Again, a recent study indicates that in an animal experiment, NOX1 plays a crucial role in the production of ONOO-in the intestines (151). Similarly, the impact of ONOO-on Na-amino acid co-transporters (*NaAAcT*) in rabbit intestinal villus cell brush border membrane during chronic intestinal inflammation. ONOO- inhibits Na-alanine co-transport (*ASCT1*) by reducing its

affinity for alanine and Na-glutamine co-transport (*B0AT1*) by decreasing co-transporter numbers, revealing potential mediation of *NaAAcT* alterations in inflammation (152).

3.3 Lipid peroxidation and lipid radicals in IBD pathogenesis

Both RNS and ROS can exacerbate lipid peroxidation. Because they are high in PUFAs, membrane lipids and lipoproteins are particularly vulnerable to oxidative damage. A hydroperoxy group is added to the hydrophobic tails of unsaturated fatty acids during lipid peroxidation. Through disruption of hydrophobic lipid-lipid and lipid-protein interactions, this change may result in structural changes to biomembranes and lipoproteins. Alternatively, it may cause the production of reactive aldehyde derivatives and hydroperoxyl radicals, which may cause secondary modifications to other cell components. Lipid peroxidation's byproducts, such as 4-hydroxynonenal or malondialdehyde, can react with lysine amino groups, histidine imidazole groups, or cysteine sulphydryl groups to damage proteins (153). These reactions can result in the formation of adducts, which can serve as biomarkers of oxidative stress and lipid modification. LOX enzymes, which catalyze the dioxygenation of polyenoic fatty acids to form hydroperoxides, are another source of lipid radicals. 5-LOXs play a significant role in the intestines by catalyzing the oxidation of arachidonic acid. GPx subsequently reduces the hydroperoxides produced by LOX enzymes (154). It has been documented that patients with CD have higher plasma levels of lipid peroxidation products, a decreased peroxidation potential, and an oxidative low-density lipoprotein level, particularly during an active phase of the disease (155). IBD patients experience lipid peroxidation, but the cause varies based on the type of IBD. The amount of lipid peroxidation products is associated with epithelial CAT expression and neutrophilic MPO activity in UC, suggesting an H_2O_2-and/or OCl-mediated mechanism. In CD, lipid peroxidation is associated with mitochondrial superoxide dismutase (SOD) activity, suggesting the involvement of OH• and O2•− (156). This is further supported by the finding that SOD activity is increased during active disease and returns to normal in remission (157).Additionally, the presence of oxidative damage and the inhibition of catalase, an antioxidant enzyme, in CD patients' immune cells further underscores oxidative stress's role in the disease (158). These results indicate a possible involvement of lipid peroxidation and SOD activity in the IBD.

3.4 Cytokines and signal pathways

Reduced cytokine synthesis, which inhibits T cell and macrophage activity, may be linked to the pathophysiology of CD. According to an earlier study, intestinal tissue from CD patients exhibits a reduced expression of IL-4 mRNA, a cytokine that postpones the formation of O_2•− in PMNS (159). Particularly in those who are genetically predisposed, external and environmental variables play a significant role in the start and progression of IBD, a complicated multifactorial illness. Similarly, IL-36, a cytokine that can induce fibrosis, is found at higher levels in fibrotic intestinal tissues of CD patients (160). Additionally, patients with CD have lower levels of antioxidative substances such as plasma ascorbic acid, α- and β-carotene, lycopene, and β-cryptoxanthin, as well as tissue GSH, which takes part in GPx-catalyzed H_2O_2 reduction (161). Antioxidative enzymes like GPx and SOD, however, tend to be dependent on the state of CD as well as the serum level; GPx activity is stable or reduced during CD remission and increases during active CD (157). In individuals with CD, oxidative stress occurs both locally and systemically. It is linked to the disease's well-documented dysbiosis and unbalanced immunological response. According to a previous study, the mice models of UC and CD demonstrate that the colon's up-regulation of GPx2 gene expression and down-regulation of aquaporin 8, which facilitates H_2O_2 diffusion, may protect against severe oxidative stress in IBD (162). In addition to IL-4, several other cytokines, including TNF-α, IL-1β, IL-6, and IL-8, contribute to CD (163). ROS and RNS are capable of inducing the release of these cytokines. It has been documented that patients with active CD had up-regulated NOS mRNA expression in their colonic mucosa and peripheral blood mononuclear cells (164). Similarly, it also suggested a positive correlation between NOS-derived NO• and plasma levels of IL-6, IL-17A, and IL-23 in Sjögren's syndrome(SS), as documented (165).

Also, some of the environmental risk factors linked to CD may be caused by oxidative stress. The precise etiopathology of CD is still unknown, but oxidative stress is widely acknowledged to play a critical role in the disease's pathogenesis. The aforementioned cytokines act through the mitogen-activated protein kinase (MAPK) and NF-κB signaling pathways. Aberrant NF-κB activation is implicated in the pathophysiology of IBD (166). The involvement of NF-κB and MAPK signaling pathways in IBD is shown in (Figure 4). NOX enzymes produce superoxide anion and other free radicals. The advanced glycation end products (AGE) content in the plasma membrane of epithelial cells is directly increased by the conversion of the superoxide anion to hydrogen peroxide by SOD3, as seen in (Figure 4). In summary, the NF-κB signaling pathway is activated by both AGE and NOX, as well as pro-inflammatory cytokines such as IL-6 or TNF-α. This leads to an increase in the expression of *caspase 3*, *ICAM, TNF-α*, or *IL-6 genes*. On the other hand, activation of MAPK improves the expression of AP-1 signaling molecules and increases the production of iNOS, the uninhibited source of NO. When combined, the inhibition of NF-κB or p38 MAPK may impact ROS/RNS production and reduce the generation of cytokines in patients with IBD, particularly when the disease is actively progressing (167).

Similarly, combining TLR4/NF-κB and Nrf2-ARE pathway modulation offers a comprehensive approach to managing IBD by simultaneously reducing inflammation and enhancing antioxidant defenses. For instance, a study showed Morningside from *Cornus officinalis* inhibits LPS-induced inflammation and oxidative stress in RAW 264.7 macrophages by blocking TLR4/NF-κB and activating Nrf2/HO-1 pathways (168). It reduces pro-inflammatory factors and ROS generation, and promotes HO-1 expression, suggesting its potential as an anti-inflammatory and antioxidant agent. Besides,

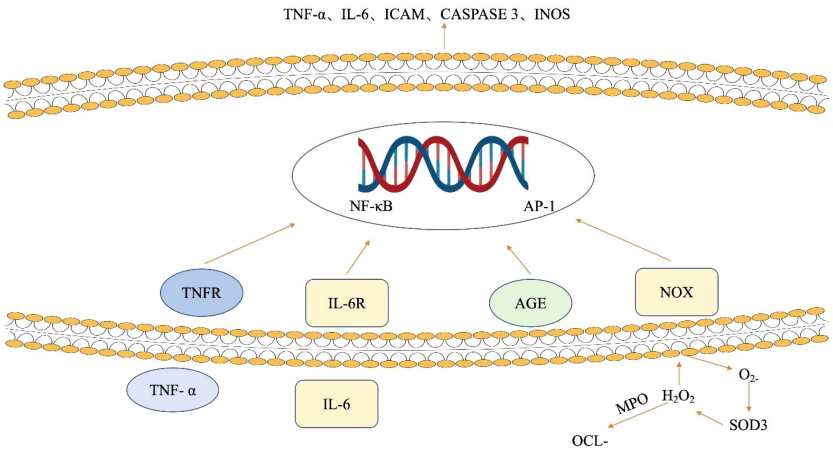

FIGURE 4
The influence of ROS and cytokines on signaling pathways in intestinal epithelial cells. NOX enzymes generate superoxide anion, elevating advanced glycation end product (AGE) in epithelial cell membranes. SOD3 converts superoxide to hydrogen peroxide, enhancing AGE content. Concurrently, NF-κB is activated by AGE, NOX, and pro-inflammatory cytokines (IL-6, TNF-α), triggering increased expression of caspase 3, ICAM, TNF-α, and IL-6 genes. Meanwhile, MAPK activation boosts AP-1 signaling and iNOS production. AGE advanced glycation end products, AP-1 activator protein 1, ICAM intracellular adhesion molecule, IL-6 interleukin 6, IL-6R interleukin 6 receptor, iNOS inducible nitric oxide synthase, NF-κB nuclear factor-kappa B, NOX NADPH oxidase, MAPK mitogen-activated protein kinases, OCl – hypochlorite ion, SOD3 extracellular superoxide dismutase, TNF-α tumor necrosis factor-alpha, TNFR tumor necrosis factor receptor.

Phosphoinositide 3-kinase (PI3K)/Akt signaling regulates cell survival and oxidative stress responses. Enhancing PI3K/Akt signaling can protect against oxidative damage in IBD. In UC mice, glutamine (Gln) reduced oxidative stress-induced injury by inhibiting the PI3K/Akt signaling pathway (169). The study showed that Gln administration improved superoxide dismutase and glutathione peroxidase activity, decreased malondialdehyde content, and ameliorated colitis symptoms and histological damage. These findings suggest that targeting oxidative stress via these molecular pathways like MAPK, TLR4/NF-κB, Nrf2, and PI3K/Akt may offer new therapeutic strategies for managing IBD.

4 Targeting oxidative stress in IBD diagnosis

IBD poses a significant challenge in gastroenterology due to its complex and multifactorial nature. The dysregulation of the immune system in the gut is a key factor in the development of IBD (170). Recent findings indicate that oxidative stress is crucial to the disease's onset and progression (108). A study (171) emphasized the critical role of oxidative stress in the development and progression of IBD, as well as the production of ROS and antioxidant defense systems. Similarly, the involvement of oxidative stress in sepsis, which disrupts redox signaling, leading to molecular damage, has been highlighted (172). The dysregulation between ROS and antioxidants contributes to sepsis progression, impacting cellular function and mortality. This emphasizes the role of ROS in sepsis pathophysiology and the potential of antioxidant adjunct therapies (172). These studies collectively contribute new insights into understanding the role of oxidative stress in IBD and potential therapeutic avenues targeting ROS-mediated pathways in the management of this condition. Presently, extensive research is being conducted to investigate the significance of oxidative stress in diagnosing IBD. These studies examine the importance of oxidative stress markers as essential indicators and explore their potential utility for diagnostic applications. ROS and RNS are key components of the oxidative stress event implicated in IBD (173). These molecules, produced at abnormally high levels in individuals with IBD, exert destructive effects on the intestinal lining. This damage not only contributes to the disease's initiation but also activates inflammatory signaling pathways, further amplifying the inflammatory cascade. Within this intricate web of events, oxidative stress markers (OSMs) such as malondialdehyde (MDA), 8-hydroxy-2'-deoxyguanosine (8-OHdG), and serum-free thiols (R-SH)emerge as valuable diagnostic targets for IBD diagnosis. MDA, an aldehyde with reactive properties formed through the peroxidation of polyunsaturated fatty acids, serves as a prominent OSM in IBD, reflecting lipid peroxidation and cellular damage and functions as a marker for cellular damage caused by free radicals (174). According to this study finding (175), there is a positive correlation between elevated lipid hydroperoxide (LOOH) levels and heightened MDA levels, suggesting MDA as a potential biomarker for lipid peroxidation and indicative of the influence of endogenous oxidative stress in individuals with CD. Numerous studies have consistently emphasized the increased levels of MDA in patients with IBD. This highlights MDA's role as a reliable marker for assessing oxidative stress in the IBD. For instance, Elevated levels of MDA in Tunisian patients, as observed in biopsies from individuals with CD, suggest the involvement of oxidative stress in the pathophysiology of IBD (176). Similarly, these studies (155, 177–179) show that MDA levels were higher in the serum and saliva of IBD patients. This specifies that MDA can be used as a valuable marker for assessing oxidative stress in these patients, with its levels positively correlating with disease activity and inflammation. MDA plays a crucial role in diagnosing and

tracking the effectiveness of treatment and the progression of the disease (175). Assessing MDA levels offers valuable information about the degree of lipid peroxidation and oxidative harm, helping guide therapeutic approaches. The substantial evidence from these studies solidifies MDA as a pertinent biomarker for oxidative stress in IBD, contributing to our understanding of disease pathogenesis and management.

On the other hand, 8-OHdG emerges as a crucial biomarker reflecting oxidative DNA damage (180). It is a modified form of guanine, one of the four nucleotide bases that make up DNA. The formation of 8-OHdG, a modified nucleoside, is a consequence of oxidative DNA damage, particularly guanine residues (181). It has been documented in a meta-analysis study and a systematic review that elevated levels of 8-OHdG in biological samples, such as urine, serum, or tissue, are indicative of increased oxidative stress (182, 183). Notably, Elevated levels of 8-OHdG in individuals with IBD have been consistently documented in research, indicating its promising role as a dependable OSM in the context of this disease (123–125, 158, 184). Similarly, a recent study revealed elevated levels of 8-OHdG in individuals with CD patients compared to those in the healthy control group (185). This suggests that 8-OhdG is a valuable indicator for evaluating oxidative stress in individuals with CD. The inflammatory milieu in IBD leads to enhanced ROS generation, causing DNA damage and subsequent 8-OHdG formation (186). Numerous studies have documented the association between elevated 8-OHdG levels and the severity of IBD symptoms, linking this marker to the progression of the disease. For instance, Assessing 8-OHdG levels provides valuable insights into the extent of oxidative damage, aiding in disease prognosis and therapeutic strategies (187). Again, elevated levels of 8-OHdG have been linked to disease severity in various conditions, including cardiovascular disease (188), chronic periodontitis (189), Huntington's disease (190), chronic kidney disease (191), and colorectal tumors (192–194). These findings suggest that 8-OHdG may serve as an OSM for disease activity and progression in various inflammatory and degenerative diseases. Utilizing 8-OHdG as a diagnostic instrument aligns with the increasing focus on personalized healthcare and targeted therapeutic strategies in the context of IBD (195). Moreover, interventions targeting oxidative stress, informed by 8-OHdG levels, could hold promise in mitigating IBD progression. 8-OHdG stands out as a robust marker for oxidative stress in IBD, offering a molecular insight into disease pathology and potential avenues for therapeutic interventions.

Besides, R-SH consistently indicates systemic oxidative stress because they are easily oxidized by ROS, making them a reliable biomarker for oxidative stress in IBD and other diseases (196, 197). For example, a study (182) examined oxidative stress in IBD and found that R-SH levels were significantly lower in IBD patients compared to healthy individuals. These free thiols, which indicate systemic oxidative stress, showed a strong correlation with endoscopic disease activity and were more effective in distinguishing disease severity than fecal calprotectin levels. Likewise, another research (183) documented significantly lower levels of R-SH in CD and UC patients compared to controls, with these lower levels correlating with increased inflammation severity and reduced in corticosteroid-treated patients, identifying systemic thiol stress as a key marker of oxidative stress and inflammation in IBD. Additionally, a recent study (198) suggests that R-SH, an OSM, could serve as a biomarker for IBD, proving more sensitive than C-reactive protein (CRP) in detecting moderate endoscopic activity, though less sensitive than fecal calprotectin, with age and albumin levels as potential confounding factors, and indicates that R-SH may improve IBD monitoring. Again, the intervention of Leucine-rich alpha-2 glycoprotein (LRG), a serum biomarker for inflammation in IBD, has shown greater accuracy than CRP in assessing clinical and endoscopic disease activity in UC, suggesting it may be a more reliable marker for inflammation in IBD (199–201).

As such, measuring MDA, 8-OHdG,and R-SH levels provides a quantitative and qualitative assessment of oxidative stress, providing clinicians with a valuable tool in the diagnostic armamentarium for IBD. The diagnostic utility of OSMs goes beyond simple identification, extending to the monitoring of disease progression and assessing the effectiveness of treatment interventions. The correlation between OSM levels and disease severity implies that tracking these markers over time can provide insights into the dynamic nature of IBD. Overall, these three OSMs play a crucial role in the pathogenesis and progression of IBD and have been suggested to serve as potential diagnostic, differential, progression, and prognostic markers in the disease. Further research is needed to fully understand the role of OSMs in IBD and their potential as biomarkers in clinical practice.

Furthermore, Antioxidant strategies may prove beneficial in alleviating oxidative stress and mitigating the progression of IBD. Antioxidants, such as vitamin C (Vit-C), vitamin E (Vit-E), glutathione, and N-acetylcysteine, represent a potential therapeutic strategy. Vitamin C, known for its potent antioxidant properties, is crucial in alleviating oxidative stress and potentially mitigating the progression of IBD. Oxidative stress, marked by an imbalance between free radicals and antioxidants, is implicated in IBD pathogenesis. Vit-C acts as an antioxidant by scavenging free radicals, thereby reducing oxidative damage to cells and tissues (202). This helps reduce inflammation and oxidative damage, improving IBD patients' outcomes (203). Several studies have explored the correlation between Vit-C and OSMs in various inflammatory conditions, highlighting its protective role. Combining Vit-C into the treatment regimen may offer therapeutic benefits by countering oxidative stress in IBD (204). Similarly, Vit-E, a fat-soluble antioxidant, primarily protects cell membranes from oxidative damage by interrupting the chain reaction of lipid peroxidation (205). Vit-E's antioxidant properties combat ROS and reduce MDA, with various studies indicating a correlation between OSMs, including MDA, and the severity of IBD (206). For instance, a current study demonstrates that the combination of pentoxifylline (PTX) and Vit-E exhibits notable anti-fibrotic effects in human primary intestinal myofibroblasts (HIMFs) and murine models of IBD. This combination treatment suppresses the expression of fibrogenic markers induced by *TGF-β1*, showing efficacy in preventing colonic fibrosis. The findings suggest that PTX and Vit-E co-administration could be a promising therapeutic approach for IBD (207). The positive correlation between Vit-E levels and a reduction in oxidative stress suggests its potential as a therapeutic agent for managing IBD.

Incorporating Vitamin E-rich foods or supplements may be beneficial in supporting the treatment of IBD. Also, Glutathione, a tripeptide composed of cysteine, glutamate, and glycine, is a crucial endogenous antioxidant that plays a key role in detoxification and free radical scavenging. In the gut, glutathione is essential for maintaining the redox balance and protecting against oxidative stress-induced damage (208). The correlation between reduced glutathione (GSH) levels and OSMs, such as MDA, is explored in studies. For instance, a significant decrease in GSH levels in hypertensive patients was found, which was associated with an increase in MDA (209). Also, it has been documented that glutathione is regulated by the transcription factor Nrf2 and is vital in protecting cells from various stressors. Its forms, reduced GSH and oxidized GSSG, along with associated enzymes like GPx and GST, contribute to detoxification and redox balance, influencing cell survival under stress and impacting cancer chemoprevention and treatment sensitivity (210). Similarly, another study reveals a significant increase in postprandial reduced GSH levels compared to postabsorptive levels, emphasizing the importance of postabsorptive specimen collection for accurately assessing the basal level of reduced glutathione (211). These studies collectively highlight the importance of reduced GSH in mitigating oxidative stress and its potential as a biomarker for oxidative damage. Notably, maintaining an optimal balance of antioxidants, including glutathione, may be key in managing inflammation and disease progression in patients with IBD. Moreover, N-acetylcysteine (NAC), a precursor to glutathione, has been extensively studied for its antioxidant properties. It acts by replenishing intracellular glutathione levels and directly scavenging free radicals. Research suggests that NAC supplementation may help protect the gut from oxidative stress-related injuries and inflammation (212). Collectively, these antioxidants contribute to the overall defense against oxidative stress in the gut, preventing cellular damage and inflammation. Hence, integrating antioxidant therapies into the diagnostic framework not only adds a layer of precision to IBD management but also emphasizes the interrelation of diagnosis and treatment in the context of oxidative stress. Hence, targeting oxidative stress in IBD diagnosis emerges as a promising avenue with far-reaching implications. By understanding the intricate interplay between OSMs and disease pathophysiology, can help enhance diagnostic precision, monitor disease progression, and tailor therapeutic interventions for individuals with IBD. The integration of antioxidant therapies further solidifies the role of oxidative stress as a key player in IBD, bridging the gap between diagnosis and treatment in the pursuit of more effective and personalized patient care.

5 Application of oxidative stress in IBD therapeutics

Oxidative/nitrosative stress is a significant pathophysiologic aspect involved in the development and course of IBD. Since inflammatory cells secrete a large number of cytokines and chemokines, oxidative stress is triggered during inflammation, and overproduction of ROS is stimulated. In light of this, treatment strategies involving compounds with anti-inflammatory and antioxidant qualities might be considered. As IBD involves oxidative stress and inflammation, these diverse antioxidants collectively act as a shield against cellular damage, offering a multifaceted approach to treatment. The complex interplay between the endocrine system, redox balance, and oxidative stress requires understanding how hormones like melatonin, estrogen, and insulin act as antioxidants, while others like thyroid hormones and corticosteroids increase oxidative stress. Plant compounds like alkaloids and flavonoids show potential in combating oxidative stress in diabetes. Extracts from pomegranate peel and grapeseed, rich in polyphenols, are studied for their effects on ovarian cancer and female reproductive health. Nutritional antioxidants such as selenium and vitamin C are known to counter adrenal hormone-induced oxidative stress. Research supports the role of melatonin in improving testicular health and fertility by reducing oxidative stress. These findings emphasize the importance of antioxidants in managing various endocrine-related conditions (213). In exploring the potential therapeutic agents for IBD, a study reports a variety of polyphenolic substances, phenolic compounds, alkaloids, storage polysaccharides, phytochemicals, and antioxidant hormones, such as resveratrol, curcumin, quercetin, berberine, tamarind xyloglucan, sulforaphane, ginger, and melatonin (196). Synthetic antioxidants provide targeted support, while natural oxidants, derived from plant sources, contribute to a holistic and sustainable therapeutic strategy since they are used within regulations' parameters (197). Additionally, micronutrient antioxidants, such as vitamins C and E, further bolster the body's defense mechanisms. Moreover, adjunctive therapies such as prebiotics, probiotics, and postbiotics are also used to manage oxidative stress and help treat IBD. Prebiotics are dietary components crucial for mammalian nutrition. They can positively impact enteric diseases and oxidative stress by altering gut microbiota composition and producing short-chain fatty acids (SCFAs) (214). This can enhance immune function, improve the gut barrier, and stimulate beneficial microorganisms, potentially preventing disease and oxidative stress. Probiotics are live microorganisms that, when consumed correctly, boost health, create competition in the gut against harmful bacteria, and promote a healthier environment (215). Combined as synbiotics, prebiotics, and probiotics show promise in treating IBS by modulating microbiota, gut barrier function, immune responses, and the gut-brain axis (216). Clinical studies demonstrate their efficacy in alleviating IBS symptoms. Postbiotics mainly refer to biologically active components secreted by bacteria (217). Their advantages over probiotics include a reduced risk of infection or potential side effects triggered by the administration of viable microorganisms to immunocompromised individuals. The most important postbiotics are organic acids, SCFA, tryptophan (Trp), and bacteriocins. Understanding the synergy among these antioxidant modalities holds promise for enhancing IBD management and improving patient outcomes.

5.1 Natural antioxidants used in IBD therapy (polyphenols)

Examples of the phytochemical family known as polyphenols present in many plant diets are flavonoids, phenolic acids, lignans, and stilbenes. An increasing number of studies have shown that in the early stages of IBD, natural polyphenols can effectively reduce the severity of intestinal inflammation and oxidative stress (218). Diets high in polyphenols may improve the pathophysiology of conditions in which the overproduction of ROS contributes to the development of the illness (219). Table 1 displays polyphenols and various plant-derived compounds possessing antioxidant properties.

5.2 Synthetic antioxidants used in IBD therapy

Synthetic antioxidants used in IBD therapy include medications, hormones, enzymes, and other biochemical substances that are presented in Table 2.

5.3 Micronutrient antioxidants used in IBD therapy

Micronutrient antioxidants in IBD therapy include vitamins E and C, reduced glutathione, and selenium, as shown in Table 3.

TABLE 1 IBD treatments with natural antioxidants.

Antioxidant	Model of study	Mechanism of action	Clinical Manifestations	Ref.
Resveratrol (RSV)	BALB/C mice model.	Upregulation of *Arg1* and *Slc6a8* and downregulation of iNOS through arginine metabolism.	Reduces colitis, modulates cytokines, promotes anti-inflammation.	(220)
	BALB/c mice model.	Alleviates colitis via cytokine modulation and ANRIL-miR-34a pathway.	Reduced colitis by modulating cytokines, miR-34a, MUC2, GLNAT7, and ANRIL.	(221)
	BALB/c mice model.	Inhibition of SUMO1 and Wnt/β-catenin pathway.	Reduces colitis, modulates cytokines, promotes anti-inflammation.	(222)
	TNBS-induced colitis murine model.	Inhibition of pro-inflammatory cytokines.	Reduces inflammation, MDA levels, and increased GPX activity.	(223)
	Randomized double-blind, placebo-control.	Decrease the severity of the disease and increase quality of life.	Decrease the severity of the disease and increase quality of life.	(223–225)
Curcumin (CUR)	DSS colitis Mice model.	Stabilization of the gut-liver axis.	Improvement of DAI, colonic mucosal injury, and inflammatory infiltration.	(226)
	Primary rat VSMCs model.	Inhibition of the TLR4-MAPK/NF-κB pathways.	Reducing the overexpression of inflammatory mediators, NO production, and the activation of TLR4, MAPK/NF-κB pathways.	(227)
	DSS-induced colitis mouse model.	Inhibits NLRP3 inflammasome activation.	Mitigates colitis symptoms and reduces inflammation.	(228)
	Randomized Controlled Trial.	Decrease the severity of the disease and increase quality of life.	Higher clinical and endoscopic remission rates. Adverse events were rare.	(228, 229)
	A Randomized, Double-Blind, Multicenter Study.	Decrease the severity of the disease and increase quality of life.	No adverse events and reduction in clinical disease activity.	(230)
Quercetin (QCT)	(TNBS) induced rat model.	Weakens the clinical, morphological, and biochemical alterations via its antioxidant mechanism.	Mitigates TNBS-induced changes with antioxidant action.	(231)
	(TNBS) induced colitis model.	Eupatilin and QCT quercetin both mitigate IBD.	Reduces MPO activity, elevates GSH levels, and attenuates lipid peroxidation.	(232)
	DSS-induced colitis model.	Modulating gut microbiota and its metabolites SCFAs.	Increasing goblet cell density and mucus protein, Reducing the overexpression of inflammatory mediators, and MPO levels.	(233)

(Continued)

TABLE 1 Continued

Antioxidant	Model of study	Mechanism of action	Clinical Manifestations	Ref.
Catechines	Randomized double-blind, placebo-control.	Polyphenon E resulted in a therapeutic benefit for patients refractory to 5-aminosalicylic and azathioprine.	Active treatment remission rate. Minor side effects.	(234)
	(TNBS)-induced colitis model.	Ameliorating colitis through the NF-κB pathway.	Effective anti-inflammatory and antioxidant impact, and stabilizing mast cells.	(235)
	C57BL/6J mice model.	Inhibition of pro-inflammatory cytokine.	Improved DAI score, reduced intestinal score.	(236)
Anthocyanins	Clinical trial. (UC patients)	Decrease the severity of the disease and increase quality of life	Improved clinical symptoms.	(237)
Silymarin	A randomized, double-blinded, placebo-controlled clinical trial.	Decrease the severity of the disease and increase quality of life.	Improvement in hemoglobin level, Improved DAI score, and High remission rate.	(238)

5.4 Adjunctive therapies used in IBD treatment (prebiotics, probiotics, and postbiotics)

Adjunctive therapies such as prebiotics, probiotics, and postbiotics are used in managing oxidative stress and the treatment of IBS and IBD, as presented in Table 4.

5.5 Kelch-like ECH-associated protein 1 (KEAP1) inhibitors used in IBD treatment

Therapeutic approaches targeting the KEAP1-NRF2 pathway primarily utilize KEAP1 inhibitors (259). These inhibitors block KEAP1 from binding to NRF2, resulting in the stabilization and activation of NRF2. Consequently, NRF2 activity increases, leading to the elevated expression of antioxidant enzymes such as glutathione S-transferase, NAD(P)H quinone oxidoreductase 1, and heme oxygenase-1 (260). These enzymes play a crucial role in reducing oxidative stress and inflammation in IBD. Currently, extensive studies are being carried out to explore the potential of KEAP1 inhibitors as treatments for IBD. For example, the intervention of KEAP1 inhibitors, such as natural coumarins, promotes Nrf2 activation, which reduces oxidative stress and inflammation in IBD by inhibiting NF-κB and enhancing antioxidant responses, as documented (261). Further studies on coumarin derivatives are essential for developing Nrf2 activators with intestinal anti-inflammatory activity. Similarly, CPUY192018, a potent small-molecule inhibitor of the Keap1-Nrf2 PPI, demonstrated cytoprotective effects in NCM460 colonic cells and a DSS-induced UC model by activating Nrf2 signaling (262). This suggests that direct inhibition of Keap1-Nrf2 PPI might be beneficial for UC treatment. By combining Keap1 inhibitors with H2S-donor moieties via molecular hybridization, DDO-1901 showed enhanced efficacy in alleviating colitis by mitigating oxidative stress and inflammation, outperforming parent drugs alone (263). Additionally, a recent research study investigates how 4-Octyl itaconate (OI), a form of itaconate functioning as a KEAP1 inhibitor, affects DSS-induced UC in mice (264). OI diminishes oxidative stress and cell death, boosts the gut barrier's function, and

TABLE 2 IBD treatments with synthetic antioxidants.

Antioxidant	Model of study	Mechanism of action	Clinical manifestation	Ref.
Melatonin	Randomized clinical trial.	Decreased level of anxiety and depression.	Help sustain remission in UC patients. Steady CRP levels.	(239)
	DSS-induced mice model.	Increased antioxidant capacity. Improve oxidative stress resistance of mice with colitis.	Regulate microbial flora. Improve intestinal health.	(240)
N-acetylcysteine (NAC)	TNBS-induced colitis model.	Suppressed COX2 and E (2) (PGE (2) levels.	Reduced iNOS activity.	(241)
Modified Superoxide Dismutase (SOD)	TNBS-induced mouse model.	Recombinant Lact. Fermentum reduces oxidative stress via the NF-κB pathway.	Higher survival rate and lower DAI score.	(241)
	Pilot study.	Improved UC therapy.	Less severe side effects.	(242)
	Double-blind control trial.	Safe treatment.	Improve serum level markers.	(243)
Propionyl-L-carnitine (PLC)	Mildto moderate UC/CD patient's study.	Improve clinical symptoms.	Decrease DAI. No side effects.	(244)
	Clinical trial	Higher safety profile. Improve clinical symptoms.	Mild digestive system adverse reaction.	(245)

TABLE 3 IBD treatments with micronutrient antioxidants.

Antioxidant	Model of study	Mechanism of action	Clinical manifestation	Ref.
Vitamin E	Clinical trial.	Novel therapy for mild to moderate active UC.	No side effects.	(246)
	Pilot study.	Lower infection frequency and disease severity.	Improved neutrophil count and function.	(247)
	C57BL/6 mice model.	PTX and Vit-E suppressed TGF-β1 induced expression of fibrogenic markers.	Exhibit significant anti-fibrotic effects on both human primary intestinal myofibroblasts (HIMFs) and *in vivo* IBD models.	(207)
Vitamin C	C57BL/6 and BALB/C.	Boost Antioxidant enzymes (SOD, CAT, GPx).	Lowers the expression of pro-inflammatory cytokines (iNOS, TNF-α). Lowers MDA levels.	(246, 247)
	Clinical trial.	Reduce corticosteroid dosage for disease control.	Significant improvement in clinical symptoms.	(248)
Selenium	DSS-induced mice model.	Has minimal impact on inflammatory processes and disease progression.	Alleviate inflammation and Lower disease severity.	(249)

lessens inflammation. It lowers the activity ofKEAP1, increases NRF2, and promotes the production of protective enzymes. This study highlights OI's promising role in treating IBD. Hence, using KEAP1 inhibitors is crucial for treating IBD, where ROS plays a significant role.

6 Conclusion and future perspective

IBD represents a significant health challenge characterized by chronic inflammation of the digestive tract. The role of oxidative stress in the pathogenesis of IBD is well-documented, with high

TABLE 4 IBD treatments with adjuvant therapies.

Adjuvant therapy	Model of study	Treatment	Clinical manifestation	Ref.
Prebiotics	A Prospective Observational Study.	Oral microencapsulated sodium butyrate (BLM).	BLM supplementation appears to be a valid add-on therapy for remission in UC patients.	(250)
	A randomized, double-blind, placebo-controlled study.	scFOS	Improved rectal discomfort, IBS symptoms, and quality of life. scFOS reduced anxiety and increased Bifidobacteria in feces.	(251)
Probiotics	A pilot, randomized, double-blind, placebo-controlled study.	Lactobacillus and Bifidobacterium species.	Significantly induced remission in UC patients. Reduced stool frequency and improved biochemical markers like C-reactive protein, hemoglobin, and IL-10 levels.	(252)
	Randomized Controlled Trial.	Specific probiotics.	Significantly reduced oxidative stress (d-ROMs) and boosted antioxidant response (BAP), improving patient health safely and effectively.	(253)
	A randomized clinical trial.	Bacillus coagulans Unique IS2.	Improved GI symptoms like pain and bowel movements. Demonstrated safety and efficacy for adult IBS. No impact on inflammatory cytokines.	(254)
	DSS-induced colitis in mice.	Lactobacillus (Pediococcuspentosaceus, Lactobacillus plantarum, and Weissellacibaria).	Reduced DAI, pathological score, regulated cytokine secretion at the level of gene expression, and increased colon length. Potential treatment for IBD.	(255)
Postbiotics	Clinical trial with a randomized controlled design.	Sodium butyrate.	A significant increase in the colonic IL-10/IL-12 ratio was found within butyrate-treated patients. Rectal butyrate enemas had minor effects on inflammation and oxidative stress in UC patients.	(256)
	DSS IBD mouse model.	D-methionine (D-Met) and/or butyric acid (BA).	Reduced disease severity and suppressed inflammation-related gene expressions. Potential therapeutic for IBD.	(257)
	DSS-induced colitis model.	Heat-killed *Bifidobacterium bifidum* B1628 (HB1628).	Reduced DAI, histology scores, and pro-inflammatory cytokines, with increased IL-13. Reduced inflammation, improved gut microbiota balance, and enriched metabolic pathways, indicating HB1628's potential in mitigating colitis and modulating gut health.	(258)

levels of reactive ROS and RNS contributing to gut mucosal damage and the activation of inflammatory pathways. Effective management of IBD may involve the use of antioxidants to mitigate oxidative stress, as evidenced by elevated oxidative stress markers such as malondialdehyde (MDA), 8-hydroxy-2'-deoxyguanosine (8-OHdG), and serum-free thiols (R-SH). Antioxidant therapies, including vitamin C, E, glutathione, and N-acetylcysteine, have shown the potential to alleviate IBD symptoms. Future research should focus on elucidating the detailed mechanisms by which oxidative stress contributes to IBD and exploring novel therapeutic strategies targeting this pathway. Specifically, targeting oxidative stress through molecular pathways such as MAPK, TLR4/NF-κB, Nrf2, and PI3K/Akt could offer new therapeutic avenues for IBD management. These pathways play critical roles in modulating inflammation and cellular responses to oxidative stress, providing promising targets for intervention. Polyphenol phytochemicals, such as curcumin, resveratrol, and others, have shown potential in modulating the molecular pathways, thereby reducing inflammation. These compounds exhibit antioxidant properties, neutralizing ROS and reducing oxidative stress, which is critical in the pathology of IBD. Further clinical trials are needed to validate these strategies' effectiveness and establish standardized protocols for incorporating antioxidants into IBD treatment regimens.

Author contributions

PM: Writing – original draft. LZ: Funding acquisition, Writing – review & editing. SL: Software, Writing – review & editing. ZZ: Software, Writing – review & editing. TJ: Writing – review & editing. FM: Conceptualization, Writing – review & editing. ZM: Writing – review & 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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Systematic review on the role of the gut microbiota in tumors and their treatment

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Tumors present a formidable health risk with limited curability and high mortality; existing treatments face challenges in addressing the unique tumor microenvironment (hypoxia, low pH, and high permeability), necessitating the development of new therapeutic approaches. Under certain circumstances, certain bacteria, especially anaerobes or parthenogenetic anaerobes, accumulate and proliferate in the tumor environment. This phenomenon activates a series of responses in the body that ultimately produce anti-tumor effects. These bacteria can target and colonize the tumor microenvironment, promoting responses aimed at targeting and fighting tumor cells. Understanding and exploiting such interactions holds promise for innovative therapeutic strategies, potentially augmenting existing treatments and contributing to the development of more effective and targeted approaches to fighting tumors. This paper reviews the tumor-promoting mechanisms and anti-tumor effects of the digestive tract microbiome and describes bacterial therapeutic strategies for tumors, including natural and engineered anti-tumor strategies.

KEYWORDS

tumor, gut microbiota, tumorigenic effect, anti-oncogenic effect, therapy

1 Introduction

Tumors exhibit genomic instability (1), characterized by the accumulation of point mutations and structural genomic alterations throughout their development (2) (Figure 1). Additionally, tumors manifest a distinct tumor microenvironment (TME). Due to tumor-specific attributes, various clinical treatment methods, including radiotherapy, chemotherapy, surgery, among others, have inherent limitations, restricting their applicability and effectiveness, thus making the majority of tumors difficult to treat.

As a major "endocrine" organ of the human body, the digestive tract plays a vital role in regulating the physiological functions of the human body, such as participating in the

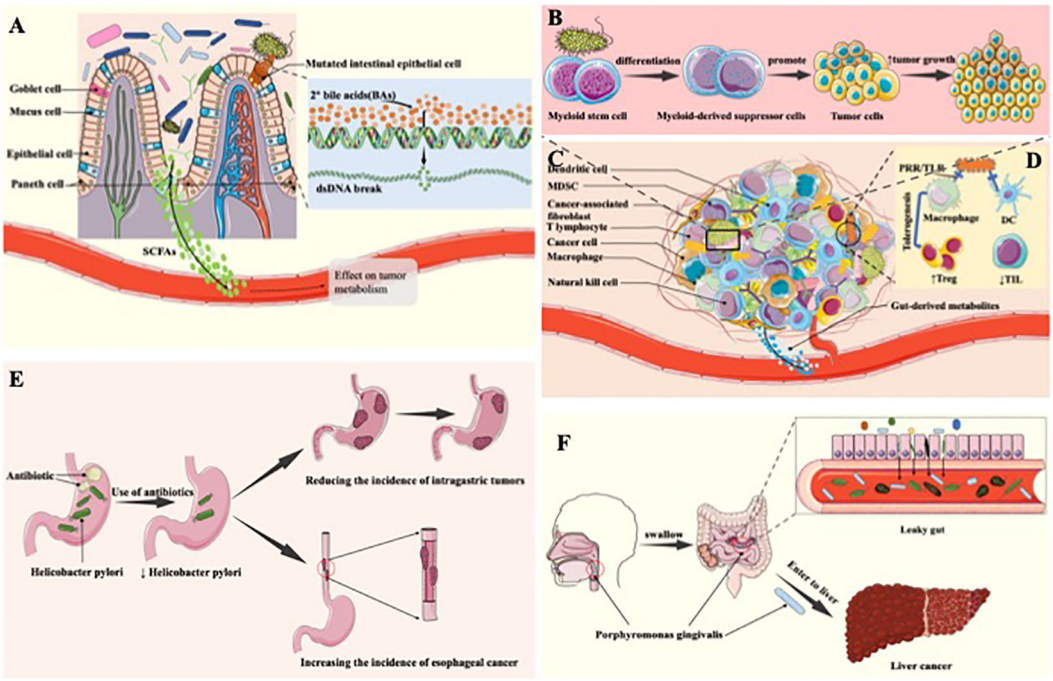

FIGURE 1
The tumorigenic effect of the gastrointestinal microbiota. **(A)** Tumor-promoting mechanism of anaerobic bacteria. Anaerobic bacteria in the gut may enzymatically convert free bile acids into secondary bile acids, which may trigger DNA damage, increase the risk of cell mutation, inhibit apoptosis, and contribute to the evolution of healthy cells into cancerous cells, thereby promoting tumour formation and progression. **(B)** The tumorigenic effect of digestive tract microbiome through induction of inflammation. The microbiome in the digestive tract triggers inflammation by releasing cytokines that stimulate the growth and proliferation of tumor cells. This inflammatory response further facilitates tumorigenesis and progression, creating a conducive environment for the development and advancement of tumors within the digestive system. **(C)** The component of TME. **(D)** The tumorigenic effect generated through change of the amount of digestive tract microbiome. In tumor patients, the digestive tract microbiome plays a role in fostering the infiltration of immunosuppressive cells by downregulating immune cells in the body. This mechanism contributes to the promotion of tumor occurrence and development, creating an environment where immunosuppression is favored and facilitating the evasion of immune responses, ultimately aiding in the progression of tumors within the digestive tract. **(E)** Antibiotic use leads to changes in digestive tract microbiome and thus tumor-promoting effects. Antibiotic use diminishes *H. pylori*, lowering the risk of gastric tumors; however, this reduction heightens the incidence of esophageal adenocarcinoma. The complex interplay highlights the dual impact of antibiotics on gastrointestinal health, underscoring the importance of considering specific cancer types when evaluating the consequences of antibiotic treatments. **(F)** Transfer of Porphyromonas gingivalis to the intestine and liver produces tumor-promoting effects. Porphyromonas gingivalis induces periodontitis in the oral cavity. Upon entering the intestinal tract, it elevates intestinal permeability, leading to "leaky gut." This facilitates the entry of certain bacteria into the liver, potentially elevating the risk of liver cancer by creating an environment conducive to hepatic complications.

synthesis of vitamins, amino acids and carbohydrates to maintain the normal function of the human body, preventing the invasion of pathogens and enhancing the biological barrier (3). The human gastrointestinal tract (GIT) is inhabited by various kind of microbial species, including bacteria, viruses, fungi, archaea, and protozoa (4), with bacteria being predominant and present in large numbers in the oral cavity, stomach, duodenum, jejunum and large intestine (5), but the highly acidic environment of the stomach results in low levels of bacteria in the stomach and upper small intestine. Conversely, the colon region is densely populated with an abundance of bacteria, and proximity to the colon correlates with increasing microbial load (3). The composition of the digestive tract microbiome bears a close association with tumors manifesting across various anatomical sites, including those arising within the GIT (6). A profound exploration of the impact of the digestive tract microbiome on tumorigenesis serves to elucidate the pathological mechanisms underlying malignant neoplasms. Furthermore, strategies leveraging bacterial entities for therapeutic purposes exhibit substantial promise in the domain of oncology due to the inherent tropism of bacteria towards the tumor microenvironment (7), which can overcome the shortcomings of current tumor therapies. Consequently, the investigation of the interplay between the digestive tract microbiome and tumors, particularly in the context of therapeutic interventions, stands as a prominent focal point in contemporary oncological research.

This comprehensive review aims to provide a meticulous and systematic examination of the intricate interrelationship between the digestive tract microbiome and tumors. It elucidates the mechanisms underpinning bacterial-associated strategies for tumor management and synthesizes the most recent advances in bacterial-based tumor therapeutics. The objective is to present a conceptual framework for the development of innovative strategies in tumor therapy.

2 The tumorigenic effect of the gastrointestinal microbiota

Microbial populations within the digestive tract including Bacteroidetes, Firmicutes, Actinobacteria, and Aspergillus (8), with this bacterial consortium encompassing not only beneficial bacteria primarily represented by Bifidobacterium and Lactobacillus, but also conditionally pathogenic organisms, notably *Escherichia coli(E. coli)*, as well as pathogenic microorganisms, prominently exemplified by Pseudomonas aeruginosa (9). In addition to the beneficial influence of beneficial bacteria on human health, both conditionally pathogenic and pathogenic bacterial organisms manifest harmful effects on the host organism. It is now widely accepted that abnormalities within the gastrointestinal microbiota may contribute to the initiation of malignant tumorigenesis (10). Simultaneously, the unique TME, characterized by internal hypoxia, low pH and increased permeability, provides an environment suitable for colonization of neoplastic lesions by a plethora of anaerobic or facultative anaerobic bacteria, take Salmonella and Escherichia (6) as examples, which in turn initiates and accelerates the process of tumorigenesis. The gastrointestinal microbiome influence tumor development through a variety of mechanisms, including releasing metabolic by-products, inducing inflammatory cascades, modulating immune responses, and altering microbial abundance and colonization sites.

2.1 The tumorigenic effect of digestive tract microbiota-derived metabolites

Distinct metabolites originating from digestive tract microbiome exhibit diverse impacts on tumor cells, with certain metabolites stimulating the proliferation and progression of neoplastic cells (11). The presence of bile acid hydrolases facilitates the production of free bile acids by anaerobic bacteria in the gastrointestinal tract, such as Bacteroides and Clostridium (12). Anaerobic bacteria in the intestinal environment have the potential to enzymatically convert free bile acids into secondary bile acids, thereby inducing DNA damage, increasing the probability of cellular mutations, inhibiting apoptosis, thereby promoting the transformation of healthy cells into cancer cells, and facilitating the onset and progression of tumors (13) (Figure 1A). Among the metabolites derived from the intestinal microbiota, short-chain fatty acids, with butyrate representing a preeminent example, have garnered extensive research attention. Investigations have unveiled that butyrate may heighten the susceptibility to tumorigenesis subsequent to genetic modifications (14). Moreover, numerous studies have revealed that butyrate amplifies the incidence of colon cancer by stimulating the proliferation of colorectal epithelial cells, resulting in the expansion of tumorigenic cell populations (15). Furthermore, certain intestinal bacteria, such as the Ruminococcaceae family within the Clostridium cluster, produce metabolites that yield β-glucuronidase, which influences estrogen levels, resulting in enhanced estrogen metabolism and free estrogen levels. Consequently, this elevation contributes to the initiation of breast tumors (11, 16, 17). It is evident that an array of GI bacterial metabolites can contribute to the initiation and advancement of tumorigenesis.

2.2 The tumorigenic effect of digestive tract microbiome through induction of inflammation

Recently, more and more research demonstrate that digestive tract microbiome has the potential to promote the formation and development of tumors by inducing an inflammatory response. Chronic inflammation is regarded as a significant contributing factor of the development of tumors. Cytokines and pro-inflammatory factors produced during inflammation, such as IL-1 and HMGB1 (18), which have the ability to activate cell proliferation, inhibit apoptosis, and promote angiogenesis, thus providing favorable conditions for tumor growth and dissemination (19). Arthur JC et al. manifest that the imbalance of digestive tract microbiome results in the increase of intestinal muscosal inflammation, thereby promote the development of colon cancer (20). A research article published in *Science* revealed that inflammation triggers the generation of respiratory electron acceptors, including substances like nitrate, ethanolamine, and tetrasulphite. These compounds serve as substrates for a diverse range of bacteria, including *E. coli* and *Salmonella*. Moreover, these bacteria possess significant characteristics that augment the persistence of chronic inflammation, consequently fostering the progression and proliferation of tumors (21). Furthermore, enterotoxigenic bacteroides fragilis (ETBF) generate bacteroides fragilis toxin, which involved in multiple signal transduction in colonic epithelial cells, inducing the generation of an inflammatory response that promotes the genesis and development of tumor cells (22–24) Thiele and collogues illustrated that ETBF induces myeloid stem cells to differentiate into myeloid suppressor cells, which activate pathogenic inflammatory pathways and promote colorectal cancer (CRC) development and progression (25). As a result, digestive tract microbiome induces an inflammatory response by producing cytokines that promote the growth and proliferation of tumor cells, which in turn promotes tumorigenesis and progression (Figure 1B).

2.3 The tumorigenic effect of digestive tract microbiome through modulation of the immune response

Digestive tract microbiome has the potential to promote the generation and proliferation of tumor cells through modulate human's immune response, therefore generating the tumorigenic effect. Chamutal Gur et al. manifest that Fusobacterium nucleatum (FN) binds to the inhibitory receptor TIGIT on human natural killer cells and T cells through Fap2 protein, which inhibits the cytotoxicity of natural killer cells, thus inhibiting the anti-tumor immune function of the body and leading to tumor occurrence (26). Moreover, Robert F.Schwabe and collogues demonstrate that FN

can inhibit the cytotoxicity of NK cells through TIGIT, down-regulate its inhibitory effect on tumor cells, and then promote the occurrence and development of tumors, especially colorectal adenocarcinoma tumors (27). Through an investigation involving 138 volunteers, Chen Ting and fellow researchers established a significant association between TOX protein expression and $CD4^+$ T cell content within colorectal tissues. Their findings demonstrated that FN exerts a pivotal role in CRC development by reducing $CD4^+$ T cell levels and suppressing TOX protein expression (28). Moreover, for individuals with Crohn's disease exhibiting elevated FN levels leading to diminished bifidobacteria content, the susceptibility to gastrointestinal and other cancers becomes notably elevated (28). Metabolites produced by the gut microbiota, which are characterised by short-chain fatty acids like butyric acid, activate certain G protein-coupled receptors (GPCRs), especially GPR43, GPR41, GPR109A and Olfr78 (29). These bioactive compounds are vital in inducing the differentiation of nascent $CD4^+$ T cells into immunosuppressive cells Tregs (30). The gut microbiota is also implicated in the progression of hepatocellular carcinoma, in addition to its impact on CRC. Research demonstrates that disruptions in gut microbiota composition caused by low-dose antibiotics or mucosal damage can drastically accelerate the advancement of hepatocellular carcinoma (31). This accelerated progression is mainly mediated by several mechanisms, including increased expression of IL-6 and activation of the nuclear factor-kappaB (NF-κB) pathway (32). Consequently, the digestive tract microbiome of tumor patients can promote the infiltration of immunosuppressive cells by downregulating the immune cells in the body, thus promoting the occurrence and development of tumors (Figures 1C, D).

2.4 The tumorigenic effect generated through change of the amount of digestive tract microbiome

The variation of the amount of digestive tract microbiome can produce tumor-promoting effect. Significant differences in the amount and composition of digestive tract flora between cancer patients and healthy populations. The gastrointestinal microbiota of healthy adults exhibits significant diversity. It is notable that Streptococcus salivarius and Streptococcus bradycosus predominate in the oral microbiome. Moving into the esophagus, one encounters Staphylococcus, Prevotella, and Veyronella populations. Stomachs contain Firmicutes, Bacteroidetes, Clostridium, Actinobacteria, Roxella and Haemophilus. As microorganisms move into the gut, diversity increases, with Proteus, Clostridium, Streptococcus and Oxalacidobacterium leading the gut environment (33). Probiotics exist in GIT possess a vital role in maintain human health (11). To be specific, an increased presence of Lactobacillus Johnsoni is linked to a decrease in genotoxicity, a reduction in pro-inflammatory factor levels, and a lower frequency of inflammatory responses. Therefore, the absence of this bacterial strain is associated with a higher susceptibility to lymphoma development in murine models (34). Furthermore, relevant reviews reported that use of antibiotic enables the imbalance of flora is digestive tract, following the influence of digestive tract tumors (35, 36). For example, *Helicobacter pylori* (*H. pylori*) has the potential to induce gastric adenocarcinoma with characteristics including lymphovascular infiltration, lymph node metastasis, and an unfavorable prognosis. These features are related to the ability of the microbe to inactivate the ARIR1A gene (37). However, Anderson and collogues manifest that the use of antibiotic leads to the decline of *H. pylori*, which reduces the incidence of gastric tumors, but increases the incidence of esophageal adenocarcinoma (38) (Figure 1E).

2.5 The tumorigenic effect generated through the transference of digestive tract microbiome

Bacteria transference also influence the generation and development of tumors. The amount of saliva swallowed by normal adults can reach 0.75–1.5L per day (39), which provides an opportunity for oral flora to flow into the digestive tract and even the intestines. At the same time, through the chewing process, the microorganisms in the mouth can be swallowed into the stomach and intestines. Porphyromonas gingivalis (P. gingivalis) causes periodontitis in the mouth, but when it enters the intestinal tract, it increases the permeability of the intestines, thus causing "leaky gut" (gut-oral axis), through which some bacteria enter the liver and increase the incidence of liver cancer (gut-liver axis) (40) (Figure 1F). Moreover, gut microbiome has been linked not only to gastrointestinal disorders, but also to mental illness wich can be known as Microbiota-Gut-Brain Axis (41). Metabolic, endocrine, neural and immunological pathways form the bidirectional link between the gut and the brain. These include the vagal nerve, the HPA axis, the production of bacterial metabolites, immune mediators and entero-endocrine signals (42, 43). The gut microbiota has a significant impact on neurological and psychiatric disorders such as major depressive disorder (MDD), schizophrenia (SCZ), bipolar disorder (BD), and autism spectrum disorder (ASD). Changes in the gut microbiome influence inflammation and depressive symptoms in MDD, neurotransmitter dysregulation in SCZ, immune-inflammatory activity in BD, and behavioral and sensory responses in ASD, highlighting the complexity of the gut-brain axis (41). Simultaneously, P. gingivalis can also bind to C5aR1 and TLR2 receptors on intestinal cells (44, 45), activate PI3K channel, inhibit normal apoptosis, promote intestinal inflammation, and ultimately, even promote intestinal tumourigenesis and development. Klebsiella pneumoniae coexists with the normal oral flora but carries a risk of inducing inflammatory bowel disease and potentially causing colon cancer (46). Additionally, Koji Atarashi et al. through gnotobiotic tech proved that Klebsiella pneumoniae, when isolated from the oral flora and colonized in the intestine, may act as a potent inducer of Th1 cells, thereby promoting the development of severe colon cancer through its genotoxic effects (45). It is worth mentioning that the colon of Klebsiella pneumoniae in healthy rats does not cause disease, however, when it comes to the rats used antibiotic, severe intestinal diseases may be caused, such as CRC.

2.6 Others

Physiological responses of the body can also promote changes in the bacterial composition of certain parts of the body that can have carcinogenic effects. For example, reflux may result in chronic oesophageal damage and promote cancer in Barrett's oesophagus (47). Yang and colleagues revealed alterations in the oesophageal microbiota caused by reflux disease through a comparison of healthy humans' microbiota with that of individuals who had reflux oesophagitis or Barrett's oesophagus. The experiment found significant amounts of Streptococcus spp. in the inflamed oesophagus. The healthy oesophagus was dominated by certain bacteria, while the reflux or Barrett's oesophagus group had a higher abundance of the Bacteroides, Aspergillus and Clostridium phylum. This is most likely due to the physiological changes caused by excess stomach acid (48). In addition, it has been demonstrated that the virulence of *H. pylori*, host genetics and environmental factors contribute to the development of gastric cancer (49). Secondly, colonization by a wide range of bacteria also promotes tumor development to some extent. Oropharyngeal or intestinal commensal bacteria (Streptococcus, Bifidobacterium, Lactobacillus, Serratia, Klebsiella, Escherichia, Pseudomonas, Neisseria, Staphylococcus and Bacillus) have been reported to be associated with gastric cancer (50–53). Additionally, *H. pylori* can modulate the microbial composition in the distal gut. Most seriously, *H. pylori* can cause low gastric acidity, which can promote the entry of acid-sensitive bacteria into the distal gastrointestinal tract, leading to changes in the colonic microbiome that may promote colon cancer development (47).

3 The anti-oncogenic effect of the gastrointestinal microbiota

3.1 The anti-oncogenic effects of digestive tract microbiota-derived metabolites

Certain metabolic byproducts of the gastrointestinal microbiota have a "biphasic" effect on tumors, which means it not only promotes the development of tumors, but also exerts anti-tumor effects. The primary gut fermentation products, short-chain fatty acids (SCFAs), play a role in regulating colonic epithelial cell growth and differentiation (54). Butyrate, which serves as a primary energy source for colonic cells, is the most extensively studied of the SCFAs (30). Butyrate can exert its anti-colon cancer effects by targeting Fas and p21 in animal tumor models, as well as by inhibiting enzymes with pro-carcinogenic activities in the intestine, such as histone deacetylases (55) (Figure 2A). These actions result in suppressing tumor cell proliferation and help to inhibit further CRC progression (46). Additionally, butyrate also contains the role of maintaining intestinal barrier function, which plays a significant role in the maintenance of intestinal function. Disruption of intestinal barrier function is the foundation of numerous diseases (56). The protection of intestinal function can be regulated by SCFAs. By activating 5'-adenosine monophosphate-activated protein kinase (AMPK) and the TLR4 pathway, butyrate in SCFAs may enhance the defensive function of intestinal epithelial cells by promoting mucin secretion (57) (Figure 2A). Additionally, sodium butyrate, a short-chain fatty acid, has been shown to increase the transcription of Claudin-1 by promoting binding between SP1 and the Claudin-1 promoter region (55). This enhancement of intestinal barrier function aids in maintaining overall intestinal homeostasis, thereby preventing the onset and development of tumors. Polyphenol metabolites are a type of microbial metabolite in the gut that help prevent CRC by modifying phytanic acid synthesis, downregulating inflammatory cascades, regulating DNA synthesis, and inducing luminal detoxifying enzymes (58). These actions aid in DNA repair, inhibit colonic pathogens, and regulate apoptosis, thus supporting the prevention of CRC (59). Quercetin-related metabolite indole-3-propionic acid acts as an AhR agonist. It suppresses inflammatory responses in colonic epithelial cells, thereby inhibiting carcinogenesis and exhibiting anticancer effects (60). Conjugated linoleic acid has the ability to inhibit DNA synthesis and induce apoptosis in human colon adenocarcinoma cell lines, and subsequently to alter the cell cycle of colon cancer cells, and consequently to reduce the incidence of colon cancer (61). Lactocin, a bacteriocin synthesized by Lactobacillus, has been extensively investigated in numerous studies for its anti-cancer effects on cell proliferation. It has been shown to increase the Bax/Bcl-2 ratio, an indicator of increased apoptosis index, and to promote apoptosis in colorectal cancer cells (62–64) In summary, the gut microbiota generates a variety of metabolites that exhibit anti-tumor activity through the inhibition of tumor cell proliferation, the regulation of tumor cell apoptosis, and the suppression of inflammatory responses. It is noteworthy that butyrate, as the most extensively studied SCFA, has been proven to have a dual role, with the ability to both promote tumorigenesis and produce anti-tumor effects through pathways such as maintaining intestinal barrier function.

3.2 The anti-oncogenic effect of digestive tract microbiome through modulation of the immune response

Bacteria exhibit anti-tumor immune responses through their ability for targeted, specific colonization of tumors and their immunogenic properties (65). The immunogenic capabilities of bacteria primarily manifest in their components, including peptides, polysaccharides, lipopolysaccharides, lipoteichoic acids, flagella, DNA, RNA, and others, which can be recognized by pattern recognition receptors on dendritic cells (DCs), macrophages, and neutrophils. This recognition subsequently triggers the appropriate immune responses, activating both the innate and adaptive immune systems, thus generating an anti-tumor effect (66, 67). To be specific, by increasing the expression of tumor necrosis factor-α (TNF-α) and IL-1β in the TME, the LPS component of Salmonella can enhance the functionality of immune cells, including $CD8^+$ T cells, and generate an anti-tumor immune response (Figure 2B). Chen et al. have demonstrated that Salmonella flagella activate immune cells via the TLR5 signaling pathway, triggering a host immune response and resulting in a

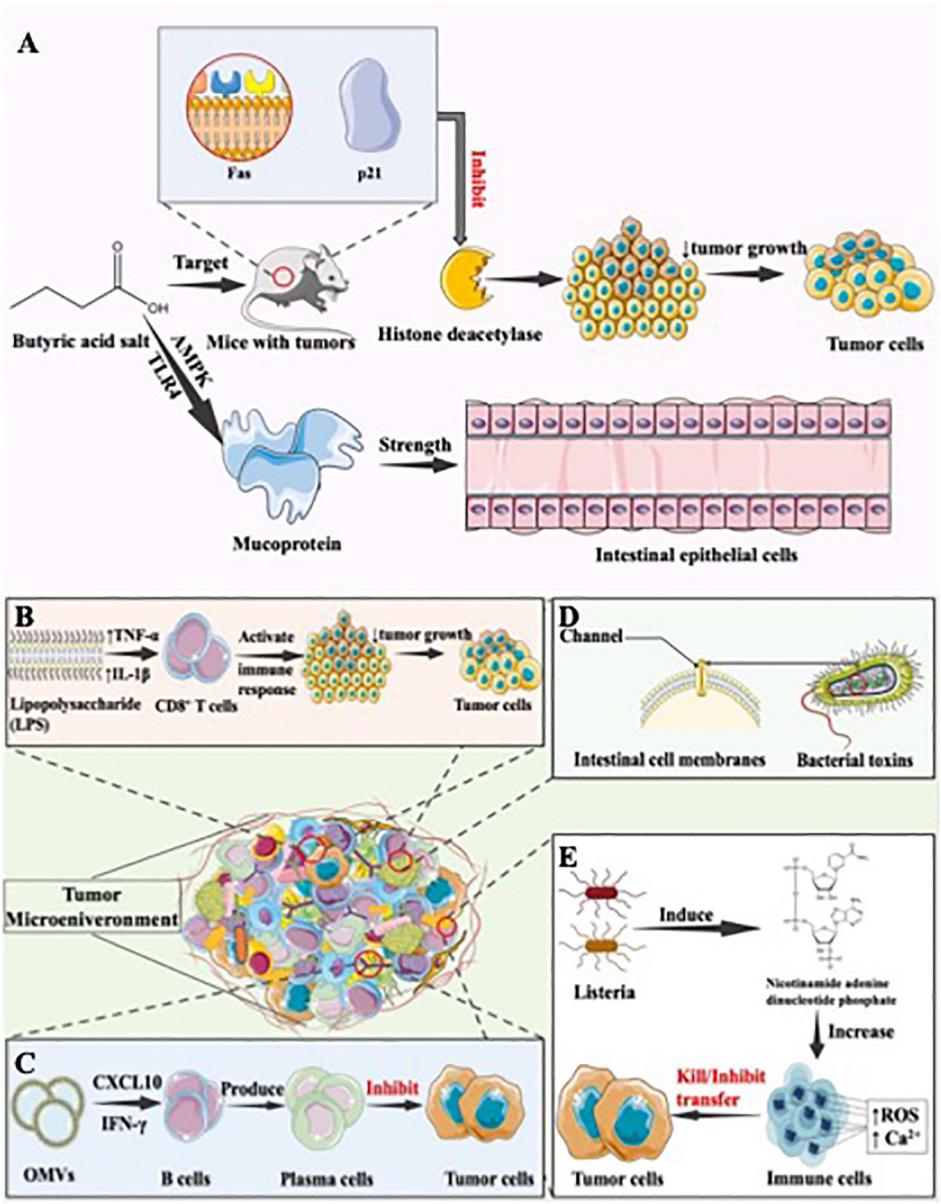

FIGURE 2
The anti-oncogenic effect of the gastrointestinal microbiota. **(A)** Antitumor mechanism of butyrate. Butyrate demonstrates anti-colon cancer effects in animal tumor models by targeting Fas and p21. It also inhibits pro-carcinogenic enzymes like histone deacetylases in the intestine. Additionally, butyrate, a short-chain fatty acid, within SCFAs, enhances the defensive role of intestinal epithelial cells by stimulating mucin secretion, further contributing to its protective influence against colon cancer, **(B)** Antitumor mechanism of Salmonella. Salmonella's LPS component elevates the expression of TNF-α and IL-1β in the TME. This augmentation enhances the functionality of immune cells, notably $CD8^+$ T cells, fostering an anti-tumor immune response with the potential to contribute to tumor suppression and immune-mediated control of cancer, **(C)** Antitumor mechanism of bacterial outer membrane vesicles. OMVs exhibit the ability to specifically target tumor tissues and prompt their rapid internalization by tumor cells. This internalization triggers the production of anti-tumor factors, such as CXCL10 and IFN-γ, and activates the human immune response. This immune stimulation not only inhibits existing tumors but also hinders the metastasis of tumors, **(D)** Antitumor mechanisms of bacterial toxins. Bacterial toxins, generated during bacterial metabolism, possess evident toxicity to the human body. These toxins have the ability to create channels in the cell membranes of eukaryotic cells, disrupting their normal barrier functions. This interference can lead to various physiological consequences, illustrating the significant impact bacterial toxins can exert on cellular processes and overall health in the context of microbial infections. **(E)** Antitumor mechanisms of inhibiting tumor metastasis. Infection with Listeria monocytogenes can activate the enzyme NADPH oxidase, which plays a critical role in the generation of ROS. Increased levels of ROS can trigger a cascade of cellular events. One of these is an increase in intracellular calcium (Ca^{2+}). The increase in Ca^{2+} levels can lead to the dysfunction of the mitochondria and the activation of various apoptotic pathways, thereby leading to cell death. In the context of tumour cells, these mechanisms can lead to direct tumour cell death through the induction of oxidative stress and apoptosis, thereby effectively reducing tumour burden.

therapeutic effect against melanoma (68). Moreover, PAMPs expressed by Listeria infecting tumor tissues can be recognized by Toll-like receptors on antigen-presenting cells. This recognition activates the NF-κB pathway, subsequently clearing tumor cells (69). Attenuated Listeria has been used as antigen-presenting vectors, leading to the development of several malignant vaccines, including cervical cancer vaccines (70), with studies demonstrating efficacy. Additionally, Kuugbee's research team investigated the effect of low-fructo-

oligosaccharide maltodextrin-enriched Lactobacillus acidophilus, Bifidobacterium bifidum, and Bifidobacterium infantum (LBB) on the progression of CRC. They discovered that LBB boosts intestinal mucosal epithelial barrier integrity and decreases tumor incidence by promoting epithelial cell apoptosis and inflammation through the TLR2 pathway in the host (71).

3.3 The anti-oncogenic effect of bacterial outer membrane vesicles

Bacterial outer membrane vesicles (OMVs) are generated by gram-negative bacteria, composed by bacterial outer membrane component, and carry the essential antigenic components required to induce protective immune responses (72). They facilitate antigen presentation, activate the immune system, and generate anti-tumor effects. OMVs contain numerous PAMPs, capable of activating adaptive immune responses, generating antigen-presenting cells and interacting with pattern recognition receptors, ultimately activating antigen-presenting cells (73), leading to inhibitory effects on tumors. Kim et al. discovered that OMVs had the potential to target tumor issues and accelerate in tumor cells, following by inducing the production of anti-tumor factor CXCL10 and γ interferon (IFN-γ), as well as activating human immune response. This stimulation of the immune response leads to the inhibition of existing tumors and also inhibits tumor metastasis (74) (Figure 2C). Also, OMVs can produce intrinsic anti-tumor effects by delivering various toxic factors. Additionally, OMVs can cause the extravasation of red blood cells in the tumor area, leading to the aggregation of hemoglobin within the tumor. This results in a noticeable darkening of the tumor's color and an increase in absorbance in the near-infrared region. Near-infrared lasers can be utilized to eliminate tumor cells (75). OMVs, as a promising means of treating tumors, can be enhanced in their anti-cancer effects through engineering modifications or their use as a drug carrier.

3.4 The anti-oncogenic effect of bacterial toxins

Bacterial toxins, synthesized during the metabolism of bacteria, are the material that have apparent toxic function to human body. They can form channel on the cell membrane of eukaryotic cells, thereby disrupting its barring function (Figure 2D), resulting in the anti-tumor effect (76). Research indicates that Salmonella typhimurium or *E. coli* can produce bacterial toxins, making their inhibitory effects on tumor onset and development more pronounced (77, 78). Additionally, Jiang and collogues proved that a cytolysin A produced by *Salmonella typhimurium*, *E. coli*, and *Shigella flexneri* can exert anti-tumor effects through two pathways: direct killing of tumor cells and promotion of tumor cell apoptosis by releasing membranous vesicles (78). Wang et al. indicated that Salmonella carrying the cell-killing expansin toxin B could inhibit tumor growth and extend survival by inducing apoptosis in tumor cells (79).

3.5 The anti-oncogenic effect of digestive tract microbiome through inhibition of tumor metastasis

Tumor metastasis is one of the main causes of the death of cancer patients, involving the capability of transference from primary tumors to other parts of the body. Conversely, bacteria, as organism, can affect the physiological functions of host cells, thus have the potential to inhibit tumor metastasis. Zheng et al. has proposed a method for using Salmonella to treat tumors, wherein the expression of Vibrio vulnificus flagellin B by attenuated Salmonella can reduce immune suppression, alleviate subcutaneous colorectal tumors in mice, and inhibit tumor growth and metastasis, leading to an extended lifespan (80). In addition, research proved that Listeria infection can activate nicotinamide adenine dinucleotide phosphate and increase the intracellular Ca^{2+} content (81), leading to the direct killing of tumor cells. Both mechanisms can generate a highly cytotoxic free radical - reactive oxygen species (ROS) (Figure 2E) (82). ROS can initiate immunogenic cell death in tumor cells, subsequently activating CD8 T cells to eliminate residual tumor cells, ultimately inhibiting tumor cell metastasis (83).

3.6 Others

Apoptosis is a cell-autonomous, orderly mode of cell death that occurs in nucleated cells under genetic regulation. Research by Hiroaki and colleagues has demonstrated that iron-siderophores in Lactobacillus could induce apoptosis through the Jun N-terminal kinase pathway, thereby inhibiting the growth of CRC (84). Yaser et al. manifested Bifidobacterium infantis had the ability to activate p53 and inhibit NF-κB, thus inducing apoptosis in colorectal cancer cells (85). Furthermore, angiogenesis provides malignant tumors with a rich nutrient supply, further promoting their development. Therefore, inhibiting angiogenesis is a direction for treating malignant tumors. By inhibiting angiogenesis, downregulating inflammatory responses, bacteria such as Lactobacillus and Bifidobacterium can therefore preventing CRC and reducing carcinogenic metabolites like SCFAs, ultimately enhancing the function of the intestinal barrier (86). In summary, digestive tract bacteria can play a role in treating tumors through immunomodulatory effects, the secretion of bacterial outer membrane vesicles, the production of bacterial toxins during metabolism, inducing apoptosis in tumor cells, and inhibiting tumor angiogenesis.

4 Natural bacterial-based tumor therapies

Digestive tract microbiome can be divided into three categories based on its function to human body. The first class comprises commensal bacteria, such as Lactobacillus and Bifidobacterium, which have a positive impact on human health and also hold potential for tumor therapy, often referred to as "anti-tumor bacteria." The purpose of treating tumor can be achieved by

increasing the amount of these "anti-tumor bacteria." The second category is conditionally pathogenic bacteria, like *E. coli*, which, under normal circumstances, do not produce adverse effects on the host organism but can become harmful when the host's immune defenses are compromised. Finally, pathogenic bacteria are the last class, involving Staphylococcus aureus, which have detrimental effects on the host's health, including the potential to induce inflammatory responses and facilitate tumor initiation and progression, earning them the title of "pro-tumor bacteria." Furthermore, Helicobacter pylori (*H. pylori*) is a bacterium that causes inflammation of the stomach lining, which can lead to stomach ulcers. Untreated, it can be a lifelong infection or a predisposition to stomach cancer (87). Consequently, the modulation of the gut microbiota has emerged as a novel approach for addressing diseases associated with gastrointestinal dysbiosis. Potential strategies for targeting the gut microbiota encompass the use of probiotics, fecal microbiota transplantation (FMT), the administration of anti-tumor antibiotics, and dietary interventions, among others.

4.1 Treating tumors by increasing the levels of "anti-tumor bacteria" within the body

Probiotics beneficial to the human body mainly include yeast, probiotic spores, lactobacillus, bifidobacterium, and actinomycetes (88). With the advancement of modern research, the study of probiotics has become more and more extensive. Research has demonstrated that probiotics can colonized in the intestinal of human body and promote overall health, through regulating the body's immune response or modulating the balance of GIT microbiome, thereby maintaining intestinal homeostasis, among other mechanisms (89) (as shown in Table 1). Extensive studies retrieved by Stephanie et al. indicate that probiotics play a crucial role in preventing the development of colon cancer through mechanisms such as enhancing the function of intestinal barriers, suppressing and preventing colorectal carcinogenesis, reducing the metabolism of carcinogens, and inhibiting the growth of pathogenic bacteria (96). Consequently, probiotics have proven to be crucial in the field of cancer therapy.

In phase I clinical trials, influence of probiotics on gastrointestinal tumors have primarily been focused on (90, 97). *In vitro* experiments suggest that the inhibitory effect of probiotics on gastrointestinal tumors primarily relies on the production of SCFAs by probiotics, which subsequently (97). Additionally, probiotics also exhibit inhibitory effects on extraintestinal tumors such as liver cancer, breast cancer, lung cancer, and melanoma (71, 90–95). Imani and colleagues have confirmed that oral administration of Lactobacillus acidophilus, a type of probiotic, can stimulate the generation of IFN-γ while reducing the production of IL-4, thereby enhancing the immune response, inhibiting breast cancer cells, and strengthening the anti-tumor effect (Figure 3A) (90). Furthermore, probiotics also demonstrate inhibitory effects on extraintestinal tumors. Jun Li et al. have developed a novel probiotic mixture, which can slow down tumor growth and reduce tumor volume and size (93). In conclusion, probiotics hold great promise and untapped potential in the treatment of cancer, providing new research directions in the field.

TABLE 1 Representative probiotics and their tumor control mechanisms.

Probiotics strain ("anti-tumor bacteria")	Types of tumor	Mechanisms that produce therapeutic oncological effects	Ref
Lactobacillus and Bifidobacterium mixture	Gastrointestinal tumors	Associated with improved intestinal integrity, TLR2-mediated cellular pathways, reduced size of gastrointestinal tumors and reduced tumor incidence	(90)
Lactobacillus casei BL23	Colon Cancer	Promoting the production of a balanced adjuvant Th-17 biased immune response	(91)
Pediococcus pentosaceus GS4	Colon Cancer	Reduces chronic inflammation and decreases NF-κB activity associated with cell proliferation	(92)
Lactobacillus and Bifidobacterium	CRC	Reduces the size and incidence of tumors	(71)
Probiotics mixture	liver cancer	Reduces the level of Th 17 cells in the gut and the extent of Th17 recruitment at the tumor site, changes the composition of the gut microbiota and reduces the size of liver tumors	(93)
Lactobacillus acidophilus	Breast cancer	Promotes the production of IFN-γ and reduces the production of IL-4, thereby enhancing the body's immune response and producing an anti-breast cancer effect	(90)
Lactococcus lactis NK34	Breast and lung cancer	Inhibits proliferation of tumor cells	(94)
Bifidobacterium	Melanoma	Reduces tumor volume of Melanoma	(95)

FMT involves extracting microbial communities from healthy individuals' feces that are effective against a specific disease and transplanting them into patients to restore the patients' gut microbiota, thereby achieving therapeutic effects (Figure 3B) (98, 99). Rosshart et al. demonstrated that laboratory mice transplanted with gut microbiota from wild mice exhibited better resistance to CRC than control mice with their native bacteria (100). Meanwhile, gut bacterial metabolites have been shown to promote the development of chronic liver disease and hepatocellular carcinoma through the gut-liver axis (101). Recent research strongly supports the role of FMT in controlling the progression of liver cancer, such as its potential to prevent alcohol-induced liver injury (102). Moreover, FMT holds promise for enhancing the anti-tumor immune response in melanoma patients by transferring a beneficial gut microbiota community (101). Gopalakrishnan and colleagues transferred fecal samples from melanoma patients to mice and observed that FMT could enhance the effectiveness of tumor immunotherapy (103).

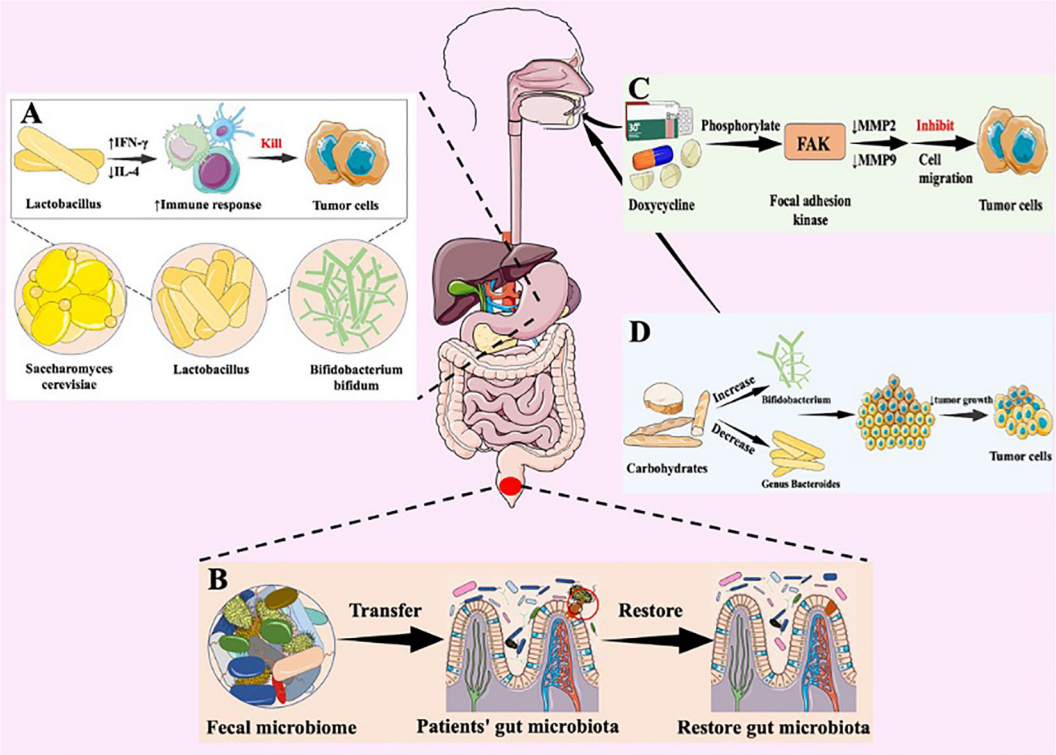

FIGURE 3
Natural bacterial-based tumor therapies. Various strategies designed to target the gut microbiota and modulate its composition for potential therapeutic benefits. **(A)** Antitumor mechanism of probiotics. Lactobacillus acidophilus is a probiotic that boosts the immune response through an increase in interferon-gamma (IFN-γ) production and a decrease in interleukin-4 (IL-4) levels. This balance leads to an increase in Th1 responses, which is critical for robust anti-tumour activity. Stimulating IFN-γ helps activate immune cells to target and destroy cancer cells, including breast cancer cells, thereby boosting the body's anti-tumour defences. **(B)** therapeutic effect of fecal microbiota transplantation. FMT symbolized by the transfer of healthy fecal material, aims to enhance microbial diversity and establish a healthy microbial community within the gut environment. FMT are introduced into the gut to restore microbial balance and promote a symbiotic relationship. **(C)** Antitumor mechanism of anti-tumor antibiotics. Doxycycline (DOX) inhibits leukaemia cell migration through phosphorylation of FAK and reduction of MMP-2 and MMP-9 expression. These enzymes are involved in the degradation of the extracellular matrix, which aids cell migration. DOX therefore reduces the ability of leukaemia cells to move and spread. **(D)** Dietary interventions. These interventions selectively nourish beneficial bacteria and contribute to the modulation of microbial composition.

4.2 Treating tumors by decreasing the levels of "pro-tumor bacteria" within the body

In the human digestive system, in addition to probiotic colonization, numerous harmful bacteria can trigger inflammatory responses that may lead to the onset and development of tumors. Therefore, the use of antibiotics to inhibit or eliminate these bacteria have been proved to provide therapeutic effect on tumor. Antibiotics have the capability to exhibit cytotoxicity against tumor cells and can exert inhibitory effects on tumors through various mechanisms. Doxycycline (DOX) have been proved possess cytotoxicity and anti-proliferative properties against various cancer cells (104, 105). Moreover, DOX exhibits broad therapeutic characteristics, such as controlling invasive and metastatic cancer cells, including inhibiting tumor growth and suppressing tumor cell migration (106). DOX inhibits leukemia cell migration by phosphorylating focal adhesion kinase, resulting in decreased expression of matrix metalloproteinases MMP2 and MMP9, which contribute to cell migration (Figure 3C) (106, 107). Yang and colleagues have demonstrated that DOX induces apoptosis in cervical cancer cell lines, inhibits tumor cell invasion, and reduces cancer stem cell (CSC) markers in cell culture, such as SOX-2 and OCT-4 (108, 109). Also, salinomycin (SAL), isolated from Streptomyces albus, has been shown not only to inhibit the proliferation of various tumor cells but also to suppress multidrug resistance (110, 111). Simultaneously, SAL is capable of targeting multiple malignant tumor CSCs while enhancing the effectiveness of radiotherapy and chemotherapy. The application of antibiotics can eliminate numerous bacteria in the gastrointestinal tract, ultimately decreasing the toxic adverse consequences that arise after cancer treatment.

4.3 Treating tumors through well-balanced diet

A well-balanced diet is essential in delaying the onset and progression of tumors. Therefore, it is necessary to follow a diet that includes a wide range of food items to prevent the growth and spread of tumors. Consuming various foods not only alters the

composition of the gut microbiota but also impacts the relative changes in gut metabolites (11). For example, the intake of carbohydrates can increase the relative abundance of Bifidobacterium in the gut, while decreasing the abundance of the genus Bacteroides (112). Also, Bifidobacterium belongs to the group of probiotics and has been associated with potential benefits in preventing inflammatory bowel diseases and CRC (Figure 3D) (113). Mehta et al. have illustrated that consuming grains and foods rich in dietary fiber may increase the likelihood of CRC FN infection (114). In addition, in patients with chronic liver disease, including those with hepatocellular carcinoma, consumption of fermented dairy products has a therapeutic effect in reducing symptoms of abdominal distension (112). Intake of probiotics enables the increase of diversity of *in vivo* microbiome, thus improving anti-tumor immune response (11). Consequently, a well-balanced diet is crucial in regulating the composition of GIT microbiome, thereby to some extent beneficial to the treatment of tumors.

5 Strategies for artificially modifying bacteria to treat tumors

The unique TME renders normal tissue blood vessels inadequate to support tumor growth during its sustained proliferation and expansion. Consequently, tumor cell growth factors activate to generate novel vascular structures (115). Such disordered vascular structure and TME characteristics present several constraints to current tumor treatment methods. These include the challenge of chemotherapeutic drugs penetrating tumor cells and their proneness to off-target effects (116). Conversely, the complexity of tumor vascular structure not only enables bacteria been trapped inside the tumor, but also provide nutrients to it, contributes to promoting the proliferation of bacteria. Consequently, bacteria can undergo artificial modification, including using engineered bacteria or bacterially incorporated nanomaterials, to enhance safety and targeting, resulting in improved anti-tumor effects.

5.1 Engineered bacteria

Genetic engineering and biological synthesis are employed to modify bacteria, aiming to achieve tumor targeting and reduce toxicity. This emerging strategy boasts numerous advantages, including a considerable enhancement in safety and specificity, as well as the inhibition of angiogenesis for anti-tumor effects, and the ability to interfere with tumor growth through RNA interference (117). Yu and colleagues employed a unique synthetic biology technique to reprogram Salmonella typhimurium to survive solely under anaerobic conditions, retaining its functionality. The team experimentally confirmed its ability to hinder tumor growth in mice, while leaving healthy cells unharmed. The engineered bacterium outperformed standard Salmonella typhimurium by reducing its toxicity and exhibiting anti-tumor properties (118). Simultaneously, engineered bacteria can be designed to target the increased angiogenesis characteristic of solid tumors and inhibit their growth, thereby offering therapeutic benefits. Jia et al. utilized genetic modifications to create attenuated Salmonella typhimurium, which delivered STAT3-specific siRNA and endostatin to mice with liver cancer. Their combination therapy demonstrated a more significant effect in treating tumors (119). As a result, engineered bacteria, as an incredibly promising method for treating tumors, are currently the subject of extensive research.

5.2 Bacteria combined with nanomaterials to exert anti-tumor effects

Nanomaterials' unique high penetrance and retention effect of nanomaterials enable them to accumulate at the tumor site, enhancing their targeting capabilities. Therefore, they find wide applications in cancer therapy (120). Currently, research indicates that bacteria can be chemically attached to nanomaterials, offering a straightforward and efficient technique. Moreover, Liu et al. have proposed innovative binding methods. To be specific, Quantum dots, due to their remarkable photostability and luminescence traits, demonstrate considerable promise in bioimaging and diagnostic applications (121). Then, Liu's team utilized bacteria as carriers to deliver quantum dots specifically into solid tumors, introducing a novel method of combining bacteria and nanomaterials (121). In order to achieve precise tumor therapy, Chen and colleagues associated Salmonella enterica YB1 to nano-photosensitizers by loading indocyanine green nanoparticles through an amide bond (122). Zhang's team devised a technique that entails loading composite nanoparticles of gold and platinum (Bac-Au@Pt) onto bacteria's surface to overcome the hindrance of antioxidant effects by tumors during chemodynamic therapy, as well as to decrease negative consequences on neighboring cells (123). Figure 4 provides an overview of the approaches to treating tumors through the bacteria's collective action.

5.3 Bacteria in combination with conventional therapies for treating tumors

Currently, radiation therapy, chemotherapy and immunotherapy are the main non-surgical approaches to tumor treatment. However, these methods lack malignancy specificity and often cause adverse effects (78, 124). By contrast, if combine bacterial treatment to conventional therapies can overcome the disadvantages like poor targeting and specificity of conventional therapies. Table 2 summarizes part of research progress associated with the combination of bacterial treatment and conventional therapies.

Radiotherapy is a focused cancer treatment using radiation to eradicate cancer cells. Its principle is to target the tumor and avoid damage to healthy adjacent cells. Combining bacteria with radiation therapy for cancer treatment can enhance the targeting of bacteria to cancer cells, prolong the duration of bacterial action, and improve the overall effectiveness of radiation therapy. This approach holds promise in improving the outcomes of cancer treatment (131). Chandra and colleagues utilized a starvation response to introduce the radioactive isotope (32) P into Listeria bacteria. This allowed for

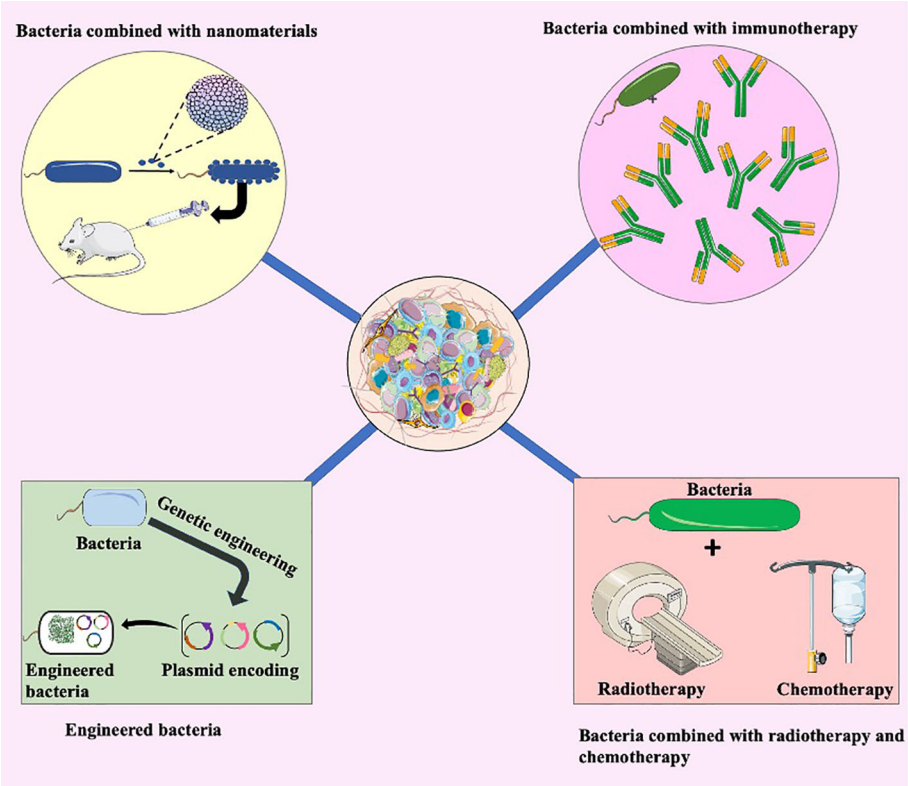

FIGURE 4
Bacterial combination therapy. The integration of bacteria with nanomaterials represents an innovative approach. Nanomaterials enhance the delivery of therapeutic agents, optimizing their interaction with tumors. This synergy holds potential for more targeted and effective tumor treatment. Bacteria combined with immunotherapy illustrates another promising avenue. The interaction between bacteria and the immune system can be harnessed to enhance the body's natural defense mechanisms against cancer cells. This combination approach aims to amplify the immune response for improved tumor recognition and elimination. Engineered bacteria involve genetic modifications to enhance their therapeutic properties. These modifications may include the production of anti-tumor agents or the expression of specific receptors for targeted delivery within the tumor microenvironment. Additionally, the integration of bacteria with traditional cancer treatments such as radiotherapy and chemotherapy. Bacteria can potentially augment the efficacy of these treatments, creating a synergistic effect for more potent tumor eradication.

the induction of ionizing radiation and bacteria-induced reactive oxygen species at the tumor site, ultimately resulting in the targeted eradication of cancer cells (132).

Chemotherapy drugs can spread throughout the body via the bloodstream and are a primary treatment method for inhibiting metastatic cancer (133). The disruption of tumor-specific vascular structures by chemotherapeutic agents can be enhanced by using bacteria in combination with chemotherapy for cancer treatment. Studies indicate that a combination of Salmonella VNP20009 with cyclophosphamide significantly reduces tumor micro-vessel density and vascular endothelial growth factor content compared to using cyclophosphamide alone (134). Meanwhile, numerous studies suggest that bacteria can act as chemotherapy drug carriers, facilitating targeted cancer therapy and reducing drug side effects (135).

Immunotherapy is the method that enhancing the immune system of immunocompromised patients. Recent research indicates that tumor cells have developed numerous mechanisms to evade immune detection, including recruiting immunosuppressive cell populations, modulating signaling pathways, and altering the TME, reducing immunotherapy efficacy (136). To be specific, tumor cells can evade host immune surveillance by expressing PD-L1 and activating PD-1 (137). whereas the microbiome is intimately involved in the regulation of checkpoint interactions, for example, bifidobacterium bifidum in combination with anti-PD-L1 therapy improves efficacy through activation of dendritic cells and enhanced accumulation of $CD8^+$ T cells (95). In addition, immune checkpoint inhibition is the key to anti-cancer immunotherapy, which enhances the immune response by blocking immune checkpoints such as CTLA-4 and PD-1 (138, 139), such as Ipilimumab. The monoclonal antibody is currently used in the treatment of melanoma, renal cell carcinoma, hepatocellular carcinoma, non-small cell lung cancer and colorectal cancer. Bacteria, genetically engineered to increase the expression of tumor antigens, can be combined with immunotherapy to boost the body's immune response, resulting in an anti-tumor response.

5.4 Others

Photothermal therapy (PTT) and photodynamic therapy (PDT) have a huge potential to become a vital method to treat tumors (140). However, the mechanism of such two treatments not fully understood yet, making it difficult to achieve optimal treatment results. At the same

TABLE 2 Bacteria combined with traditional methods to treat tumors.

No.	Traditional oncology therapy in combination with bacteria	Name of bacteria	Drug	Types of tumor	Mechanism of action	Ref
1	Radiotherapy	*Salmonella*		systemic antitumor effects	Salmonella activates peripheral DCs in tumor marginal tissues, resulting in increased DC numbers and prolonged survival to produce good tumor therapeutic effects.	(125)
2	Radiotherapy	*E. coli*		CRC	Significant reduction in tumor size and production of cytolysin A	(78)
3	Chemotherapy	*E. coli* Nissle 1917 (EcN)	doxorubicin (DOX)	Breast cancer	Adriamycin is coupled to EcN via the acid instability of cis-aconitic anhydride and this coupling not only increases its viability in tumor tissue but also has no effect on bacterial motility	(126)
4	Chemotherapy	*Salmonella*	doxorubicin	CRC	With the help of high intensity focused ultrasound heating, the release of adriamycin from colon cancer cells is induced, thus allowing the drug to take effect in the cytoplasm and nucleus of the cancer cells.	(127)
5	Immunotheraoy	Listeria	gemcitabine	pancreatic ductal adenocarcinoma	Selectively targets tumors and can be delivered to the tumor area to act	(128)
6	Immunotherapy	*L. acidophilus*		Lung cancer	Increase in serum levels of IFN-γ, IL-10, CD4 and CD8 cells	(129)
7	Immunotherapy	*L. monocytogenes*		Melanoma	Enhanced infiltration of CD4 and $CD8^+$ T cells	(130)

time, these two methods all approaches confront challenges such as limited penetration depth and the suboptimal tumor-targeting capabilities of photosensitizers. However, obligate or facultative anaerobic bacteria are naturally endowed with specific targeting capabilities for hypoxic regions within the tumor. Furthermore, the increased permeability of tumors enables enhanced binding between internal chemotactic factors and bacterial-specific receptors, promoting bacterial colonization within the tumor. Once established, bacteria can inhibit tumor growth through mechanisms such as immunomodulation (141). Due to the innate targeting capabilities of bacteria towards tumor cells, bacteria can be conjugated with photosensitizers to facilitate the delivery of these photosensitizers to the tumor site (122). Simultaneously, bacteria activate the host immune response, thereby significantly improving the effectiveness of PDT and PTT in treating tumors.

5.5 Clinical research based on bacterial tumor treatment

Bacterial-based tumor therapy has plenty of advantages compared to conventional drug delivery systems. Firstly, bacteria such as Bifidobacterium, Salmonella, Escherichia and Clostridium can selectively target and proliferate within tumour tissue due to

TABLE 3 Clinical trials related to the treatment of tumors with bacteria.

No.	Company	Bacteria	Indications	Clinical Phase	Clinical Trials No.	Ref
1	Jianbin Xiang, Huashan Hospital	intestinal maladjusted flora	Rectal Cancer	Early Phase 1	NCT05759741	(142)
2	Mayo Clinic	Engineering Gut Microbiome	Breast and Lung Cancer	Early Phase 1	NCT04857697	(143)
3	National Cancer Institute (NCI)	gut microbiome	melanoma	Phase I Phase II	NCT03819296	(144)
4	Parker Institute for Cancer Immunotherapy	oral microbiome	Metastatic Melanoma	Phase 1	NCT03817125	(145)
5	Shanghai Zhongshan Hospital	intestinal flora	Non-Small Cell Lung Cancer	Phase 1	NCT05008861	(146)
6	Michael Dill, University Hospital Heidelberg	Fecal Microbiota	hepatocellular carcinoma	Phase 2	NCT05690048	(147)
7	National Institutes of Health Clinical Center (CC) (National Cancer Institute (NCI))	gut bacteria	Metastatic Hepatocellular Carcinoma	Phase 2	NCT04025567	(148)
8	National Institutes of Health Clinical Center (CC) (National Cancer Institute (NCI))	gut bacteria	Hepatocellular Carcinoma	Phase 2	NCT03785210	(149)

their innate ability to thrive in the hypoxic and acidic environments characteristic of solid tumours. These bacteria can penetrate the blood-tumour barrier and deliver therapeutic agents directly to the tumor site by exploiting the unique conditions of the tumour microenvironment. In addition, the anti-tumor effects of bacteria can be enhanced by their inherent immunogenicity and toxin production. Recent advances in genetic engineering have reignited interest in bacterial therapy as a promising modality for cancer treatment by further optimising these bacterial vectors, enabling precise targeting and minimising off-target effects. Based on the huge progress in laboratory research of bacterial tumor treatment, numerous clinical research has also been undertaken (Table 3).

6 Future perspective

The gastrointestinal tract encompasses the human digestive process, from ingestion to excretion, and contains a diverse array of bacteria. The gut microbiota has been implicated in several gastrointestinal diseases. The connection between the gut microbiota and the development and progression of tumors is intricate, with different gastrointestinal bacteria exerting varying influences on tumorigenesis and growth. Some bacteria have potential in tumor therapy because they promote tumorigenesis, while others inhibit tumor cell growth. Apart from their intrinsic anti-tumor effects, bacteria can be genetically engineered to achieve specific targeting. Bacteria targeting tumors can overcome the unique tumor microenvironment and overcome the limitations of current clinical methods of treating tumors, which fail to penetrate deep into the tumor. Accordingly, bacteria hold great promise as a modality for the treatment of tumors. More complex goals in tumor therapy can be achieved by combining bacteria with lightweight nanomaterials. Furthermore, bacterial combination therapy is a prominent area of research seeking to overcome the limitations of current tumor treatment methods. Presently, several clinical trials related to bacterial therapy of tumors have entered either phase I or phase II. Consequently, bacterial therapy for tumors has the potential to become a novel frontier in future tumor treatment.

Author contributions

YS: Writing – original draft, Writing – review & editing. XL: Writing – review & editing, Funding acquisition. JZ: Writing – review & editing, 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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Reduction of hyperglycemia in STZ-induced diabetic mice by prophylactic treatment with heat-killed *Mycobacterium aurum*: possible effects on glucose utilization, mitochondrial uncoupling, and oxidative stress in liver and skeletal muscle

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Background: In addition to conventional treatment and modifications in physical activity and diet, alternative strategies have been investigated to manage, prevent, or delay diabetes in humans. In this regard, one strategy has relied on the immunomodulatory properties of mycobacteria, whereby Bacillus Calmette–Guerin, an attenuated live strain of *Mycobacterium bovis*, has been shown to improve glycemic control in patients with diabetes and to alleviate hyperglycemia in selected murine models of diabetes. A novel heat-killed (HK) whole-cell preparation of *Mycobacterium aurum* (*M. aurum*) is currently under development as a potential food supplement; nevertheless, its potential bioactivity remains largely unknown. Thus, the present study investigated the potential prophylactic anti-diabetic effects of HK *M. aurum* in streptozotocin (STZ)–induced diabetic mice.

Methods: Mice were divided into three groups: the STZ-induced diabetic group was injected with a single intraperitoneal high dose of STZ, the HK *M. aurum*–treated diabetic group was prophylactically treated with three doses of HK *M. aurum* 6 weeks before STZ injection, and the control non-diabetic group was given three intradermal injections of borate-buffered saline and an intraperitoneal injection of citrate buffer. Liver lactate dehydrogenase (LDH), uncoupling protein 2 (UCP2), and glucose transporter 2 (GLUT2) and skeletal muscle LDH, UCP3, and GLUT4 protein expression levels in different mouse groups were determined by Western blot.

Results: Our results indicated that HK *M. aurum* did not cause any significant changes in glycemic levels of normal non-diabetic mice. Prophylactic administration of three doses of HK *M. aurum* to diabetic mice resulted in a significant reduction in their blood glucose levels when compared to those in control diabetic mice. Prophylactic treatment of diabetic mice with HK *M. aurum*

significantly restored their disturbed protein expression levels of liver UCP2 and LDH as well as of skeletal muscle UCP3. On the other hand, prophylactic treatment of diabetic mice with HK *M. aurum* had no significant effect on their liver GLUT2 and skeletal muscle GLUT4 and LDH protein expression levels.

Conclusions: Our findings provide the first evidence that HK *M. aurum* possesses a hyperglycemia-lowering capacity and might support its future use as a food supplement for the amelioration of diabetes.

KEYWORDS

diabetes, *Mycobacterium aurum*, uncoupling protein 2 (UCP2), uncoupling protein 3 (UCP3), GLUT2, GLUT4, oxidative stress, LDH

Introduction

According to the International Diabetes Federation, diabetes has become a disease of pandemic proportions whereby it is projected to reach more than 1.31 billion people worldwide by 2050 (1). Given the global burden of diabetes, several studies have been looking for novel strategies to alleviate or prevent the symptoms and complications of diabetes such as the use of herbal products or probiotics (2, 3). Type 2 diabetes (T2D) can be largely prevented through diet and lifestyle changes; however, prevention of type 1 diabetes (T1D) remains challenging. Bearing in mind that T1D is an autoimmune disease during which the immune system attacks the pancreatic β-cells and insulin auto-antigens, researchers have attempted to identify an immunomodulatory agent for the prevention or treatment of this disease.

Mycobacterial species are known to hold a powerful immunomodulatory capability, and this is mainly attributed to their complex cell wall composition (4). In this context, exploiting such immunomodulatory properties might represent one innovative approach to reverse, delay, or prevent the onset of diabetes. Bacille Calmette–Guérin (BCG), a live attenuated strain of *Mycobacterium bovis* (*M. bovis*), has been administered to humans for more than 100 years as a vaccine against tuberculosis and later as a therapeutic agent for non-invasive bladder cancer (5). The potential use of BCG to prevent or manage diabetes has been demonstrated in several studies that were conducted in both humans and mice, whereby there were several variations among those studies in terms of BCG's used strains, doses, as well as the route and timing of its administration (6–10). Recent epidemiological data strongly support a possible role for BCG in the prevention of T1D, but not T2D (11). Moreover, BCG has been previously shown to lower hyperglycemia in patients with T1D as well as in single high-dose streptozotocin (STZ)–induced diabetic mice (12). BCG exerts its hyperglycemia lowering effects in humans by inducing the expansion of regulatory T cells (Tregs) and reducing the numbers of cytotoxic T cells (Tcs). Interestingly, BCG also acts on T lymphocytes by promoting glycolysis, which leads to the consumption of high amounts of glucose, thus lowering hyperglycemia (12, 13).

Considering the uncommon adverse effects and rare complications (i.e., local abscess formation, regional lymphadenitis, and disseminated BCG disease) experienced with multiple BCG administration (5) and the current sporadic shortage in BCG production (14), an alternate immunomodulatory-based approach that relies on the use of an environmental mycobacterium may prove to be efficient in preventing or treating diabetes. *Mycobacterium vaccae* (*M. vaccae*) is an environmental, rapidly growing mycobacterium that has been used in the form of a heat-killed (HK) whole-cell preparation as an immunotherapeutic/immunomodulatory agent in various disorders and disease settings (15–17). Similar to BCG, HK *M. vaccae* has been revealed to induce the expansion of Tregs (18) and to immunomodulate innate immune cells (19). In a study employing whole-genome sequencing, the environmental and rapidly growing *Mycobacterium aurum* (*M. aurum*; also known as *Mycolicibacterium aurum* Aogashima) was positioned in close proximity to *M. vaccae* (20). Little is known about the immunomodulatory properties of *M. aurum*; however, a HK whole-cell preparation of this mycobacterial strain is currently undergoing development as a potential psychobiotic (21). HK *M. aurum* was previously reported to exhibit no toxicity or pathogenicity in rats following its oral administration, thus justifying its potential use as a food ingredient (22). Given its phylogenetic proximity to *M. vaccae*, *M. aurum* may also hold a yet undiscovered similar immunomodulatory potential that may be beneficial in the prevention or management of diseases, such as diabetes.

The present study aimed to evaluate the efficacy of HK *M. aurum* in preventing the development of diabetes in STZ-induced diabetic BALB/c mice. Therefore, we examined the effect of HK *M. aurum* on blood and urine glucose levels in diabetic mice. Moreover, we assessed in diabetic mice the effect of HK *M. aurum* on the expression of key proteins involved in glucose transport and metabolism in the liver and

skeletal muscle which are insulin-sensitive tissues significantly influenced by insulin insufficiency and hyperglycemia (23).

Materials and methods

Antibodies

The following antibodies were used: goat anti-mouse uncoupling protein 2 (UCP2), rabbit anti-mouse UCP3 antibodies (Santa Cruz Biotechnology), rabbit anti-mouse lactate dehydrogenase (LDH) A subunit antibody (Abcam), rabbit anti-mouse glucose transporter 2 (GLUT2) antibody (EMD Millipore), mouse anti-mouse GLUT4 antibody (Cell Signaling), goat anti-mouse actin antibody, horseradish peroxidase (HRP)–conjugated donkey anti-goat antibody (Santa Cruz Biotechnology), HRP-conjugated donkey anti-rabbit antibody (Abcam), and HRP-conjugated goat anti-mouse antibody (Invitrogen).

HK mycobacteria

Briefly, sterile HK *M. aurum* whole-cell preparations (stock: 200 mg/mL in water) were manufactured by autoclaving for 15 min at 121°C (kindly provided by Immune Boost Clinic Limited). Sub-dilutions were prepared in borate-buffered saline (BBS) to make solutions of 1 mg/mL and 10 mg/mL that were stored at 4°C for later administration to mice.

STZ preparation

STZ (Sigma-Aldrich) was freshly made by dissolving in ice-cold citrate buffer (CB; pH 4.5), whereby a preparation of STZ (150 mg/kg), was prepared and administered to mice, within the first 30 min of STZ preparation (12).

Animals

Adult male BALB/c mice (4–6 weeks in age) ranging in weight from 18 g to 25 g were used in all experiments. Mice were housed at the University of Balamand animal facility that is set with a 12-h dark/light cycle and fed regular chow *ad libitum*. The animal experimental protocols were approved by the research committee at the Faculty of Medicine and Medical Sciences, University of Balamand. The experiments with animals were performed in accordance with the guidelines for ethical conduct in the care and use of animals set by the institution. Experimental mouse groups were randomly assigned into nine groups that were matched by weight (20.8 ± 0.98 g) and age.

Experimental design

Mice were randomly divided into nine matched groups. The control non-diabetic "BBS" group received three doses of 100 µL of BBS given 2 weeks apart over 6 weeks. The control non-diabetic "BBS + CB" group received three doses of 100 µL of BBS given 2 weeks apart over 6 weeks and a single dose of 100 µL of CB given on the sixth week after the first BBS dose. The "Ma (0.1)" group received three doses of 100 µL of 0.1 mg of HK *M. aurum* given 2 weeks apart over 6 weeks. The "Ma (0.1) + CB" group received three doses of 100 µL of 0.1 mg of HK *M. aurum* given 2 weeks apart over 6 weeks and a single dose of 100 µL of CB given on the sixth week after the first *M. aurum* dose. The "Ma (1)" group received three doses of 100 µL of 1 mg of HK *M. aurum* given 2 weeks apart over 6 weeks. The "Ma + CB" group received three doses of 100 µL of 1 mg of HK *M. aurum* given 2 weeks apart over 6 weeks and a single dose of 100 µL of CB given on the sixth week after the first HK *M. aurum* dose. The diabetic "BBS + STZ" group received three doses of 100 µL of BBS given 2 weeks apart over 6 weeks and a single dose of STZ (150 mg/kg body weight in 100 µL of CB per dose) given on the sixth week after the first BBS dose. The "Ma (0.1) + STZ" group received three doses of 100 µL of 0.1 mg of HK *M. aurum* given 2 weeks apart over 6 weeks and a single STZ dose (150 mg/kg body weight, in 100 µL of CB) given on the sixth week after the first HK *M. aurum* dose. The "Ma + STZ" group received three doses of 100 µL of 1 mg of HK *M. aurum* given 2 weeks apart over 6 weeks and a single STZ dose (150 mg/kg body weight, in 100 µL of CB) given on the sixth week after the first HK *M. aurum* dose. Mice pre-treated with BBS or HK *M. aurum* received intradermal (at the base of the tale) injections of either treatment. BBS was given as a vehicle control for HK *M. aurum* to the aforementioned assigned mouse groups. A single high-dose injection of STZ (150 mg/kg) was administered intraperitoneally to STZ-treated mice so as to induce hyperglycemia (Kuhtreiber et al., 2018). CB was given intraperitoneally as a vehicle control for STZ to mice in non-diabetic groups. Mouse groups injected with STZ received 10% sucrose water for the first 48 h after STZ injection. Experimental design and procedures are summarized in Supplementary Figure 1.

Measurement of body weight, urine, and blood glucose levels

Mice body weights as well as their blood glucose levels were monitored every 2 weeks for a period of 6 weeks before STZ injection and on a weekly basis for a period of 6 weeks after STZ injection. Blood was collected from the tail vein of mice for measurement of blood glucose levels using the human Accu-Chek Guide glucometer (Roche Diagnostics). Mice were allowed to fast for 4 h prior to blood glucose measurements. Urine glucose levels in mice were measured before STZ injection at week 0 and on weeks 2 and 4 after STZ injection using urine colorimetric strips (Acon Laboratories).

Western blot analysis

Total protein (50 µg) isolated from the liver and skeletal muscle 6 weeks after STZ injection was electrophoresed through 12% SDS–polyacrylamide gel and then electro-blotted to polyvinylidene difluoride (PVDF) membranes (GE Healthcare). The membranes were blocked with 5% bovine serum albumin (BSA) in 1% tris-buffered saline (TBS) containing 1% Tween 20 (1% TBS-T) and probed overnight with the primary antibody of choice (anti-UCP2, anti-UCP3, anti-GLUT2, anti-GLUT4, anti-LDH, or anti-actin antibody). Membranes were then washed five times for 10 min with 1% TBS-T, before incubation with the respective secondary antibody for an hour and a half under constant shaking. Membranes are then washed again (five times for 5 min). Finally, the membranes were incubated with Clarity Western enhanced chemiluminescence substrate (Bio-Rad) for 5 min at room temperature and visualized using the Chemidoc system (Bio-Rad). The bands were quantified using the Image lab software (version 5.2.1; Bio-Rad).

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (version 6; GraphPad Software). Data were presented as mean values ± standard error of the mean (SEM). Unpaired t-test with Welch correction was used to compare the means of two different experimental groups. One-way or two-way ANOVA test followed by the Tukey's multiple comparison *post-hoc* test was used to compare the means between three different experimental groups. Multiple unpaired t-tests were used to compare the means between two different experimental groups at different time points. Each P-value is adjusted to account for multiple comparisons. Differences between groups were considered to be statistically significant at $p < 0.05$. The schematic diagram was created using Biorender website.

Results

HK *M. aurum* prophylactic treatment does not induce hypoglycemia or weight change in non-diabetic mice but reduces hyperglycemia and glucosuria in STZ-induced diabetic mice

The primary set of experiments was carried out to evaluate the potential effect of HK *M. aurum* administration to non-diabetic BALB/c mice on their body weight and blood glucose levels. Non-diabetic mice were injected with three doses of 0.1 or 1 mg per injection of HK *M. aurum* given 2 weeks apart. Body weight and blood glucose levels were monitored on a weekly basis up to week 6 and compared to control non-diabetic mice that were injected with BBS as vehicle. Both parameters were found to be similar between the two mouse groups, whereby neither body weights (Figure 1A) nor blood glucose levels (Figure 1B) were significantly altered in non-diabetic mice treated with 0.1 mg or 1 mg per injection of HK *M. aurum* mice.

Next, we investigated the potential prophylactic effect of HK *M. aurum* in restoring weight gain and reducing hyperglycemia in STZ-induced diabetic mice. Mice were pre-treated with three doses of 1 mg per injection of HK *M. aurum* given 2 weeks apart prior to STZ injection. STZ was administered 6 weeks after the first *M. aurum* dose. As expected, the diabetic group (BBS + STZ) was unable to gain weight on a weekly basis as it did prior to STZ injection and HK *M. aurum* did not seem to prevent the STZ-induced halt in body weight gain (Figure 2A). Blood glucose levels were monitored on a weekly basis in *M. aurum*–pre-treated group (Ma + STZ) and compared to those of control diabetic group (BBS + STZ). The HK *M. aurum*–pre-treated group showed a trend toward decreased blood glucose levels for 5 weeks after STZ injection, whereas, at week 6, the HK *M. aurum*–pre-treated group had a

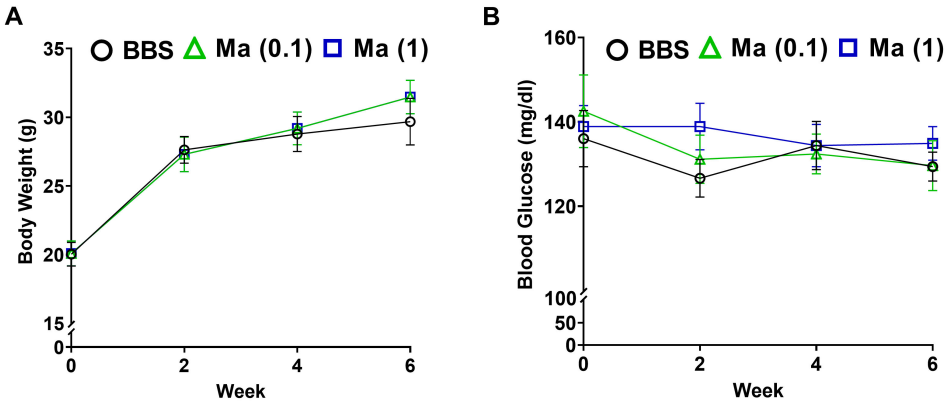

FIGURE 1
Effect HK *M. aurum* treatment on body weight and blood glucose levels in non-diabetic mice. Non-diabetic BALB/c mice were treated with three doses of borate-buffered saline (BBS) or HK *M. aurum* (Ma; 0.1 mg or 1 mg per injection) given 2 weeks apart. Mice **(A)** body weights and **(B)** blood glucose levels were recorded on a weekly basis for different mouse groups. Each symbol represents the mean value ± SEM of body weight or blood glucose level for each mouse group (n = 8 mice per group). Statistically significant differences were determined by two-way ANOVA followed by Tukey *post-hoc* test.

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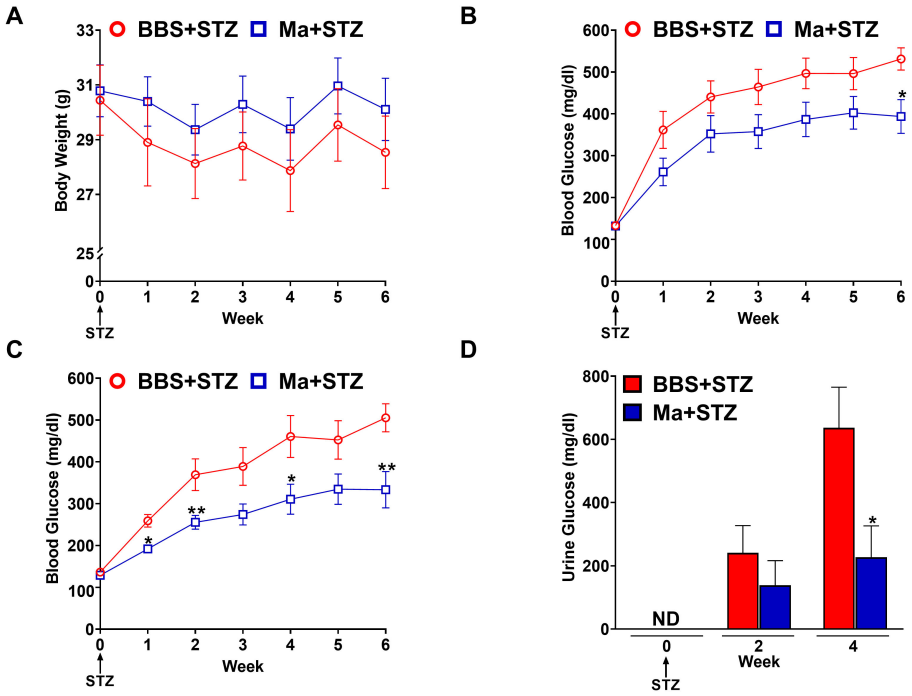

FIGURE 2
Prophylactic effects HK *M. aurum* on body weight, blood, and urine glucose levels in STZ-induced diabetic mice. Non-diabetic BALB/c mice were treated with three doses of borate-buffered saline (BBS) or HK *M. aurum* (Ma; 1 mg per injection) given 2 weeks apart. After 6 weeks of prophylactic treatment (at week 0), diabetes was induced in both groups of mice through injecting them with STZ (150 mg/kg). The control non-diabetic group received citrate buffer (BBS + CB). Mice **(A)** body weights and **(B, C)** blood glucose levels were measured on a weekly basis up to 6 weeks after STZ, whereas their **(D)** urine glucose levels were analyzed only at weeks 2 and 4 after STZ. **(A–C)** Each symbol represents the mean value ± SEM of body weight or blood glucose level for each mouse group (A, B: n = 12 mice per group; C: n = 7 mice per group). **(D)** Bar graphs represent the mean value ± SEM of urine glucose levels for each mouse group (n = 12 mice per group). For body weights and blood glucose levels, statistically significant differences were determined by two-way ANOVA followed by Tukey *post-hoc* test. For urine glucose levels, statistically significant differences were determined by multiple unpaired t-tests. *$p < 0.05$ and **$p < 0.01$ *versus* the BBS + STZ group. ND, not detected.

significantly ($p < 0.05$) lower (~26%) blood glucose level than its corresponding diabetic control mice group (Figure 2B). Further analysis of mice subpopulations in both groups revealed that HK *M. aurum* pre-treatment exerted a more evident blood glucose–lowering effect in terms of statistical significance ($p < 0.05$) at weeks 1, 2, 4, and 6 when severely diabetic mice (blood glucose > 300 mg/dL) (24) within the first week after STZ injection were excluded from analysis. Moreover, a statistically significant ($p < 0.01$) decrease of ~34% in glycemic levels was observed at week 6 after STZ injection in mice pre-treated with HK *M. aurum* (1 mg per injection) as compared to that in the BBS pre-treated group (Figure 2C). In confirmation to the results observed on blood glucose levels, urine glucose levels were monitored for 4 weeks after STZ injection. The *M. aurum*–pre-treated diabetic group (Ma + STZ) showed a trend toward a decrease in urine glucose levels at week 2 that became statistically significant ($p < 0.05$) at week 4 after STZ injection with urine glucose levels that were 2.8-fold lower than those of the diabetic group pre-treated with BBS (BBS + STZ) (Figure 2D). We also noted that pre-treating mice with a lower dose of HK *M. aurum* (0.1 mg per injection) failed to restore weight gain and to reduce hyperglycemia in STZ-induced diabetic mice (Supplementary Figure 2).

Prophylactic treatment with HK *M. aurum* prevents the dysregulation of skeletal muscle UCP3 and liver LDH and UCP2 but fails to restore liver GLUT2 and skeletal muscle GLUT4 protein expression levels in STZ-induced diabetic mice

Western blot was performed to assess the effect of HK *M. aurum* prophylactic treatment on the expression of key metabolism–related proteins that are involved in glucose transport (GLUT2 and GLUT4), glycolysis (LDH), and mitochondrial uncoupling (UCP2 and UCP3). Therefore, the expression levels of the aforementioned proteins were evaluated in the skeletal muscle and/or liver of normal control mice (BBS + CB), untreated diabetic mice (BBS + STZ), and *M. aurum*–treated diabetic mice (Ma + STZ). In the skeletal muscle, LDH and UCP3 protein levels were shown to be significantly elevated in the untreated diabetic group (BBS + STZ) as compared to that in the non-diabetic control group (BBS + CB) (Figures 3A, B). Prophylactic treatment with HK *M. aurum* significantly reduced the STZ-induced elevation in UCP3 protein expression in the Ma + STZ group (Figure 3B); however, such treatment did not affect

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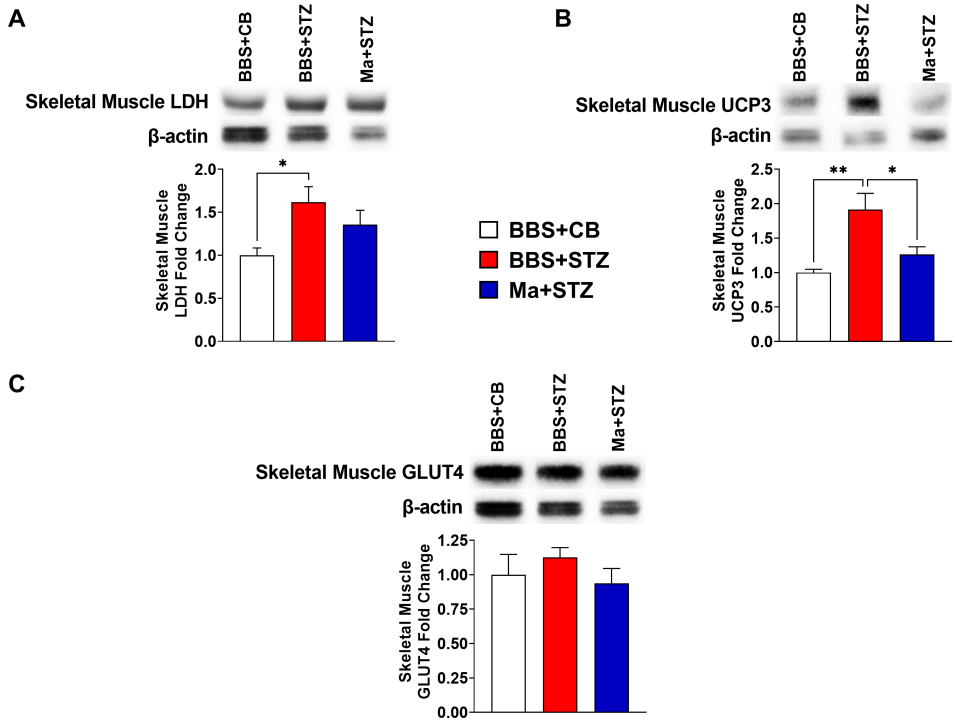

FIGURE 3
HK *M. aurum* prophylactic treatment prevents the upregulation of skeletal muscle UCP3 expression in STZ-induced diabetic mice. Non-diabetic BALB/c mice were treated with three doses of borate-buffered saline (BBS) or HK *M. aurum* (Ma; 1 mg per injection) given 2 weeks apart. After 6 weeks of prophylactic treatment (at week 0), diabetes was induced in both groups of mice through injecting them with STZ (150 mg/kg). The control non-diabetic group received citrate buffer (BBS + CB). Representative Western blots showing protein expression levels and quantification of skeletal muscle **(A)** LDH, **(B)** UCP3, and **(C)** GLUT4 protein expression levels were performed in normal non-diabetic (BBS + CB), untreated diabetic (BBS + STZ), and Ma-treated diabetic (Ma + STZ) mice at week 6 after STZ. Bar graphs show the relative protein density of **(A)** LDH, **(B)** UCP3, and **(C)** GLUT4 after normalization with β-actin protein. Data are expressed as mean ± SEM (n = 5–10 mice per group). One-way ANOVA test followed by the Tukey's multiple comparison *post-hoc* test was used to compare the means between the three different experimental groups. Differences between groups were considered to be statistically significant at $*p < 0.05$ and $**p < 0.01$.

LDH protein expression (Figure 3A). In comparison to control non-diabetic mice (BBS + CB), the protein expression of skeletal muscle GLUT4 was not significantly affected by STZ treatment in the diabetic group (BBS + STZ) as well in the HK *M. aurum*–pre-treated diabetic group (Ma + STZ) (Figure 3C). Results showed that hepatic LDH and UCP2 protein expression levels were significantly ($p < 0.05$) downregulated in the untreated diabetic group (BBS + STZ) as compared to that in the control non-diabetic group (BBS + CB) (Figures 4A, B). Prophylactic treatment of diabetic mice with HK *M. aurum* (BBS + STZ) prevented the STZ-induced downregulation in LDH and UCP2 levels, whereby the expression levels of both proteins were comparable to those of control non-diabetic mice (BBS + CB) but significantly ($p < 0.05$) higher than those of untreated diabetic mice (STZ + BBS) (Figures 4A, B). GLUT2 expression in the liver was found to be reduced by ~33% in the untreated diabetic group (BBS + STZ) as compared to the control non-diabetic group (BBS + CB) (Figure 4C). However, HK *M. aurum* pre-administration to diabetic mice (Ma + STZ) did not affect GLUT2 protein expression as compared to the untreated diabetic mice (BBS + STZ) (Figure 4C).

Discussion

Our study explored the potential prophylactic anti-diabetic effects of multiple doses of HK *M. aurum* in STZ-induced diabetic mice. HK *M. aurum* was administered to mice 6 weeks prior to diabetes induction by a single high-dose STZ treatment, which induces T1D. This approach was designed to evaluate whether HK *M. aurum* could prevent or ameliorate hyperglycemia in STZ-induced diabetic mice. We have demonstrated for the first time that prophylactic treatment with three doses of HK *M. aurum* is able to significantly reduce hyperglycemia and glycosuria in STZ-induced diabetic mice. Meanwhile, prophylactic treatment with HK *M. aurum* corrected the dysregulated protein levels of liver LDH and UCP2 and skeletal muscle UCP3 in STZ-induced diabetic mice. To model diabetes in BALB/c mice, we utilized STZ, a compound widely used to induce diabetes in rodents by selectively targeting pancreatic β-cells (25). STZ is a glucosamine-nitrosourea compound that enters pancreatic β-cells via the GLUT2 glucose transporter. Once inside the cells, STZ causes β-cell death through multiple mechanisms that involve the induction of DNA damage and oxidative stress, thus

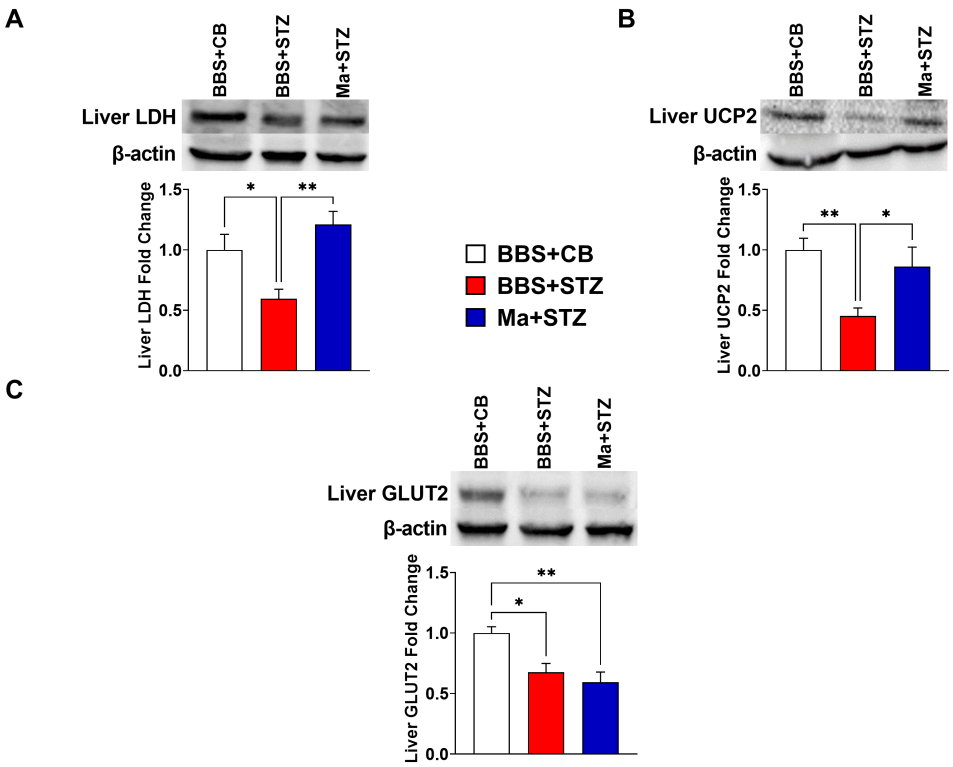

FIGURE 4
HK *M. aurum* prophylactic treatment prevents the downregulation of liver LDH and UCP2 expression in STZ-induced diabetic mice. Non-diabetic BALB/c mice were treated with three doses of borate-buffered saline (BBS) or HK *M. aurum* (Ma; 1 mg per injection) given 2 weeks apart. After 6 weeks of prophylactic treatment (at week 0), diabetes was induced in both groups of mice through injecting them with STZ (150 mg/kg). The control non-diabetic group received citrate buffer (BBS + CB). Representative Western blots showing protein expression levels and quantification of liver **(A)** LDH, **(B)** UCP2, and **(C)** GLUT2 in normal non-diabetic (BBS + CB), untreated diabetic (BBS + STZ), and Ma-treated diabetic (Ma + STZ) mice at week 6 after STZ. Bar graphs show the relative protein density of **(A)** LDH, **(B)** UCP2, and **(C)** GLUT2 after normalization with β-actin protein. Data are expressed as mean ± SEM (n = 5–11 mice per group). One-way ANOVA test followed by the Tukey's multiple comparison *post-hoc* test was used to compare the means between the three different experimental groups. Differences between groups were considered to be statistically significant at $*p < 0.05$ and $**p < 0.01$.

contributing to the apoptosis and necrosis of pancreatic β-cells (26). These mechanisms collectively lead to a severe reduction in insulin secretion and the development of subsequent hyperglycemia and therefore mimicking aspects of T1D when a single high dose (150 mg/kg) of STZ is injected into BALB/c mice.

The complex configuration of the cell wall of various mycobacterial species has granted them potent immunomodulatory activities (4). The live attenuated strain of *M. bovis*, BCG, has been reported to hold immunomodulatory effects that target both the innate and adaptive arms of the human immune system (27). In that manner, BCG is able to reduce hyperglycemia in patients with diabetes and in STZ-induced diabetic mice by inducing Treg and decreasing Tc cell numbers. Furthermore, it is able to reprogram Tregs' metabolism to favor glucose take-up and oxidation through glycolysis (12, 13). Another mycobacterial species, *M. vaccae*, that was used in the form of a HK whole-cell preparation, has gained attention as an effective immunomodulator with evident effects on adaptive immune cells, via Treg expansion (18), as well as on innate immune cells (19, 28). Currently, a HK preparation of *M. aurum* is being marketed as a promising "tuner" of the immune system, whereby it might aid in coping with anxiety, managing undesired inflammatory responses, and enhancing stress resilience (21). Whole-genome sequencing coupled with phylogenetic analysis revealed a high degree of similarity between *M. vaccae* and *M. aurum*; thus, it is highly likely that the latter species might possess comparable immunomodulatory properties and probably a potential benefit in the management of diabetes. Therefore, lessons learned from studies highlighting the prophylactic anti-diabetic effects of BCG have helped us to construct our study design in the hope to uncover similar properties of HK *M. aurum* in preventing or lowering hyperglycemia in STZ-induced diabetic mice.

In the present study, non-diabetic mice prophylactically treated with three intradermal doses of HK *M. aurum* did not display any significant changes in their glycemic levels or weight gain patterns, thus strengthening the safety profile of this HK mycobacterial preparation. This is in accordance with a previous toxicity evaluation study in rats that confirmed that HK *M. aurum* was safe and tolerable when administered via the oral route, hence qualifying it as a possible food ingredient (22).

As expected, data from the current study revealed that untreated STZ-induced diabetic mice displayed an increase in their blood glucose levels (> 300 mg/dL) starting week 1 after STZ injection as well as a retardation in their body-weight gain. However, prophylactic treatment of diabetic mice with three doses of HK *M. aurum* (1 mg per injection) resulted in a trend toward a

decrease in blood glucose levels as of week 1 until week 5 after STZ injection with a significant reduction in urine and blood glucose levels at week 4 and week 6, respectively, after STZ injection. A similar hyperglycemia-lowering effect has been previously reported with BCG, whereby a single footpad prophylactic administration of BCG to STZ-induced diabetic BALB/c mice led to a significant decline in their blood glucose levels as compared to those in untreated STZ-induced diabetic mice (12).

Further examination of subpopulations of different mouse groups in this study resulted in an intriguing finding, whereby when severely hyperglycemic mice (blood glucose levels ≥ 300 mg/dL) (24) were excluded from data analysis at week 1 after STZ injection, the blood glucose–lowering effect of HK *M. aurum* became more evident and statistically significant ($p < 0.05$). In this case, diabetic mice prophylactically treated with HK *M. aurum* had blood glucose levels that were constantly lower by an average of ~30% throughout the 6 weeks after STZ injection, as compared to untreated diabetic mice. This observation might be attributed to the fact that HK *M. aurum* pre-treatment would be less effective in delaying or reducing severe hyperglycemia in mice exhibiting a sharp and rapid rise in their blood glucose levels.

HK *M. aurum* was more effective at lowering hyperglycemia when administered at higher doses (1 mg per injection *versus* 0.1 mg per injection). Lower doses might not be able to generate an immune response capable of inducing systemic modifications to the host's metabolic status and therefore improving glycemic control. Dose-dependent effects have been also reported in studies that employed BCG to prevent diabetes in non-obese diabetic (NOD) mouse (8). This proposed process might involve but is not restricted to the induction of TNF-α, an essential cytokine that indicates that an active and sufficient mycobacterial-induced immune response is being mounted (10, 17, 29, 30). We opted to use multiple doses of HK *M. aurum* so as to maximize the possible desirable effects. Multiple therapeutic doses of BCG have been shown to be more effective in human diabetes studies, as they probably increase the likelihood of "resetting" the host's immune system (13, 31). In addition, a lag time between prophylactic treatment with BCG and STZ-induced hyperglycemia has been found to be crucial for BCG to exert its hyperglycemia-lowering effect in STZ-induced diabetic mice (12, 32). Similarly, BCG therapy has been shown to improve HbA1c levels and to decrease insulin usage in patients with T1D following a lag period of 3 years after BCG injection (12). Given that our study is the first to use HK *M. aurum* in the context of preventing or ameliorating hyperglycemia, we factored in the possibility of a similar lag time being required for its efficacy. Our study revealed the benefit of administering three doses of HK *M. aurum* 6 weeks prior to STZ injection.

Although HK *M. aurum* treatment resulted in lower blood glucose levels in the treated diabetic group as compared to those in the untreated diabetic group, blood glucose levels still remained ≥ 200 mg/dL. This observation indicates that, although HK *M. aurum* appears to mitigate the severity of hyperglycemia, it does not completely normalize blood glucose levels. The observed partial hyperglycemia-lowering efficacy suggests that HK *M. aurum* may help to reduce the extent of glucose elevation but may not be sufficient to fully restore normal glucose homeostasis under the given treatment conditions. This warrants further experiments that investigate the dosing, timing and alternative routes of administration so as to gain a better understanding of HK *M. aurum*'s beneficial properties in diabetes prevention or management.

Insulin sensitive tissues such as the liver and skeletal muscle are naturally the most affected from insulin insufficiency and hyperglycemia (33). In the liver, LDH and GLUT2 are the two important proteins involved in glucose metabolism and regulation. When blood glucose levels are high, LDH levels in the liver can increase to support the higher demand for glucose oxidation. However, prolonged hyperglycemia can lead to oxidative stress and damage to liver cells, which can further result in decreased LDH levels and impaired liver function. Furthermore, insulin insufficiency in the liver leads to a decrease in glucokinase and fructose 2,6-bisphosphate that inhibits glycolysis while increasing enzymes related to glycogen breakdown and gluconeogenesis such as glucose-6-phosphatase (34–36).

GLUT2 is a high-capacity, low-affinity glucose transporter predominantly expressed in the liver, pancreas, intestine, and kidneys (37). It plays a crucial role in the regulation of glucose homeostasis. In hepatocytes, GLUT2 facilitates the bidirectional transport of glucose, allowing the liver to uptake glucose during hyperglycemia and release glucose during hypoglycemia (38). Its activity is primarily regulated by the concentration gradient of glucose rather than insulin levels. Although GLUT2 is considered insulin-independent, its expression and function can be modulated by the metabolic state of the organism. For instance, hyperglycemia can lead to an increase in GLUT2 expression as part of the body's adaptive mechanism to manage elevated blood glucose levels (39). Given that STZ-induced diabetes leads to significant hyperglycemia, assessing GLUT2 expression helps us understand how HK *M. aurum* prophylactic treatment might influence hepatic glucose handling under diabetic conditions.

In our study, liver GLUT2 and LDH levels were found to be reduced in untreated diabetic mice 6 weeks after STZ injection. GLUT2 provides a major route for excess blood glucose to access the liver; thus, any reduction in its expression would contribute to further elevation of hyperglycemia (36, 37). Moreover, any decrease in the glycolytic activity in the liver, as evidenced by a reduction in LDH levels, would eventually decrease glucose usage and promote hepatic glucose production and output that is further translated by the release of glucose into the blood stream despite the elevated blood glucose levels. HK *M. aurum* prophylactic treatment of diabetic mouse group prevented the downregulation in liver LDH levels. This is indicative of increased glucose utilization by the liver and would probably reflect an improved liver function. However, the noted increase in LDH levels was not accompanied with a correction (upregulation) of liver GLUT2 levels in the *M. aurum*–pre-treated diabetic group. In other words, the decrease in hepatic glucose production, which leads to a reduction in glucose export out of the liver, possibly explains why the *M. aurum*–pre-treated diabetic group had lower blood glucose levels than the untreated diabetic group. In addition, *M. aurum* might exert its hyperglycemia-lowering effect on liver glucose production independently of changes in GLUT2-dependent glucose import/export.

UCP2 is a mitochondrial transporter protein that is able to dissipate the proton gradient across the inner mitochondrial membrane in the presence of certain activators such as fatty acids, coenzyme Q, superoxide, and lipid peroxidation products (40). This process uncouples oxidative phosphorylation from ATP production (40). UCP2 is also known to act as a regulator of mitochondrial reactive oxygen species (mitoROS) by means of a negative feedback loop (41–43). Stimulation of murine macrophages by the mycobacterial cell wall component, muramyl dipeptide, rapidly upregulates the expression of UCP2 and pre-treatment with vitamin E attenuates the upregulation of UCP2 (44). An increase in mitoROS upregulates UCP2 expression, which, in turn, dissipates the membrane potential, making the electron transport chain (ETC) less reduced and therefore less prone to produce free radicals. Under conditions of prolonged hyperglycemia, UCP2 expression in the liver can be downregulated (45). Chronic hyperglycemia can lead to the production of ROS, which can impair mitochondrial function by oxidative stress and consequently results in a decrease in liver enzymes and function. In this study, liver UCP2 expression was found to be downregulated in control diabetic mice. Nevertheless, prophylactic treatment of diabetic mice with HK *M. aurum* protected the liver from the disruptive effects of prolonged hyperglycemia. Just like liver LDH, the expression of UCP2 in the treated diabetic group was comparable to the normoglycemic group. Being a negative regulator of ROS, an increase in UCP2 levels in the HK *M. aurum*–pre-treated diabetic group indicates that the mitochondrial anti-oxidative mechanisms in the liver are maintained despite chronic hyperglycemic conditions.

In the skeletal muscle, UCP3, another member of the uncoupling protein family, and LDH levels were markedly increased in the control diabetic group as compared to those in the non-diabetic group. This goes in accordance with similar findings in the literature where upregulated UCP3 and LDH levels have been reported during states of insulin insufficiency, diabetes, and fasting (46, 47). Research on UCP3 suggests its involvement in metabolic regulation, particularly in the utilization of fatty acids as a fuel source. UCP3 may play a role in protection against lipid-induced oxidative stress and in regulation insulin sensitivity (48). On one hand, insulin insufficiency was found to cause an upregulation in key enzymes involved in glycolysis and glycogenolysis, justifying the observed increase in LDH levels in the skeletal muscle of diabetic mice (33, 49–51). On the other hand, free fatty acid (FFA) uptake is increased along with a partially inactive β-oxidation pathway, thus leading to the FFA accumulation near the mitochondria and subsequently triggering ROS-induced peroxidation and elevation in the levels of lipid peroxide, a known positive activator of UCP3 (52). Therefore, an increase in UCP3 levels serves as a type of feedback inhibition to export excess FFAs and to reduce ROS levels through UCP3-mediated uncoupling (53–55).

In our study, we observed that LDH expression in the skeletal muscle was elevated in the untreated diabetic group, whereas it was decreased in the liver. Elevated LDH expression in the skeletal muscle could be attributed to increased anaerobic glycolysis, which serves as a compensatory mechanism for energy production in the absence of adequate insulin. This suggests that skeletal muscle tissue adapts to the insulin-deficient state by enhancing anaerobic pathways to meet its energy demands. Conversely, the decrease in LDH expression observed in the liver may reflect altered metabolic functions in hepatic tissue under diabetic conditions. This could be due to compromised liver function or shifts in metabolic pathways that reduce the reliance on glycolysis. The liver, being central to various metabolic processes, might exhibit a different adaptive response to diabetes compared to skeletal muscle, potentially prioritizing gluconeogenesis or other metabolic pathways over glycolysis. Our results showed that HK *M. aurum* prophylactic treatment can prevent the STZ-induced upregulation in UCP3 expression with no effect on LDH expression. The observed *M. aurum*–induced reduction in the skeletal muscle UCP3 expression in diabetic mice might be also attributed to a diminished level of ROS-induced peroxidation or translocation of FFAs to the mitochondria.

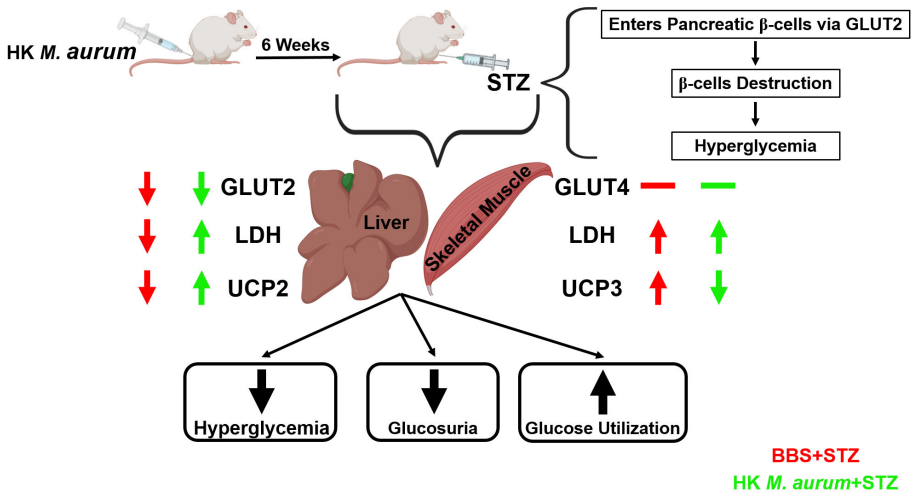

FIGURE 5
Schematic diagram summarizing the prophylactic anti-diabetic effects of HK *M. aurum* in STZ-induced diabetic mice.

Another GLUT member, GLUT4, is predominantly found in the skeletal muscle and adipose tissue, where it mediates insulin-stimulated glucose uptake (56). Under basal conditions, GLUT4 resides in intracellular vesicles and translocates to the plasma membrane in response to insulin signaling (57). Evaluating GLUT4 in basal conditions (i.e., without acute insulin stimulation) provides insights into the chronic regulation of GLUT4 expression and its potential contribution to overall glucose homeostasis during diabetic states. In STZ-induced diabetic mice, insulin levels are significantly reduced due to β-cell destruction, which impacts GLUT4 translocation and glucose uptake. By measuring GLUT4 expression, we aimed to determine whether HK *M. aurum* treatment could modulate GLUT4 levels and potentially enhance glucose uptake in the skeletal muscle despite the low insulin environment. Our results showed that the protein expression of skeletal muscle GLUT4 was not regulated either in untreated or in *M. aurum*–pre-treated diabetic mice as compared to that in non-diabetic mice.

It is important to note that STZ-induced diabetes can involve insulitis, an inflammatory response in the pancreatic islets (58). This response, although different from the autoimmune attack observed in T1D, can contribute to β-cell dysfunction and death. Although our current results do not directly demonstrate an anti-inflammatory effect of HK *M. aurum*, it is plausible that the observed reduction in hyperglycemia could be partially attributed to an anti-inflammatory effect induced by HK *M. aurum*. In fact, *M. vaccae*, which is phylogenetically very close to *M. aurum*, has been reported to possess potent anti-inflammatory properties when used as a HK preparation (59, 60). In these studies, HK *M. vaccae* was shown to induce Tregs and anti-inflammatory cytokine production. Therefore, the possible modulation of inflammation by HK *M. aurum* remains a hypothesis that requires further investigation.

In summary, HK *M. aurum* exerted a hyperglycemia-reducing effect in STZ-induced diabetic mice via a selective mechanism that was only evident under hyperglycemic conditions. This highlights HK *M. aurum* safety and its potential use for the amelioration of hyperglycemia and diabetes. Prophylactic treatment with HK *M. aurum* was shown to increase glucose utilization in hyperglycemic conditions independently from the impact of glucose import to the liver via GLUT2 and as evidenced by LDH expression. It was also able to regulate the expression of UCP2 and UCP3 in the liver and skeletal muscle, respectively, of STZ-induced diabetic mice. This might reflect an improvement in liver and skeletal muscle metabolic function and anti-oxidative mechanisms (Figure 5). These findings underscore a potential prophylactic value of HK *M. aurum* in managing diabetes development and warrant additional investigation into its underlying mechanisms of action for later clinical development. Future research should be conducted to further evaluate the therapeutic effects of HK *M. aurum* in STZ-induced diabetic mice.

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 research committee at the Faculty of Medicine and Medical Sciences, University of Balamand. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

FA: Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. SB: Formal analysis, Investigation, Supervision, Validation, Writing – review & editing. CA: Conceptualization, Resources, Writing – review & editing. GB: Conceptualization, Formal analysis, Resources, Supervision, Validation, Writing – review & editing. KE: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

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Conflict of interest

CA serves as the chief medical officer at Immune Boost Clinic Limited.

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

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Supplementary material

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Intestinal flora and bile acid interactions impact the progression of diabetic kidney disease

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In recent years, with the rapid development of omics technologies, researchers have shown that interactions between the intestinal flora and bile acids are closely related to the progression of diabetic kidney disease (DKD). By regulating bile acid metabolism and receptor expression, the intestinal flora affects host metabolism, impacts the immune system, and exacerbates kidney injury in DKD patients. To explore interactions among the gut flora, bile acids and DKD, as well as the related mechanisms, in depth, in this paper, we review the existing literature on correlations among the gut flora, bile acids and DKD. This review also summarizes the efficacy of bile acids and their receptors as well as traditional Chinese medicines in the treatment of DKD and highlights the unique advantages of bile acid receptors in DKD treatment. This paper is expected to reveal a new and important potential strategy for the clinical treatment of DKD.

KEYWORDS

diabetic kidney disease, intestinal flora, bile acids, farnesoid X receptor, G protein-coupled bile acid receptor 1, exosomes

1 Introduction

With the increasing prevalence of diabetes mellitus worldwide, DKD, which is a serious complication of diabetes, has become one of the top ten causes of death around the world. The global prevalence of diabetes mellitus was reported to be 463 million in 2019, and it is expected to increase by 25% by 2030 and 51% by 2045. Approximately 20–30% of all diabetic patients around the world develop DKD, and approximately 50% of these patients progress to end-stage renal disease (ESRD) (1, 2). The pathogenesis of DKD is complex. The inflammatory response, renal haemodynamic disorders and the imbalance of immune homeostasis can cause glomerular histiocyte and endothelial cell injury, glomerular basement membrane thickening, and glomerulosclerosis, ultimately leading to renal

fibrosis and exacerbating renal injury. Glycolipid metabolism disorders are also important causes of DKD. High glucose levels increase AGE production, and the interaction of AGEs with their receptors causes oxidative stress, which increases the production and release of inflammatory factors and causes an inflammatory response. Hyperglycaemia interferes with cholesterol synthesis and bile acid metabolism, exacerbating renal lipid accumulation; this renal lipid accumulation, in turn, causes Extracellular matrix (ECM) accumulation, further worsening glomerulosclerosis and renal fibrosis (3). Increasing evidence suggests that the gut flora and bile acids synergistically regulate host energy metabolism and immune responses via the "gut-liver-kidney axis", thereby accelerating the progression of DKD (Figure 1). However, the exact mechanisms involved remain unclear. Therefore, understanding interactions between the intestinal flora and bile acids and elucidating the pathological mechanisms involved in DKD progression are important for the treatment and prevention of DKD. In this paper, we aim to provide a detailed review of this issue, focusing on the effects of bile acids and receptors on DKD that are mediated by different metabolic and inflammatory pathways, interventions and mechanisms that regulate these pathogenic factors, and the use of traditional Chinese medicine to slow DKD progression. Our goal is to provide a theoretical basis for DKD treatment and intervention and offer new ideas to improve the clinical outcomes of DKD patients.

2 Disturbance of the intestinal flora disrupts bile acid homeostasis in DKD patients

2.1 DKD is characterized by disruption of intestinal flora homeostasis

Approximately 500 different bacterial species are present in the intestinal tract of healthy individuals, with a predominance of anaerobic bacteria, including Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria and Verrucomicrococcus; among these phyla, Firmicutes and Bacteroidetes account for 90% of the bacteria in the intestinal tract. The intestinal flora plays an important role in regulating human metabolism and maintaining immune homeostasis, and it is involved in the progression of many metabolism-related diseases (4). Previous studies have shown that DKD causes changes in the structure of the intestinal flora. Compared with healthy individuals, DKD patients exhibit significant differences in the structure of their microbiota; these differences are characterized by notable changes in α and β diversity (5–7), and exacerbate the disruption of gut microbial homeostasis. In recent years, the intestinal flora has been shown to be involved in the development of DKD. Li et al. (8) conducted 16S rDNA gene sequencing on the intestinal flora of DKD model mice and reported a decrease in the

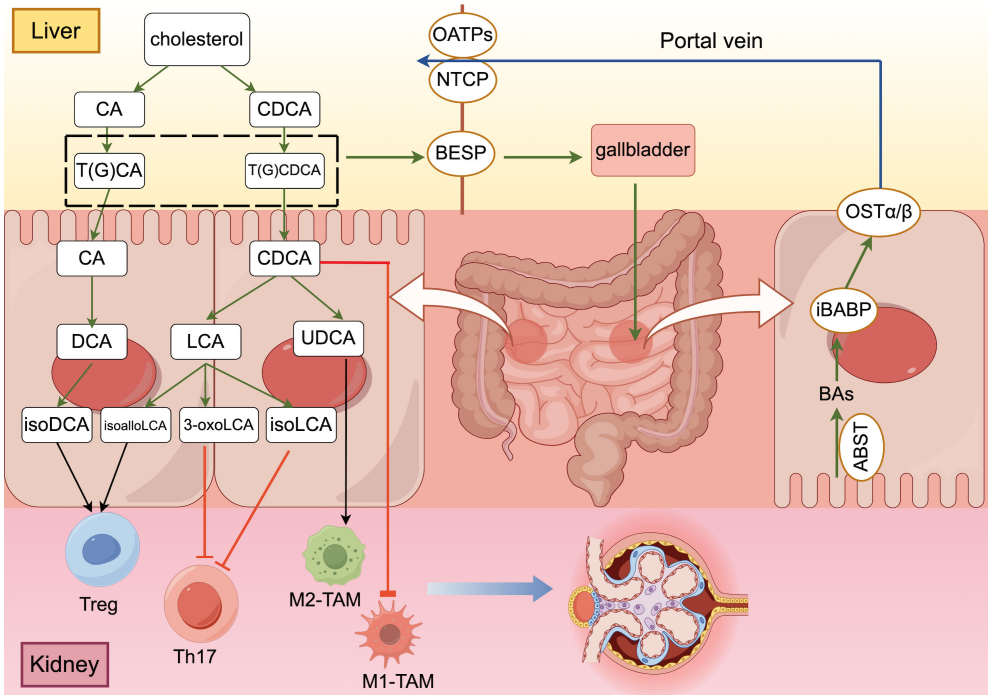

FIGURE 1
The "gut-liver-kidney axis" in patients with DKD ((generated by Figdraw 2.0). Primary conjugated bile acids are synthesized into secondary bile acids by deconjugation, dehydroxylation, oxidation and differential isomerization, and the intestinal flora participates in this process. Bile acids that are synthesized and stored in the liver are transported to the intestine via transporter proteins and transferred to hepatocytes with the help of other transporter proteins, completing the "enterohepatic cycle" in the human body. After bile acids perform their functions in the intestine, they promote the polarization of Treg cells and M2 macrophages and inhibit the differentiation of Th17 cells and the polarization of M1 macrophages through different signaling pathways to maintain the homeostatic environment of cellular immunity.

abundance of Firmicutes, such as Lachnospiraceae, Lactococcus, Fusobacterium, and Lactobacillus, whereas the abundance of Bacillus increased, exhibiting a positive correlation with albuminuria levels. Tao et al. (9) r reported that the abundance of Prevotella and Bifidobacterium was lower in DKD patients than in healthy individuals. Wang et al. (6) reported a reduction in the abundance of Roseburia, which is an Actinobacteria, and Akkermansia muciniphila, which is a member of the phylum Verrucomicrococcus, in the intestinal tracts of DKD patients. Moreover, other studies revealed increased abundances of Klebsiella and *Escherichia coli*, as well as the phylum Aspergillus (10–14). All the aforementioned studies indicate that DKD is closely associated with disordered gut microbial ecology and that DKD patients have a decreased diversity of gut flora compared with healthy individuals, as evidenced by a decrease in the abundance of beneficial bacteria and an increase in the abundance of pathogenic bacteria (Table 1).

2.2 Imbalances in intestinal flora homeostasis can lead to disturbances in bile acid metabolism in DKD patients

Bile acid (BA) is an amphiphilic substance that is synthesized from cholesterol in the liver and plays crucial roles in glycolipid metabolism and immune regulation (14). In the human body, BA produces cholic acid (CA) and cyanodeoxycholic acid (CDCA) through classical pathways, while CDCA is also produced through alternative pathways (15, 16). In rodents, generated CDCA is immediately converted into rhombocholic acid (αMCA and βMCA) (16). Unlike that in humans and mice, primary BAs in pigs are dominated by hyocholic acid (HCA). The resultant primary free BA combines with glycine and taurine at a ratio of 3:1 to form binding BAs (17), such as glycincholic acid (GCA), taurocholic acid (TCA), glycochenodeoxycholic acid (GCDCA), and taurochenodeoxycholic acid (TCDCA). An increasing number of studies have shown that there is a significant correlation between dysregulation of the serum bile acid profile in DKD patients and disruption of the intestinal flora and that the interaction between the two affects the development of DKD. Xiao et al. (18) analyzed the serum bile acid components of 184 patients with biopsy-confirmed DKD and reported that low bile acid levels were significantly correlated with increased proteinuria and decreased eGFR levels. Li et al. (19) observed an increase in the proportion of bound bile acids (TCA, GCA, TCDCA, and GCDCA) in the serum of ESRD patients, while the content of free bile acids (CA, CDCA and UDCA) decreased. Mantovani et al. (20) reported that the level of deoxycholic acid (DCA) was increased in patients with glucose metabolism disorders, and Li et al. (21) reported that the level of lithocholic acid (LCA) was decreased in rats fed a high-fat diet (Table 1). As mentioned in section 1.1, intestinal flora homeostasis in DKD patients is disrupted, resulting in a decrease in the level of enzymes involved in bile acid biotransformation; in turn, this decrease affects secondary bile acid synthesis, bile acid cyclic metabolism and bile acid receptor expression.

2.2.1 Disruption of the intestinal flora affects secondary bile acid synthesis

Under the action of the intestinal flora, the primary BA in the physiological state is first uncoupled by bile saline hydrolysase (BSH) (22) and then forms secondary BA after 7α-dehydroxylation; this product is transformed into DCA and LCA in humans, into mouse deoxycholic acid (MDCA) in mice, and pigs generate hyodeoxycholic acid (HDCA) (23). In the human body, certain bacteria produce hydroxysteroid dehydrogenase (HSDH) to isomerize CDCA to

TABLE 1 Evidence from studies of changes in intestinal flora in patients with DKD and effects on bile acids.

Phylum	Changes of genus level in DKD	Influence on BAS	Changes of BAs DKD	Ref.
Firmicutes	Lachnospiraceae↓	The number of bai operators required for 7α-dehydroxylation is reduced	LCA↓	(8, 21)
	Lactococcus↓			
	Fusobacterium↓			
	Lactobacillus↓			
	Bacillus↑			
Bacteroides	Bifidobacterium↓	Decreased BSH activity	conjugated BAs(TCA/GCA/TCDCA/GCDCA)↑ Unconjugated BAs(CA/CDCA)↓	(9, 19)
Actinobacteria	Roseburia↓	Decreased HSDH activity and the isomerization of bile acids 3α-, 7α- and 12α- was inhibited from α- to β- direction	UDCA↓	(6, 19)
Verrucomicrobia	Akkermansia muciniphila↓			(6)
Proteobacteria	Klebsiella↑	Decreased 7α/β-dehydrogenase activity and inhibit dehydroxylation	DCA↑	(12, 20)
	Escherichia coli↑	ASBT transporter function is inhibited	Obstruction of enterohepatic circulation caused by cholestasis	(10)

↓: decreased; ↑: increased.

UDCA, which can also convert LCA to iso-stone cholic acid and oxystone cholic acid. In the mouse body, β-MCA is hetero-isomerized to form ω-MCA at the C-6 position (25–27).

Studies have shown that BSH is widely active in gram-positive bacteria such as Bifidobacterium, Lactobacillus, Enterococcus, and Brucella and can also be detected in gram-negative bacteria such as Bacteroidetes (24–27). The bai operon is a key structure that regulates gene expression, and scientists have found that the enzyme it encodes plays an important role in the dehydroxylation of cholic acid. Researchers have found that bai operators are required for 7α-dehydroxylation in Firmicutes and Clostridium, and a 7α/β-dehydrogenase capable of converting CA and CDCA into DCA and LCA after dehydroxylation has been found in Proteobacteria and Erysipelothrix (26, 28, 29). HSDH in Actinomycetes and Proteobacteria can catalyze the isomerization of the bile acid 3α-, 7α- and 12α-hydroxyl groups from the α-direction to the β-direction, thereby increasing the hydrophilicity of bile acids (25). Researchers have also shown that Egeria tarda and Clostridium perfringens are involved in bile acid oxidation and differential isomerization (26, 27). However, due to the decrease in the abundance of Bifidobacteria, Firmicutes and Actinobacteria in the intestinal tract of DKD patients, the activities of enzymes such as BSH and HSDH are reduced, and the dissociation and isomerization of conjugated bile acids are blocked, resulting in increased cholestasis and the exudation of inflammatory substances, thus aggravating the systemic inflammatory response. The above studies indicated that intestinal flora homeostasis in DKD patients affects the activity of bile acid-metabolizing enzymes, leading to disruption of secondary bile acid synthesis.

2.2.2 Disruptions in the intestinal flora affect bile acid circulation and metabolism

Synthesized BA is metabolized in the human body via interactions between the liver and the intestine and plays a crucial role in the absorption of nutrients and energy metabolism throughout the body (29). BA bound to the liver is secreted into the bile duct through the bile salt output pump (BSEP) and stored in the gallbladder. After eating, the gallbladder is stimulated to contract and release BA into the intestinal lumen to promote the emulsification and absorption of lipids and fat-soluble vitamins (22). Then, the apical membrane sodium-dependent BA transporter (ASBT) is reabsorbed into intestinal epithelial cells, the intracellular ileal BA binding protein (iBABP) is transported to the basolateral side of intestinal epithelial cells, and the organic solute transporter (OSTα/β) transfers BA through the basolateral membrane to the portal vein (15). Subsequently, sodium-taurocholic acid cotransporter peptides (NTCP) and organic anion transporter peptides (OATPs) are involved in transporting BA from the blood to liver cells, and the reabsorbed BA is again secreted into the bile duct with the newly synthesized BA to complete a complete enterohepatic cycle (30) (Figure 1). In the human body, the enterohepatic cycle occurs approximately 4-12 times a day, and only 5% of BA is excreted in the stool (31).

Intestinal flora disruption results in abnormal bile acid metabolism by affecting the expression of bile acid transporters (32). *Escherichia coli* is an opportunistic pathogen, and the abundance of *E. coli* is significantly increased in the intestines of DKD patients. Annaba et al. (33) reported that *E. coli* inhibit the transport function of ASBT by inducing the secretion of proinflammatory factors and reducing tyrosine phosphorylation. In an animal study, Out et al. (34) reported that intestinal flora disorders inhibited ASBT expression by activating Gata4, thus disrupting bile acid reabsorption and cholestasis in the intestine, worsening intestinal damage and exacerbating inflammatory responses.

2.2.3 Disruption of the intestinal flora affects bile acid receptor expression

Farnesoid X receptor (FXR) and Takeda-G-protein-receptor-5 (TGR5) are important bile acid receptors that play important roles in host metabolism and immune regulation. According to human and animal model studies, FXR is a member of the nuclear receptor superfamily and is widely expressed in the liver, kidney and small intestine (36–40), whereas TGR5 belongs to the membrane receptor family and is highly expressed in the liver, gallbladder, intestine and kidney (41–43). A recent study revealed that FXR-/TGR5- db/db mice presented elevated levels of creatinine, uric acid, and proteinuria (41), suggesting that limited bile acid receptor expression in DKD patients may exacerbate kidney injury.

As the endogenous ligand of FXR/TGR5, bile acids have different excitatory or inhibitory effects on this receptor. Disruption of the intestinal flora further affects FXR/TGR5 expression by altering bile acid homeostasis. In the liver, the ability of bile acids to activate FXR is as follows: CDCA>DCA>LCA>CA (42, 43); however, UDCA, Tα-MCA, and Tβ-MCA are considered natural antagonists of FXR (44–46). The secondary bile acids LCA and DCA have the strongest excitatory effects on TGR5, followed by CA and CDCA (46, 47). As mentioned in section 1.2.1, intestinal flora homeostasis in DKD patients caused a decrease in the abundance of Bifidobacterium and Lactobacillus, which produce BSH, leading to an obstruction of the dissociation of Tβ-MCA in mice and an increase in the level of Tβ-MCA *in vivo*, thus increasing the antagonism of FXR. In addition, the production of 7α-dehydrogenase by Bacteroides is decreased in the guts of DKD patients, resulting in a corresponding decrease in LCA conversion; this decrease suppresses the excitatory activity of TGR5. Furthermore, drugs affect the distribution of the intestinal flora, which in turn affects bile acid receptors. Metformin is currently the most common hypoglycaemic drug, and metformin can affect the structure of the intestinal flora and inhibit the activity of FXR in the intestine. When T2D patients take metformin, the activity of BSH produced by *Bacteroides fragilis* in the intestine decreases, resulting in increased levels of glycoursodeoxycholic acid (GUDCA) and tauroursodeoxycholic acid (TUDCA). GUDCA and TUDCA are considered effective FXR antagonists that ameliorate insulin resistance by inhibiting FXR activity in the intestine (45).

3 Disruption of bile acid metabolism can accelerate the progression of DKD

Studies have shown that bile acid metabolism disorders can result in energy metabolism and cellular immune dysfunction in DKD patients by interfering with glucose and lipid metabolism and immune cell function, including macrophage polarization and Th17 and Treg cell differentiation; these effects ultimately accelerate the progression of DKD.

3.1 Bile acid metabolism disorders affect energy metabolism in patients with DKD

Studies have shown that changes in the bile acid spectrum in DKD patients can lead to abnormal levels of glucose and lipid metabolism indicators. A study examining the correlation between changes in total bile acid (TBA) and glucose metabolism revealed that increased TBA levels are positively correlated with the HOMA-IR index (48). In another study, Haeusler et al. (49) investigated plasma BA levels and insulin sensitivity in 35 T2DM patients and reported that an increased ratio of 12α-hydroxyl/non-12α-hydroxyl BA is accompanied by decreased insulin sensitivity and increased triglyceride levels. Further tests revealed that an increase in 12α-hydroxy-BA levels exacerbates insulin resistance in T2DM patients; thus, we believe that a change in 12α-hydroxy-BA levels is the main factor that impacts DKD-related glucose metabolism indices. Studies have shown that bile acids can increase glucose uptake by tissues and help maintain systemic blood glucose homeostasis by activating FXR/TGR5 (50, 51). In an animal study, Wang et al. (52) reported that triglycerides and cholesterol accumulate in FXR-knockout mice and that glomerulosclerosis and albuminuria are exacerbated in these mice. After the administration of the FXR agonist INT-747 to diabetic mice, proteinuria is significantly reduced, and glomerulosclerosis and renal tubulointerstitial fibrosis are significantly alleviated. Thus, FXR activation can reverse kidney injury and delay DKD progression. In addition, researchers have reported that mice with FXR deficiency are more likely to develop kidney damage in the context of high glucose levels. According to section 1.2, the bile acid metabolism of DKD patients is disrupted, which suppresses FXR/TGR5 activation and prevents FXR/TGR5 from regulating energy metabolism.

3.2 Bile acid metabolism disorders affect immune function in DKD patients

3.2.1 Bile acid metabolism disorders affect macrophage polarization

Macrophages are innate immune cells that can phagocytose pathogens, present antigens and secrete inflammatory substances. There is a significant correlation between DKD and macrophages. Compared with those in healthy individuals, many macrophages are enriched in the glomerulus and renal interstitium of DKD patients, and the degree of infiltration is positively correlated with renal damage indices, such as creatinine levels and proteinuria (53). These results suggest that macrophages are involved in inducing inflammation in the early stages of DKD and mediating kidney injury. Additionally, changes in the serum bile acid concentration in DKD patients can influence the polarization of macrophages, thus affecting the immune balance within the body.

Wang et al. (54) induced M1 macrophage infiltration after DCA supplementation in mice with inflammation *in vivo*. The mechanism may include DCA targeting Toll-like receptor 2 (TLR2) through the M2-mAchR/Src pathway and activation of the downstream ERK/JNK/NF-κB signaling pathway following TLR2 phosphorylation. This activation induces the polarization of macrophages, resulting in the mass recruitment of M1 macrophages; the release of TNF-α, IL-6, IL-8, IL-12 and other proinflammatory factors; and the exacerbation of inflammatory damage in DKD patients. Cao et al. (55) reported that increasing the CDCA level in mice could stimulate FXR activity, thereby inhibiting M1 macrophage polarization and IL-6 expression and exerting anti-inflammatory effects. Other studies have confirmed that UDCA inhibits intestinal inflammation and alleviates inflammation by inducing M2 macrophage polarization (56). After UDCA supplementation in ob/ob mice, Chen et al. (57) reported significantly reduced expression of M1 markers, such as NF-κB, IL-6 and TNF-α; increased expression of M2 markers, such as CD206, CD204 and CD163; and a decrease in the M1/M2 ratio; these phenomena alleviated the kidney injury caused by inflammation. As noted in section 1.2, DKD patients have increased DCA levels and decreased CDCA and UDCA levels, which can promote the polarization of M1 macrophages and inhibit the polarization of M2 macrophages; these effects induce the production of many proinflammatory factors and exacerbate the inflammatory response in DKD patients.

3.2.2 Bile acid metabolism disruption affects the differentiation of Treg and Th17 cells

BA is also involved in the regulation of adaptive immunity and the differentiation of T cells, including Treg cells and Th17 cells. Treg cells, which are a subset of $CD4^+$ T cells (58), play crucial roles in immune regulation by suppressing immune responses. Foxp3 is an important transcription factor in the development of Treg cells. Treg cells expressing Foxp3 can secrete TGF-β and other anti-inflammatory factors, which play important roles in maintaining immune homeostasis (59, 60). Th17 cells differentiate from $CD4^+$ T cells after inducing RORγt expression and activating IL-17A in response to high concentrations of TGF-β. Th17 cells mainly secrete proinflammatory factors such as IL-17, IL-21, and IL-22, and these cytokines recruit neutrophils to the site of inflammation and exacerbate inflammation. Under physiological conditions, the relative balance between Treg cells and Th17 cells helps maintain immune homeostasis within the body (61).

Many recent studies have reported that an imbalance in bile acid homeostasis in DKD patients leads to a decrease in the proportion of Treg cells and an increase in the proportion of

Th17 cells, which exacerbates inflammation and further affects the progression of DKD (62). Song et al. (63) reported that changes in the bile acid pool affect the expression of the transcription factors RORγt and Foxp3 and further affect the regulation of immune cells, resulting in increased colon inflammation. Researchers further investigated and reported that lithocholic acid derivatives affect Th17 and Treg cell differentiation in mice. 3-oxoLCA and isoLCA inhibit RORγt transcriptional activity and thus inhibit Th17 differentiation, whereas isoalloLCA and isoDCA promote Treg cell differentiation by increasing Foxp3 expression (64–66). As stated in section 1.2.1, a decrease in the number of bacteria that produce HSDH in the intestinal tract of DKD patients prevents the isomerization of stone cholic acid and reduces the production of stone cholic acid derivatives, thus suppressing Treg differentiation and the inhibition of Th17 differentiation, disrupting the immune balance of Treg/Th17 cells, and exacerbating the disorder of the immune environment in DKD patients (Figure 2).

4 Delaying the progression of DKD through the "gut-liver-kidney axis"

4.1 Regulating the homeostatic pathway of intestinal flora delays DKD

Through the exploration of the relationship between disease and intestinal flora, it has been found that the structure and composition of intestinal flora influence the development of metabolic diseases, and intestinal flora is both a pathogenic factor and a therapeutic tool. A recent clinical trial found that the probiotic Bifidobacterium longum NCC3001 increased levels of GUDCA and free fatty acids, as well as reduced depression scores and improved anxiety responses in patients. This suggests that there is a correlation between human subjective stress and intestinal flora (67). In a recent study that sequenced the 16S rRNA gene in stool samples from 56 Tibetan Buddhist monks and neighboring residents, researchers found that Prevotella and Bacteroides were significantly enriched in the meditation group, at 42.35% and 6.21%, respectively, compared with 29.15% and 4.07% in the control group. They also found significant reductions in clinical risk factors in the meditation group, including total cholesterol and apolipoprotein B; a separate study also found that LDL levels were significantly lower in the meditation group, and inflammatory genes including NF-KB-2 and IL1-B were 0.3 and 0.2 times higher, respectively, than in the control group (68). In addition to this, most of the research on the treatment of DKD by intestinal flora has focused on probiotics, prebiotics and fecal microbiota transplantation (FMT), which can treat and prevent DKD by regulating glucose metabolism and protecting the intestinal barrier.

4.1.1 Probiotics and prebiotics

Probiotics and prebiotics have been considered effective treatments for DKD in recent years. Probiotics, which are intestinal microorganisms, can regulate the distribution of the

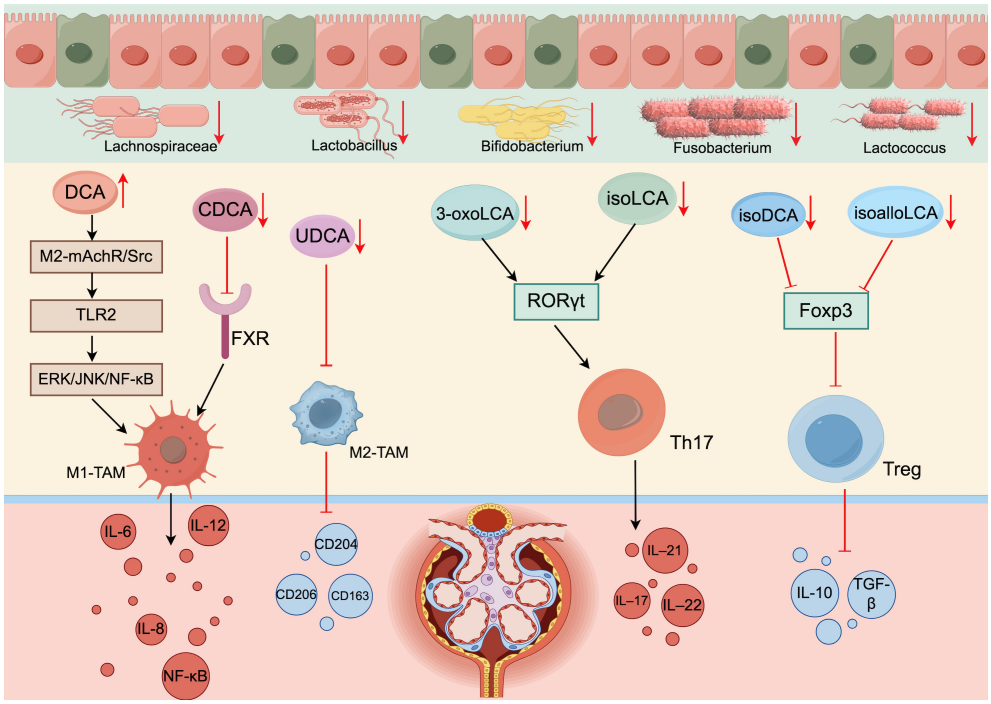

FIGURE 2
Disturbances in bile acid metabolism disrupt immune cell homeostasis (generated by Figdraw 2.0). When the intestinal flora of DKD patients is disrupted, the number of bacteria involved in bile acid synthesis and enterohepatic circulation is reduced, which affects the activity of various enzymes and reduces secondary bile acid synthesis. Bile acid acts as the endogenous ligand of FXR/TGR5; thus, reduced bile acid synthesis inhibits the activation of FXR/TGR5. In addition, an imbalance in the intestinal flora disrupts bile acid metabolism by inhibiting ABST expression.

intestinal flora and ameliorate intestinal flora disorders. Prebiotics are nondigestible food components that are usually degraded by gut microbes to provide nutrients to the gut but cannot be absorbed by the gut; these components restore gut health by stimulating the activity of beneficial bacteria in the gut (69). Both probiotics and prebiotics are significantly decreased in DKD patients, and supplementation with both can improve glucose metabolism, restore intestinal mucosal function, and reverse renal tissue structural damage in DKD patients.

Lactobacillus royale LR6 was found to increase the number of cuprocytes and protect the integrity of the intestinal mucus and lamina propria layers (70). Taverniti et al. (71) reported that *Bifidobacterium bifidum*, *Lactobacillus suis*, and *Lactobacillus casei* decrease zonulin expression and promote intestinal barrier repair. Second, probiotics have been shown to have an ameliorative effect on glucose metabolism markers, including FBG, HbA1c and HOMA-IR, in diabetic patients (72). After researchers treated diabetic mice with four strains of Lactobacillus probiotics for 8 weeks, the mice presented decreases in both FPG and HOMA-IR levels and significant increases in both GLP-1 and GLUT-2 expression (73). In addition, researchers orally administered two probiotics, *Bifidobacterium bifidum* BL21 and *Lactobacillus lactis* LRa05, to T2DM model mice, and these treatments reduced the expression of IL-17, IL-6, and endotoxin and ameliorated oxidative stress and inflammation in these mice (74). The mechanism involves blocking the TLR4/MyD88 signaling pathway by inhibiting the specific binding of LPS to TLR4, which ultimately prevents the expression of NF-κB signaling pathway components and reduces the production of proinflammatory factors, such as IL-2, IL-6, and TNF-α.

4.1.2 FMT

FMT involves the transplantation of feces from a healthy person into a patient to remodel the patient's intestinal microbial environment with the healthy flora of the donor. With in-depth research on FMT, an increasing number of studies have concluded that FMT is an effective treatment for dysbiosis, and in 2013, FMT was approved for the treatment of *Clostridium difficile* infection in the United States; this advanced the prospects of FMT to new heights (75).

Several studies have shown a significant increase in the diversity of the intestinal flora after FMT, and the levels of Ackermannia mucinophilic, Mycobacterium anisopliae and Mycobacterium-like organisms, which are important beneficial bacteria in the intestinal tract and are thought to inhibit intestinal inflammation, were increased after FMT. Zhang et al. (76) reported that the levels of fasting blood glucose, triglycerides and low-density lipoprotein cholesterol were reduced in db/db mice treated with FMT, suggesting that FMT has a positive regulatory effect on glucose and lipid metabolism in DKD patients. In a Phase 2 clinical trial predicting obesity and FMT-related traits, researchers found a significant increase in bacterial abundance in recipients after FMT and observed a significant improvement in HOMA2-IR at week 6 (77). In addition, The researchers studied FMT in 11 kidney transplant recipients and found that FMT treatment reduced drug resistance in the body by inhibiting the same strain (78). The results indicated that FMT was effective in improving intestinal bacterial richness and glucose metabolism. Zhao et al. (79) also reported that FMT reduced the level of LPS in the colons of mice, thereby inhibiting the activation and expression of NF-κB and reducing the production of proinflammatory factors such as TNF-α, IL-1β, IL-6, and IL-17. In addition, they reported that FMT improved the distribution of ZO-1 and tight junction proteins and restored intestinal barrier function in colitis model mice, suggesting that FMT plays an important role in regulating inflammatory pathways and alleviating inflammatory symptoms.

4.1.3 Exosomes

Exosomes, which are vesicular vesicles secreted by a variety of cells containing various nucleic acids, proteins, lipids, amino acids, and metabolites, are considered to be important mediators of intercellular communication because they can participate in the exchange of information and substances between different cells (80, 81). Researchers first observed that exosomes secreted by intestinal bacteria could transport biomolecules into host cells after introducing Escherichia coli into mice (82). Fizanne et al. (83) found that feces-derived extracellular vesicles were able to reduce the expression of tight junction proteins in the intestines of wild mice, increase intestinal permeability which exacerbated intestinal inflammation. The above studies demonstrated the interaction between exosomes and gut microorganisms, which together affect the development of diseases. Subsequently, Teng et al. (84) found that ginger exosomes-like nanoparticles could induce Lactobacillus rhamnosus to activate the AHR signaling pathway and thus mediate the production of IL-22, which could ameliorate intestinal inflammation and restore the intestinal barrier function. In addition, Zhu et al. (85) found that plant-derived exosome-like nanoparticles increased Lactobacillus reuteri levels and promoted indole derivative-activated reprogramming of $CD4^+$ T cells into double-positive $CD4^+CD8^+$ T cells, reducing the levels of pro-inflammatory causative cells. Yan et al. (86) also found that combining mesenchymal stem cells-derived exosomes (hUC-Exos and hFP-Exos) transplanted into colitis mice and found that hUC-Exos and hFP-Exos ameliorated intestinal inflammation by regulating the proportional balance between Th17 and Treg cells.

4.2 Reversing DKD bile acid metabolism disorder can delay the progression of DKD

Bile acid is both a pathogenic factor and a therapeutic agent. Modulation and regulation of bile acid disorders have significant effect on the treatment of DKD. On the one hand, bile acid can be supplemented externally, and on the other hand, energy metabolism disorders and immune inflammatory responses can be improved by regulating the bile acid receptor ratio (FXR/TGR5); this regulation can alleviate kidney injury and delay the progression of DKD (Figure 3).

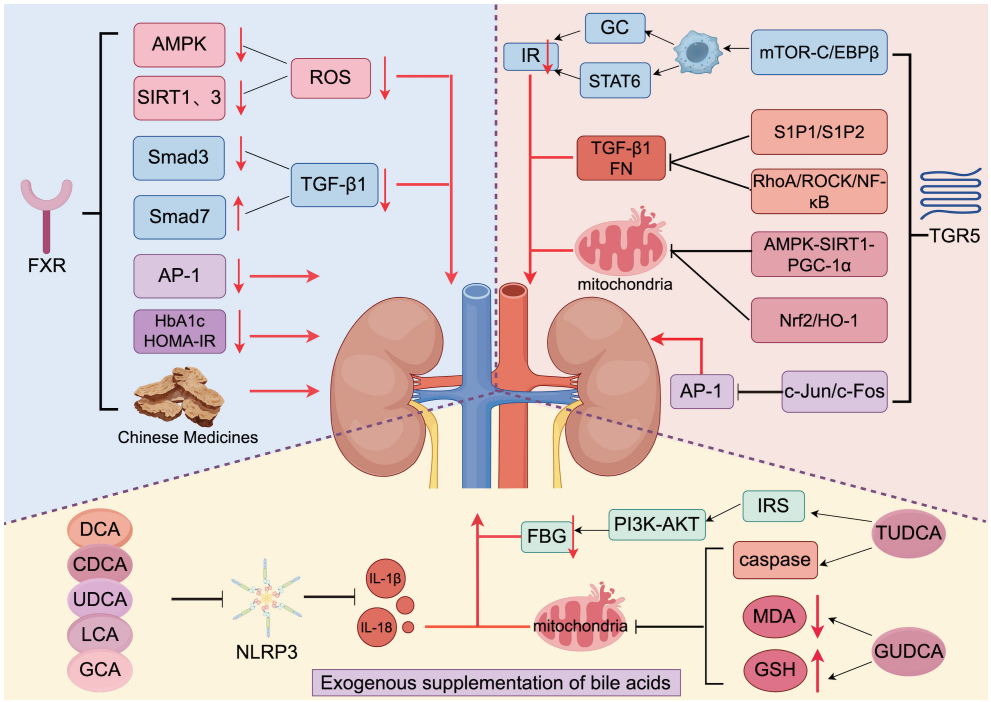

FIGURE 3
Treatment of DKD via bile acids and traditional Chinese medicines (generated by Figdraw 2.0). Exogenous supplementation with bile acids ameliorates renal inflammation in DKD patients by inhibiting NLRP3, MDA, and GSH and activating IRS. TGR5 is activated to promote the production of GC and STAT6 and reduce the production of TGF-1 and FN by regulating various pathways. Additionally, activated TGR5 inhibits the renal damage that is caused by oxidative stress. FXR is activated to reduce ROS production by inhibiting AMPK and SIRT1. When FXR is activated, it reduces ROS production by inhibiting AMPK, SIRT1, and SIRT3 expression; reduces TGF-1 production by inhibiting Smad3 and promoting Smad7 expression to slow the process of renal fibrosis; and inhibits the expression of AP-1, HbA1c, and HOMA-IR to alleviate renal injury. In addition, traditional Chinese medicine also has a protective effect on the kidneys by interacting with FXR.

4.2.1 Exogenous supplementation of bile acids can delay the progression of DKD

Bile acid, a new therapeutic agent, has been shown to play an important regulatory role in glucose metabolism and the inflammatory response (87). Jiang et al. (35) treated DKD mice with cholic acid for 16 weeks and observed significant reduction of proteinuria and glomerulosclerosis by immunofluorescence microscopy. Researchers have shown improvements in inflammation in mice after UDCA supplementation, possibly due to the significant antibacterial effect of UDCA via the TGR5-NF-κB pathway (88). Da Silva et al. (89) reported that TUDCA can improve glucose levels through the activation of IRS-1/PI3K-AKT signaling by binding to IRS insulin binding sites 1 and 2. During an 8-week clinical trial, the researchers found that after oral administration of propionate, secondary bile acid (DCA) levels were elevated, which in turn led to lower cholesterol levels (90). In DKD patients, increased malondialdehyde (MDA) levels and decreased glutathione (GSH) levels are closely related to renal tissue damage. Chen et al. (91) reported that supplementation of GUDCA in patients with glucose metabolism disorder can reduce MDA level and increase GSH level to improve oxidative stress injury and improve the glomerular filtration rate of patients. Protects kidney function. In addition, Guo et al. (92) studied DCA, CDCA, and GCA and the interaction between LCA and macrophages; they found that these bile acids can inhibit the secretion of IL-1β by macrophages through TGR5, reduce the damage to the kidney, and reduce the urine protein content and blood creatinine of patients. The researchers used a bile acid chelator (Sevelamer) in a mouse model of NASH and found that it improves liver fibrosis by regulating gut flora and bile acids. Therefore, we believe that Sevelamer may have a similar effect in reducing kidney inflammation and fibrosis (93). These results indicate that bile acid can be used as a potential therapeutic target for DKD; BA alleviates the inflammatory response, metabolic disorders, and renal tissue injury and dysfunction in DKD patients.

4.2.2 Regulation of bile acid receptors can delay the progression of DKD

4.2.2.1 Regulating FXR delays the progression of DKD

In recent years, FXR is related to regulating the ecological disturbance of intestinal flora, protecting intestinal barrier and improving intestinal antibacterial ability, and is an important way to treat DKD. Gadaleta et al. (94) found that the use of FXR agonists prevented colitius-induced increases in Caco-2 cell permeability and significantly reduced the expression of IL-1β, IL-6, and MCP-1. Xu et al. (95) found that supplementation with Muribaculum and Parasturtella can enhance gut FXR expression and then inhibit cholesterol 7α-hydroxylase (CYP7A1) and sterol 12ahydroxylase (CYP8B1) gene expression in the gut by activating FGF15 via the FXR-SHP axis, thereby promoting bile acid synthesis and reducing

cholesterol accumulation. Chen et al. (96) also found that B. fragilis inhibits NLRP3 expression and restores BSH enzymatic activity through FXR, thereby improving intestinal and kidney damage caused by intestinal flora disturbance and abnormal bile acid metabolism.

FXR has received increasing attention for its ability to regulate glycolipid metabolism and alleviate oxidative stress and renal fibrosis (92). In a randomized placebo-controlled trial, the researchers found that supplementation with aronia juice activates FXR and TGR5 through secondary bile acid signaling molecules, which together promote glucose and lipid metabolism (99). After application of the FXR agonist GW4064 to db/db mice for 3 months, the levels of HbA1c and HOMA-IR were reduced, and an insulin resistance test confirmed that FXR ameliorated insulin resistance (100). Xu et al. (98) found that the intestinal FXR antagonist effectively inhibited the expression of PGC-1α in the liver and gluconeogenesis and reduced blood glucose levels. A recent study revealed that vertical sleeve gastrectomy (VSG) altered the gut microbiome distribution and the hydrophobicity of bile acid pools in mice, thereby promoting bile acid metabolism and reducing inflammation and glucose metabolism, while FXR knockout weakened the glycemic improvement induced by VSG (101).

In terms of anti-inflammatory effects, Wang et al. (52) reported that an FXR/TGR5 double agonist (INT-767) increased the activity of AMPK, SIRT1 and SIRT3, all of which play important regulatory roles in reducing ROS production and oxidative stress. This finding provides strong evidence that FXR activation helps maintain mitochondrial homeostasis. Obeticholic acid, a typical FXR agonist, was shown to improve histological features and alleviate fibrosis progression in patients with NASH in a multicenter, randomized, placebo-controlled Phase 3 trial (102). Subsequent studies have shown that activation of FXR also plays a good role in the process of renal fibrosis. Renal fibrosis is an important pathological feature in the progression of DKD to ESRD, and TGF-β1 is an important transforming factor in the process of renal fibrosis. Abnormal expression of TGF-β1 can induce renal fibrosis through the classical Smad pathway, in which Smad3 increases fibrin transcription and promotes tissue fibrosis, while Smad7 inhibits tissue fibrosis. Studies have shown that activation of FXR can inhibit the activation of TGF-β1 pathway by down-regulating the expression of Smad3 and up-regulating the expression of Smad7, thereby alleviating renal fibrosis. FXR can also inhibit the target of AP-1 and antagonize its expression, thereby improving renal fibrosis, thereby increasing glomerular filtration rate, and improving renal injury in DKD patients.

4.2.2.2 Regulation of TGR5 delays DKD progression

Many studies have shown that in both diabetic patients and mouse models, the expression of TGR5 in the kidney is significantly reduced (97) and is inhibited to varying degrees, resulting in serious disorders of blood glucose regulation and inflammatory responses (103). Recently, the researchers reported that they found that the lead compound (77A) of the TGR5 agonist promotes GLP-1 expression, promotes insulin secretion, and lowers blood sugar *in vitro* and in mice (104). After applying TGR5 agonists to diabetic mice, researchers observed that both fasting blood glucose and HbA1c levels were significantly reduced (105, 106). The mechanism may involve the activation of TGR5 by LCA to promote GLP-1 activity and increase glucose uptake by tissues and insulin secretion to stabilize glucose homeostasis. Similarly, increased cholic 7-sulfate was observed in mice during sleeve gastrectomy (SG), and activation of TGR5 induced GLP-1 secretion (107).

Perino et al. (108) reported that TGR5 reduced M1 macrophage chemotaxis by activating mTOR-C/EBP-β but did not affect M2 macrophage polarization. Caratti et al. (109) reported that the GC receptor and STAT6 in M2 macrophages can synergically mediate insulin homeostasis in adipose tissue. Therefore, we hypothesize that TGR5 can regulate the polarization of macrophages, thereby affecting the expression of the GC receptor, preventing severe kidney injury caused by insulin resistance, and delaying the progressive development of DKD. Wang et al. (39)found that eGFR decreased by more than 0.6 ml/min/month in patients with reduced TGR5 expression, and glomerular podocin markers such as Nephrin and Podocin were also reduced, which could be reversed after supplementation with TGR5. Subsequently, Lin et al. (110) found that Smurf1 overexpression could be inhibited by TGR5 supplementation, thereby reducing UTP, Cr, BUN, and NAG activities in DKD patients.

Fibronectin (FN) is known to be an important factor in chemotactic fibroblasts, and its overexpression can cause glomerular basement membrane thickening and accelerate renal tubule fibrosis. Previous studies have shown that NF-κB signaling is mediated by RhoA/ROCK signaling and that TGR5 activation can inhibit RhoA/ROCK/NF-κB signaling in mouse mesangial cells and reduce the expression of FN and TGF-β1, thus alleviating renal fibrosis in DKD mice (111, 112).

4.3 Traditional Chinese medicines can regulate the intestinal microbiota-bile acid axis and delay the progression of DKD

At present, the application of traditional Chinese medicine in the treatment of DKD is becoming increasingly extensive, which shows the unique advantages of Chinese medicine in alleviating kidney tissue injury, regulating glucose and lipid metabolism, reducing urinary protein levels, and suppressing the inflammatory response. DKD has no exact term in Chinese medicine but can be classified as " Oedema and Urinary block and vomiting" according to its pathogenesis and clinical manifestations. The disease occurs in " Wasting-thirst", long time dry heat injury of thin fluids, Qi and Yin deficiency pattern, and eventually lead to Qi and Blood and Yin and Yang deficiency pattern. The etiology and pathogenesis of DKD is deficiency of the spleen and kidney pattern as the root, phlegm turbidity blood stasis as the tip. Treatment should pay attention to both the tip and root, kidney and spleen based, with transformation stasis and drain water retention, can effectively reduce the patient's blood creatinine and urine protein.

Research shows that many TCM monomers could improve blood sugar, lipid levels and early renal function through the "gut-liver-kidney axis", and reduce pathological damage to kidney tissue. Modern studies have shown that traditional Chinese medicine plays a

prominent role in regulating the ecological imbalance of intestinal flora. Shen et al. (113) found that Astragalus membranaceus and Salvia miltiorrhiza increased the abundance of Akkermansia, Akkermansia_muciniphila, Lactobacillus and Lactobacillus muris, among which Akkermansia and Akkermansia_muciniphila were positively correlated with the production of arachidonic acid metabolites. Lactobacillus and Lactobacillus muris are negatively correlated with glycerophospholipid metabolism, which indicates that Chinese medicine can play a positive anti-inflammatory and lipid-lowering role by improving intestinal flora distribution. Liu et al. (114) found that Zicuiyin decoction increased Prevotellaceae and Lactobacillaceae and decreased Enterobacteriaceae, Clostridium and micrococcaceae.

Astragaloside IV (AS-IV), the active ingredient in astragaloside, promotes the release of Nrf2 from Keap1-Nrf2 and reverses the mitochondrial dysfunction induced by high sugar concentrations, thus alleviating podocyte damage caused by oxidative stress (115). In a recent 24-week double-blind randomized trial, researchers found that curcumin can reduce the ratio of firmicute to Bacteroides, regulate intestinal flora homeostasis, increase serum CDCA levels, and thus stimulate the expression of TGR5 in peripheral blood mononuclear cells, playing its therapeutic role (116). Two triterpenoids (alisol M 23-acetate and alisol A 23-acetate) in Alisol can promote the binding of FXR and SHP to play an excitatory role (117). The clinical effect of TCM compounds in the treatment of DKD has been widely verified. Studies have shown that Yinchenhao decoction can improve the abnormal elevation of TCDCA, GCDCA, Tα-MCA and Tβ-MCA and persistent inflammation in mice by regulating FXR (118). Huanglian Jiedu decoction can enhance blood glucose metabolism in DKD rats by regulating the AGE/RAGE/Akt/Nrf2 pathway while reducing triglyceride and low-density lipoprotein cholesterol levels to protect kidney function (119). Danggui Buxue Decoction (120), Danggui Shaoyao SAN (121), Chaihuang Yishen Granules (122) and Yishentongluo have all been shown to inhibit TGF-β1 production and delay renal fibrosis in DKD patients.

5 Conclusions

In recent years, an increasing number of researchers have studied the relationships among the intestinal flora, bile acids, and kidney disease, especially the related mechanisms that underlie the progression of DKD. Intestinal flora and bile acids, as novel ways to treat DKD in the future, have great prospects in influencing host epigenetic modification. Bile acid is an important link between the intestinal flora and DKD and regulates energy metabolism and immune homeostasis in the host. Although studies have shown that supplementation of prebiotics and probiotics are effective in the treatment of DKD, for critically ill patients, probiotics, although not harmful, are ineffective in alleviating symptoms and improving prognosis. Although FMT has shown a superior improvement in treatment, many safety factors have not been more accurately studied, such as the risk of donor-transmitted disease and acceptability of the recipient. Many studies have shown that disordered intestinal flora in DKD patients lead to changes in the bile acid pool, metabolic disorders and abnormal expression of bile acid receptors; these phenomena subsequently affect host glucose metabolism and the immunoinflammatory response via different signaling pathways, thereby exacerbating kidney injury in DKD patients. Previous studies have shown that alleviating disordered bile acid metabolism has important potential as a strategy for the treatment of DKD; however, bile acid components are complex and diverse, and additional animal experiments and clinical studies are needed for verification. Therefore, an increasing number of studies have focused on bile acid receptors, and many exciting results have been obtained. FXR/TGR5 activation not only is a clear and highly safe target but also effectively regulates glucose and lipid metabolism, modulates the inflammatory response, and regulates immune homeostasis to delay the progression of DKD. In addition, the clinical efficacy of traditional Chinese medicine has been verified over thousands of years, and modern research has confirmed that these medicines can stimulate FXR/TGR5 and protect the kidney by exerting synergistic effects on multiple targets and pathways. However, given the differences between animals and humans, the design of FXR/TGR5 receptors for use in the clinic is still risky, and more clinical studies are needed to develop safer bile acid receptors in the future; these receptors will provide potential novel directions for the treatment of DKD.

Author contributions

JX: Writing – original draft, Writing – review & editing. NW: Writing – review & editing. LY: Writing – review & editing. JZ: Writing – review & editing. MC: Writing – review & 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.

Publisher's note

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Recent advances of traditional Chinese medicine against cardiovascular disease: overview and potential mechanisms

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Cardiovascular disease (CVD) is the leading cause of human mortality worldwide. Despite Western medicine having made encouraging results in the clinical management of CVD, the morbidity, mortality, and disability rates of the disease remain high. Modern pharmacology has confirmed that traditional Chinese medicine (TCM), characterized by its multi-component, multi-target, and integrity, plays a positive and important role in the prevention and treatment of various CVDs in China, which has notable advantages in stabilizing disease, improving heart function, and enhancing the quality of life. Importantly, TCM is gradually being accepted by the international community due to its low cost, high safety, versatile bioactivity, and low toxicity. Unfortunately, comprehensive studies on the therapeutic effect of TCM on CVD and its mechanisms are very limited, which may restrict the clinical application of TCM in CVD. Therefore, this review is performed to analyze the pathogenesis of CVD, including inflammatory response, oxidative stress, mitochondrial dysfunction, pyroptosis, ferroptosis, dysbiosis of gut microbiota, *etc.* Moreover, we summarized the latest progress of TCM (formulas, extracts, and compounds) in curing CVD according to published literature from 2018 to 2023, as well as its mechanisms and clinical evidence. In conclusion, this review is expected to provide useful information and reference for the clinical application of TCM in the prevention and treatment of CVD and further drug development of CVD.

KEYWORDS

cardiovascular disease, traditional Chinese medicine, heart function, therapeutic mechanisms, gut microbiota

1 Introduction

Cardiovascular disease (CVD) is the diseases of the circulatory system, including disorders of the heart and blood vessels. As a chronic progressive condition, CVD is characterized by high morbidity, mortality, hospitalization, and disability rates, causing a huge economic and health burden worldwide (1, 2). According to the World Health

Organization, CVD was the leading cause of the highest number of deaths in 2019 (3), and about 23 million CVD-related deaths in 2030 (4). Meanwhile, CVD remains the predominant cause of human mortality in China (5) and Western countries (6). Recent studies have confirmed that the occurrence and progression of CVD are the results of the interaction of genetic and environmental factors, and common risk factors include age, obesity, tobacco use, alcohol consumption, dyslipidemia, hypertension, diabetes (7–12), *etc.* Meanwhile, other studies have found that air pollution and circadian syndrome as contributing factors to CVD (13, 14). In addition, numerous studies have demonstrated that oxidative stress, inflammatory response, programmed cell death (such as apoptosis and autophagy, pyroptosis, and ferroptosis), and intestinal flora disorders were associated with the abnormalities of structural and functional in the cardiovascular system (15–17). Currently, surgery and drugs are commonly used in the clinical management of various CVDs, but surgical procedures are both risky and expensive. Besides, the effectiveness of cardiovascular drugs decreases with prolonged use and is accompanied by adverse side effects, which has become a major problem that needs to be urgently addressed in the Western medical treatment of CVD. Therefore, the pathogenesis of CVD needs to be further explored and effective prevention and treatment strategies need to be developed.

Traditional Chinese medicine (TCM) is an accumulation of the Chinese Nation's clinical experience for thousands of years, characterized by comprehensive resources and low cost, and has been widely used for treating various diseases in clinical practice (18, 19). TCM was an important source of modern drug development for more than 2,000 years. More interestingly, TCM has become increasingly popular in many developed countries (20), such as Australia and the United States, because of its unique advantages including low adverse effects, stable efficacy, and a wide range of targets. Modern medical studies have demonstrated that TCM (including formulas, extracts, and compounds) possessed significant effects on the treatment of CVD, and TCM treatments are well tolerated by patients with CVD (21). Currently, the "compound Dan-Shen dropping pill", which consists of three TCMs for the treatment of coronary heart disease and angina pectoris, was the first TCM formula in the world to complete a phase III randomized, double-blind, and international multicenter clinical trial approved by the U.S. Food and Drug Administration (NCT00797953) and this drug was widely used in Australia after being approved by the Australian Therapeutic Goods Administration. Meanwhile, the standard of Panax notoginseng extracts has been incorporated into the German Drug Code for the benefit of patients with CVD. Functionally, TCM can exert cardioprotective effects through multiple targets on oxidative stress, inflammation, autophagy, lipid metabolism, cardiomyocyte/vascular endothelial cell function, and gut microbiota (22–24), which compensates for the lack of a single drug model for the treatment of CVD in clinical. Several studies have confirmed that TCM combined with Western drugs can more effectively alleviate clinical symptoms and disease progression in patients with CVD (25, 26). Importantly, with the development of omics technologies such as transcriptome, proteome, metabolome, and bioinformatics, the detailed mechanisms of TCM in the prevention and treatment of CVD have been systematically and comprehensively expanded to multiple levels such as RNA, protein, and metabolites, and also extend to the single-cell microscopic level from the perspective of time and space (27). This suggests that TCM provides new perspectives and strategies to combat various CVDs in modern society.

Currently, there are few reviews on TCM for the prevention and treatment of various CVDs. In this review, the current pathogenesis of CVD was comprehensively overviewed. Moreover, the current research on TCM (including TCM formulas, extracts, and compounds) protection against CVD was summarized and discussed based on the published literature from 2018-2023 through global and local databases including PubMed, Web of Science, and China National Knowledge Infrastructure, as well as its mechanisms and clinical efficacy, which may provide a reference for the clinical application of TCM in the treatment of CVD and a theoretical basis for the development of new drugs to combat CVD.

2 The pathogenesis of CVDs

The development and progression of CVD were associated with genetic mutations, obesity, environmental factors, and poor lifestyle (28, 29). Increasing evidence has demonstrated that the possible pathogenesis of CVD includes inflammation, oxidative stress, mitochondrial dysfunction, cell death (e.g., apoptosis, ferroptosis, and pyroptosis), and gut microbiota imbalance, which would lead to cardiomyocyte injury, inflammatory response, and vascular lesions (15, 30, 31), etc.

2.1 Inflammation

Inflammation plays an important role in the pathogenesis of various CVDs (32), and anti-inflammatory therapies have proven beneficial in several recent clinical trials (33, 34). Increased incidence of cardiovascular events has also been shown in patients with chronic inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus, psoriasis, inflammatory myopathies, and inflammatory bowel disease (35). Evidence suggested that the upregulation of circulating C reactive protein resulted in a greater risk of incident acute myocardial infarction (36) or cerebrovascular events (37). Previous studies have shown that atherosclerosis is a low-grade and aseptic inflammatory disease (38). For example, Mai et al. (39) demonstrated that nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome was a key driver of atherosclerosis. Meanwhile, the inflammatory response was considered to be a trigger for the developmental process of atrial fibrillation (40). Over-activation of NLRP3 inflammasome was directly associated with hospitalization rates in patients with cardiac insufficiency and dilated cardiomyopathy, accompanied by cellular scorching of cardiomyocytes (41). In addition, it has also been demonstrated that inhibition of the inflammatory response or NLRP3 gene deletion improved cardiac remodeling and reduced proinflammatory cytokines secretion and fibrotic processes (42, 43),

as well as attenuated angiotensin II (Ang II)-induced hypertension (44). Taken together, inflammation was involved in the pathogenesis of several CVDs (Figure 1), which also provides new strategies for the prevention and management of CVD.

2.2 Oxidative stress

Oxidative stress is a pathological state of reactive oxygen species (ROS) accumulation caused by excessive production of oxygen free radicals or impaired intracellular antioxidant defense systems (45). Normal physiological state of ROS levels contributes to the maintenance of cardiovascular homeostasis (46), while excessive and/or sustained increases in ROS production play an important role in the pathological statute of CVD (Figure 2), such as atherosclerosis, hypertension, myocardial ischemia-reperfusion injury, arrhythmia, heart failure, and acute myocardial infarction (47). Of note, oxidative stress has emerged as a new target for the prevention and treatment of CVD (48). It has also been found that common CVD risk factors contribute to a sustained increase in ROS production in the vascular wall (49). Functionally, oxidative stress not only promotes lipid peroxidation, protein and enzyme denaturation, DNA damage, and severe functional impairment of vascular endothelial cells and cardiomyocytes, but also participates in the pathogenesis of hypertension, myocardial ischemia-reperfusion injury, atherosclerosis, and other CVDs by regulating inflammation and stimulating vascular smooth muscle cell proliferation (50). In addition, endogenous antioxidant enzymes (e.g., superoxide dismutase, glutathione peroxidase, catalase, glutathione S-transferase, and peroxidase) and exogenous antioxidants may act by scavenging free radicals and exerting anti-CVD activities. For example, overexpression of glutathione peroxidase 4 (GPX4) inhibited atherosclerosis progression in apolipoprotein E-deficient (ApoE$^{-/-}$) mice (51). Giam et al. (52) showed that the antioxidant NAC attenuated cardiac injury and prevented cardiac fibrosis which improved cardiac function in mice with heart failure.

2.3 Mitochondrial dysfunction

Mitochondria, a key site of cellular metabolism for ATP production, provides enough energy for the contraction and diastole of human cardiomyocytes, but mitochondrial dysfunction accelerates the occurrence and progression of CVD (Figure 3). For example, mitochondrial dysfunction in macrophages contributes to inducing inflammation and inhibiting repair after myocardial infarction, but mitochondrial-targeted ROS scavenging alleviates these phenomena and reduces death after myocardial infarction in mice (53). Currently, mitochondrial dysfunction, mitochondrial DNA and nuclear DNA gene mutation, and the presence of mutant proteins associated with mitochondria are considered to be non-negligible causes of CVD pathogenesis (54). For instance, four mitochondrial DNA mutation genes (e.g., MT-RNR1, MT-TL1, MT-TL2, and MT-CYB) have been reported to be connected with atherosclerosis progression (55). Functionally, mutations in

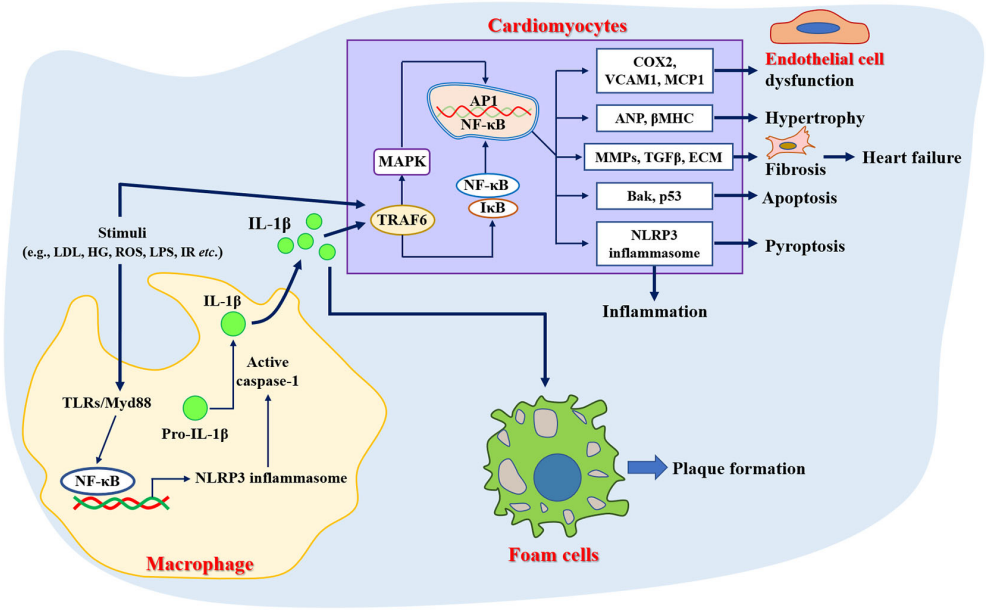

FIGURE 1
Role of inflammation in the pathogenesis of cardiovascular diseases. ANP, Atrial natriuretic peptide; Bak, Bcl-2 antagonist/killer; COX2, Cyclooxygenase 2; ECM, Extracellular matrix; HG, High glucose; LDL, Low-density lipoprotein; LPS, Lipopolysaccharide; MCP1, Monocyte chemotactic protein 1; NLRP3, Nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3; ROS, Reactive oxygen species; TGFβ, Transforming growth factor beta; TLRs, Toll-like receptors; TRAF6, Tumor necrosis factor receptor-associated factor 6; VCAM1, Vascular cell adhesion molecule 1; βMHC, Beta-myosin heavy chain.

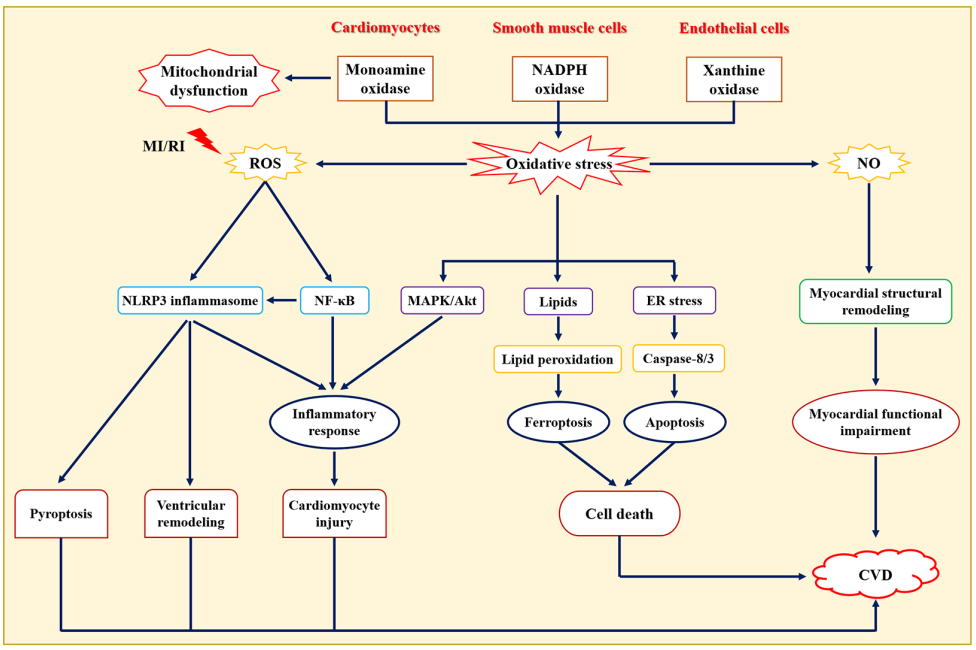

FIGURE 2
Role of oxidative stress in the pathogenesis of cardiovascular diseases. NO: one of the members of reactive nitrogen, damages cardiomyocytes through direct cytotoxicity or generates $ONOO^-$ with O^{2-} to cause cardiomyocyte damage. CVD, Cardiovascular diseases; ER, Endoplasmic reticulum; MAPK, Mitogen-activated protein kinase; MI/RI, Myocardial ischemia/reperfusion injury; NF-κB, Nuclear transcription factor-κB; NLRP3, Nucleotide-binding oligomerization domain-like receptor protein 3.

the mitochondrial genome and nuclear genome may disrupt mitochondrial homeostasis, leading to excessive ROS production and reducing oxidative phosphorylation capacity, which are risk factors for CVD (56). For example, specific targeted antioxidant treatments that reduced ROS production and enhanced ROS scavenging have been shown to alleviate impaired mitochondrial-induced oxidative stress (57). Jacinto et al. (58) showed that the overproduction of mitochondrial ROS promoted atherosclerosis progression by triggering DNA fragmentation and cell apoptosis. Moreover, mitophagy plays an important regulatory role in maintaining cellular homeostasis, whereas mitophagy damage predisposes to cause abnormal function of cardiovascular-derived cells (59). Notably, several intervention strategies ameliorate CVD by improving four important characteristics of mitochondria, such as scavenging mitochondrial ROS (60), mitochondrial DNA editing or mitochondrial replacement therapy (61), increased oxidative phosphorylation (62), and enhanced mitophagy (63). Therefore, maintaining normal mitochondrial function has the potential to be used as an effective therapeutic strategy for CVDs.

2.4 Pyroptosis

Pyroptosis, a form of programmed cell death, is closely related to the inflammatory response, mediated by the Gasdermin protein, and dependent on caspase activity (64). Pyroptosis is typically characterized by the swelling and rupture of cell membranes, the release of pro-inflammatory factors, and cell contents from the plasma membrane to the extracellular environment (65), which aggravates inflammatory response. Recent studies have shown that pyroptosis was involved in the development and progression of several CVDs (Figure 4), including atherosclerosis, diabetic cardiomyopathy, myocardial infarction, myocardial ischemia-reperfusion injury, myocarditis (66), etc. Mechanistically, NLRP3 inflammasome activated caspase-1 and triggered an inflammatory cascade, which plays an important role in pyroptosis (67). For example, NLRP3 inhibitor MCC950 has the potential to prevent NLRP3-related diseases, such as cardiac hypertrophy (68), hypertension (69), atherosclerosis (70), and myocardial injury (71). Jin et al. (72) showed that caspase-1 inhibitor VX765 ameliorated mitochondrial damage induced by the NLRP3 inflammasome activation and inhibition of vascular inflammation in both low-density lipoprotein receptor-deficient ($Ldlr^{-/-}$) and $ApoE^{-/-}$ mice. These results suggested that inhibition of pyroptosis may provide a new avenue for the treatment and management of CVDs.

2.5 Ferroptosis

Ferroptosis is a new type of cellular iron-dependent programmed cell death, and the process mainly involves the accumulation of lipid peroxidation products and lethal ROS (73). Increasing evidence has demonstrated that ferroptosis was

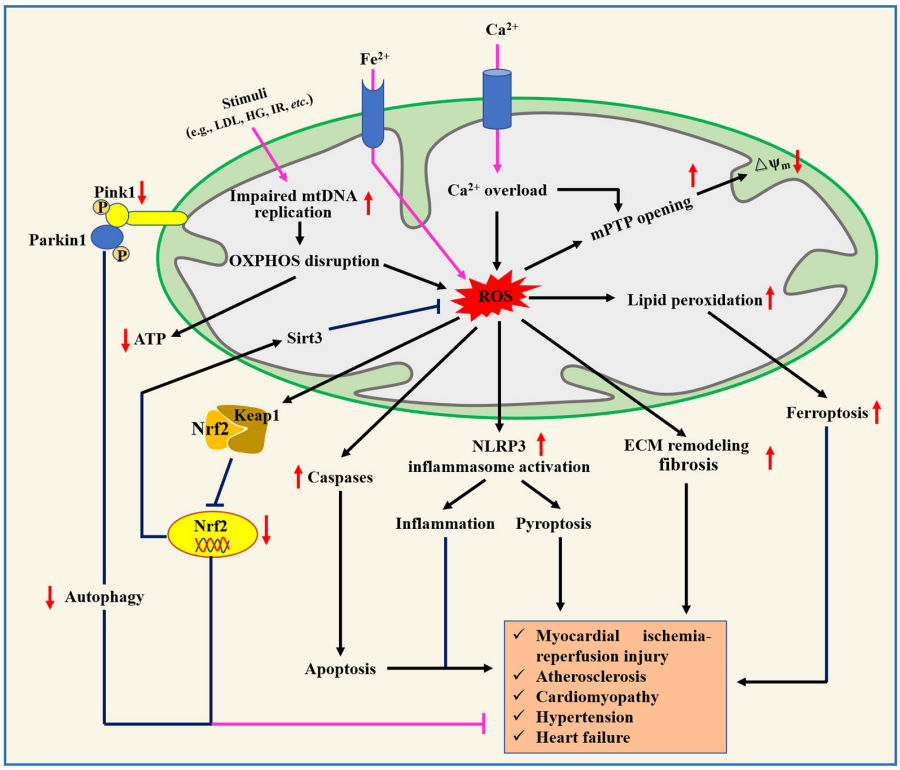

FIGURE 3
Role of mitochondrial dysfunction in the pathogenesis of cardiovascular diseases. ECM, Extracellular matrix; HG, High glucose; IR, ischemia/reperfusion; Keap1, Kelch-like ECH-associated protein 1; LDL, Low-density lipoprotein; mPTP, Mitochondrial permeability transition pore; Nrf2, Nuclear factor erythroid 2-related factor 2.

morphologically, biochemically, and genetically distinct from cell apoptosis, necrosis, and autophagy (74), which was mainly characterized by impaired cell membrane integrity, mitochondrial atrophy, normal nuclei, and a significant decrease in the levels of GPX4, glutamate-cystine antiporter system components (SLC3A2 and SLC7A11), and coenzyme II. Available studies have shown that ferroptosis was closely associated with the development of various CVDs including cardiomyopathy, myocardial ischemia-reperfusion injury, heart failure, myocardial infarction, vascular injury, and atherosclerosis (75). For example, Wang et al. (76) reported that increased levels of lipid peroxidation and reduced SLC7A11 levels were observed in the development of diabetic cardiomyopathy. Bai et al. (77) found that ferrostatin-1 (Fer-1, ferroptosis inhibitor) alleviated atherosclerotic lesions by reducing iron accumulation and lipid peroxidation, and enhancing the expression of GPX4 and SLC7A11 in a high-fat diet (HFD)-fed ApoE$^{-/-}$ mice. Another study showed that the inactivation of the Nrf2/GPX4 pathway could aggravate doxorubicin-induced cardiomyopathy by promoting cardiomyocyte ferroptosis (78). Importantly, three types of iron chelators (e.g., deferiprone, deferoxamine, deferasirox) have been used in clinical practice for the treatment of iron overload cardiomyopathy (79). Although many preclinical studies suggest that pharmacological regulation of ferroptosis and genetic inhibition of iron uptake are promising treatment strategies for CVD (Figure 5), the underlying mechanism and regulatory networks need to be fully investigated during the pathological process of CVD, which will provide new ideas and strategies for the prevention and treatment of CVD.

2.6 Gut microbiota and metabolomics

Gut microbiota refers to the large number of commensal microorganisms living in the human intestinal tract, which mainly consists of *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, and *Actinobacteria* at the phylum level, but its balance is easily disturbed by food intake, lifestyle, and environment (80). Functionally, the gut microbiota can form the intestinal epithelial barrier, regulate intestinal immunity, and prevent the invasion of pathogenic bacteria and metabolic abnormalities (81), which are essential for human health. Numerous studies have demonstrated that dysbiosis of intestinal bacteria and its metabolites, such as Trimethylamine oxide (TMAO), lipopolysaccharides (LPS), short-chain fatty acids (SCFAs), and bile acids, were closely associated with the development of CVD (82), and targeting the gut microbiota was expected to be a potential new target for the treatment of CVD (Figure 6). For example, Jie et al. (83) reported that patients with atherosclerotic cardiovascular disease (ACVD) possessed an increased relative abundance of *Enterobacteriaceae* and *Streptococcus* spp., which contributed to aggravating ACVD as well as other diseases. In another survey, high levels of *Prevotella*,

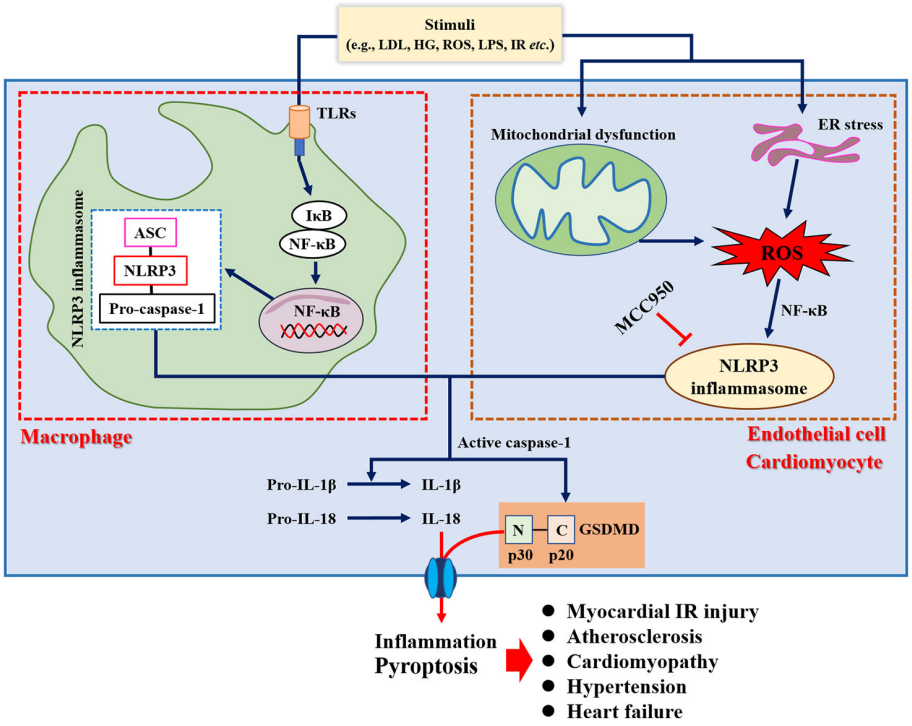

FIGURE 4
Role of pyroptosis in the pathogenesis of cardiovascular diseases.

Hungatella, and *Succinclasticum* and low levels of *Lachnospiraceae* family and *Faecalibacterium* were observed in patients with heart failure (84). Meanwhile, elevated plasma levels of TMAO were positively associated with stroke (85), hypertension (86), and atherosclerosis (87), as well as increased cardiovascular events (88), suggesting that reducing intake of dietary TMAO precursors was an effective strategy to decrease the risk of CVD. The above studies suggest that gut microbiota serves as a "microbial organ" that affects cardiovascular health and the "gut-heart" axis is a potential avenue in the prevention and treatment of CVD.

2.7 Others

Except for the pathogenesis mentioned above, researchers believe that CVD is associated with endoplasmic reticulum stress (ERS) (89), autophagy deficiency (90), diabetes (91), metabolic syndrome (92), etc. Moreover, searching for biomarkers used to determine the occurrence and progression of CVDs and revealing their mechanisms are of great clinical significance for the early diagnosis and treatment of CVD. Meanwhile, the exploration of assessment tools for the early identification of people at high risk of CVD is an important guarantee to reduce cardiovascular mortality. However, the drugs developed to address this pathogenesis can only alleviate the symptoms of CVD, but cannot inhibit or reverse CVD progression. Therefore, elucidating the pathogenesis of CVD remains a key clinical problem that needs to be addressed. Of note, understanding the pathogenesis of CVD may provide effective biomarkers and pathways for subsequent therapeutic and new drug development.

3 TCM in the treatment of CVD

With in-depth research on the pathogenesis of CVD, TCM has shown unique therapeutic advantages in CVD by virtue of its multi-component, multi-target, and integrity (93). More and more studies have demonstrated that TCM (including formulas, extracts, and compounds) exhibited a protective effect on cardiovascular (21), and mechanisms of action of TCM in preventing CVD are shown in Figure 7 and Tables 1–3. Meanwhile, the majority of Chinese patients with CVD have been treated with TCM during the diagnosis and treatment process (94). Herein, we summarized the research progress of TCM in the treatment of various CVDs to provide a reference for the research on the complex mechanism of TCM in combating CVD.

3.1 TCM formulas for CVD

Chinese herbal compounding (*fu fang* or prescription in Chinese) is the main form of TCM for the prevention and treatment of various diseases, which is the simultaneous application of multiple herbs to regulate the body as a whole for therapeutic purposes in clinical practice. A meta-analysis showed that the efficacy of Bushen Huoxue decoction in treating coronary

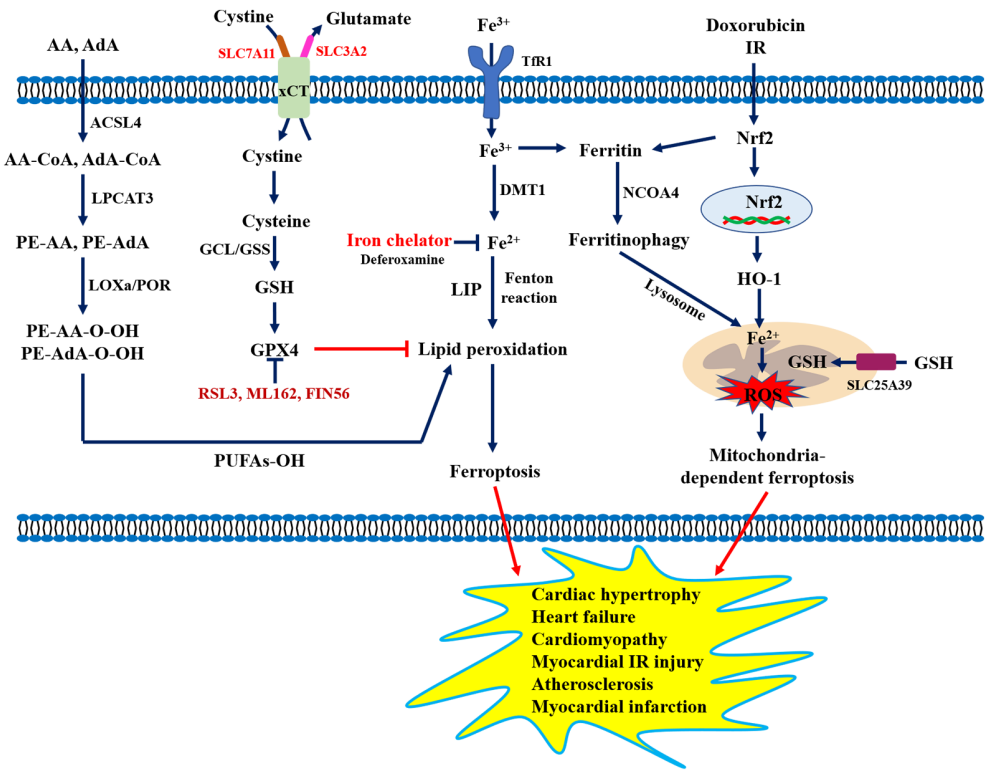

FIGURE 5
Role of ferroptosis in the pathogenesis of cardiovascular diseases. AA, Arachidonic acid; ACSL4, Long-chain fatty acyl-CoA synthase 4; AdA, Adrenal acid; DMT1, Divalent metal transporter 1; FfR1, Transferrin receptor 1; GCL, Glutamate-cysteine ligase; GPX4, Glutathione peroxidase 4; GSH, Glutathione; GSS, Glutathione synthase; HO-1, Heme oxygenase 1; LPCAT3, Lysolecithin acyltransferase 3; LOXs, Lipoxygenases; NCOA4, Nuclear receptor coactivator 4; POR, Cytochrome P450 oxidoreductase; PUFAs, Polyunsaturated fatty acids; SLC7A11, Solute carrier family 7 member 11; xCT, System X^{c-}.

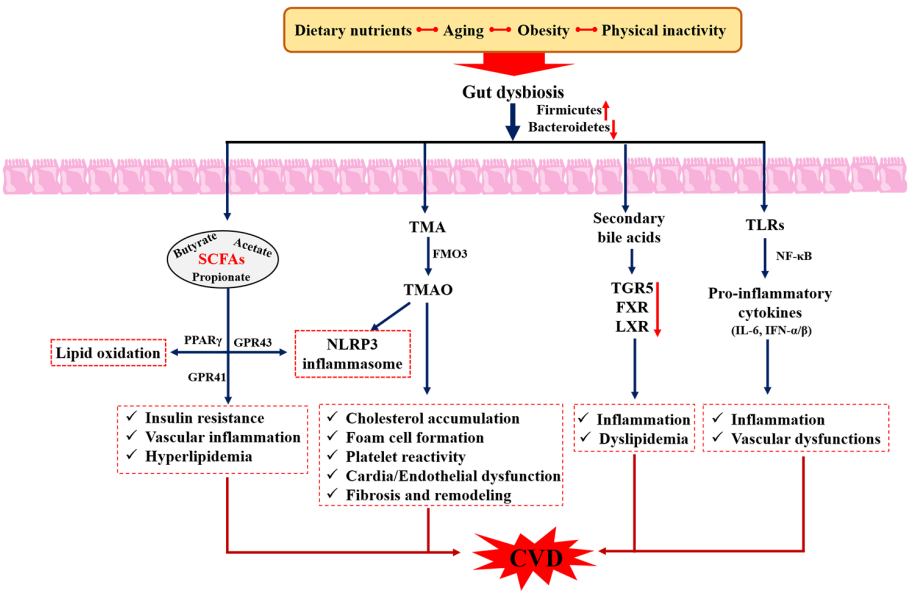

FIGURE 6
Role of gut microbiota in the pathogenesis of cardiovascular diseases. SCFAs, Short chain fatty acids; LPS, Lipopolysaccharides; TGR5, Takeda G-protein-coupled receptor 5; FXR, farnesoid X receptor; TMAO, trimethylamine-N-oxide; TMA, trimethylamine.

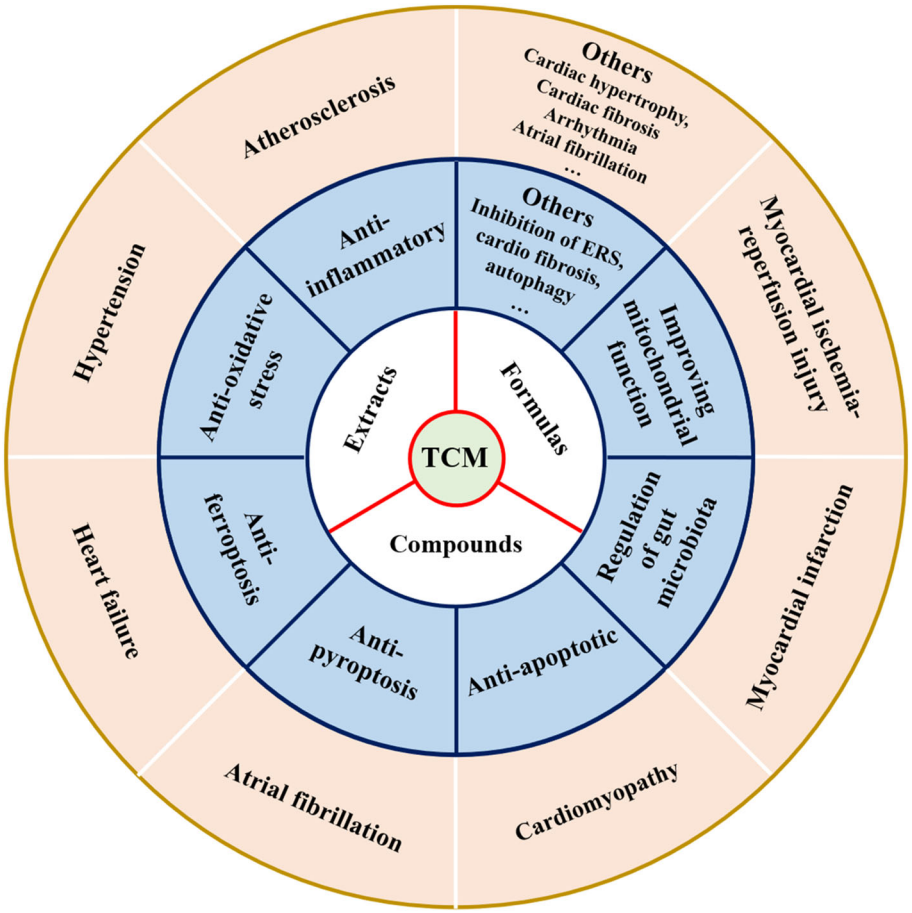

FIGURE 7
Therapeutic effects of TCM on cardiovascular diseases and its mechanism.

heart disease was superior to conventional Western medicine (95). Bi and his colleagues (96) confirmed that Qingre Huatan formulae for the phlegm-heat-stasis syndrome pattern of coronary heart disease was safe and can effectively improve vascular endothelial function. In a randomized, multicenter, double-blind, non-inferiority trial, the results showed that treatment with the Songling Xuemaikang capsule had a well-tolerated and improved total hypertension symptom score and total cholesterol in patients with essential hypertension (97). In addition, TCM prescriptions have been shown to improve sleep disorders in patients with CVD (98). Mechanistically, the Qing-Xue-Xiao-Zhi formula can alleviate the development of atherosclerosis by blocking the TLR4/MyD88/NF-κB pathway to promote lipid efflux, reducing atherosclerotic plaques in the aorta and aortic root and serum TMAO levels, and inhibiting macrophage-mediated inflammation (99). Wu et al. (100) observed that the QiShenYiQi dripping pill can inhibit myocardial ischemia-induced ferroptosis in cardiomyocytes by reducing mitochondrial ROS levels and restoring mitochondrial function (e.g., biogenesis and dynamic homeostasis). Chen et al. (101) demonstrated that Qishen granule administration exhibited cardioprotective effects by inactivation of NF-κB/NLRP3/GSDMD pathway in myocardial infarction, as evidenced by improving cardiac function, reducing inflammatory cell infiltration and collagen deposition, as well as inhibiting NLRP3 inflammasome activation and pyroptosis. Qing-Xin-Jie-Yu granule treatment contributed to the alleviation of atherosclerosis development by regulating gut microbiota composition (that is, the relative abundance of *Turicibacter* and *Roseburia* was enhanced), increasing bile acids production, and reducing metaflammation induced by HFD (102). Zhou et al. (103) showed by a comprehensive network analysis that Shenfu injection can be used to treat coronavirus disease 2019 (COVID-19) combined with heart failure. Except for the above-mentioned TCM prescriptions, there are still numerous studies reported on the use of some classical TCM formulas for the prevention and treatment of CVD according to ancient works and the modern clinical. Herein, we summarized the pharmacological effects and molecular mechanisms of TCM prescriptions on CVD based on published studies from 2018 to 2023 and listed in Table 1.

3.2 TCM extracts for CVD

Increasing evidence has proved that single TCM extracts also possessed a protective effect against CVD except for TCM preparations mentioned above (Table 2). For example, a network

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TABLE 1 Summary of traditional Chinese medicine formulas in the prevention and treatment of various cardiovascular diseases from 2018-2023.

Prescription	Composition (In Chinese)	Evaluation model	Effects and action mechanism	Ref.
Atherosclerosis				
Buyang huanwu decoction	Huangqi, Chishao, Chuanxiong, Danggui, Dilong, Taoren, and Honghua in a ratio of 120:6:4:5:3:3:3	HFD-induced ApoE$^{-/-}$ mice	Levels of TC, TG, LDL-c↓and HDL-c↑ Levels of TNF-α, IL-1β, IL-6, iNOS↓ NF-κB pathway↓	(196)
Huang-Lian-Jie-Du decoction	Huanglian, Huangqin, Huangbo, and Zhizi in a weight ratio of 3:2:2:3	HFD-induced ApoE$^{-/-}$ mice ox-LDL-induced RAW264.7 cells	Carotid lesion plaques stability↑ Levels of IL-1β, IL-6, TNF-α↓ Foam cell formation↓and M2 polarization↑	(197)
Guanxinkang decoction	Huangqi, Yimucao, Danshen, Xiebai, Banxia, and Gualou in a weight ratio of 10:10:4:4:4:5	HFD-induced LDLR$^{-/-}$ mice ox-LDL-induced RAW264.7 cells	Body weight and levels of TC, TG, LDL-c↓ Atherosclerotic plaques↓and α-SMA level↑ Levels of IL-1β, IL-6, TNF-α, LOX-1, MCP-1↓ MAPKs/NF-κB pathway↓	(198)
Qing-Xin-Jie-Yu granule	Huangqi, Danshen, Chuanxiong, Guanghuoxiang, and Huanglian in a ratio of 3:3:2:2:1	HFD-induced ApoE$^{-/-}$ mice	Body weight and levels of TC, TG, and LDL-c↓ Levels of HDL-c↑and IL-1β, IL-6↓ The abundance of *Turicibacter* and *Roseburia*↑ The abundance of *Alistripes*, *Rikenella*, *Blautia*↓	(102)
Qing-Xin-Jie-Yu granule	Huangqi, Danshen, Chuanxiong, Guanghuoxiang, and Huanglian in a ratio of 3:3:2:2:1	HFD-induced ApoE$^{-/-}$ mice	TC, TG, LDL-c levels, and ferroptosis↓ Levels of IL-6, IL-1β, TNF-α, Fe^{2+}, ROS↓ Expression of GPX4/xCT in aorta tissues↑	(199)
Yiqihuoxue decoction	Chuanxiong, Chishao, and Xiyangshen in a ratio of 40:20:1	HFD-induced ApoE$^{-/-}$ mice	Blood glucose and levels of TNF-α and IL-6↓ Aortic arch plaque area↓	(200)
Wu-Zhu-Yu decoction	Wuzhuyu, Shengjiang, Renshen, and Dazao in a ratio of 1:2:1:1	HFD-induced ApoE$^{-/-}$ mice	Aortic lesion areas↓ Levels of TC, TG, LDL-c↓and HDL-c↑	(201)
Tongqiaohuoxue decoction	Shaoyao, Chuanxiong, Taoren, Honghua, Onion, Wuchizao, Ginger, and Yunmuxiang in a ratio of 16:16:48:48:12:8:48:20	HFD-induced ApoE$^{-/-}$ mice ox-LDL-induced THP-1 cells ox-LDL-induced HUVECs	Lipid deposition, plaque formation, lipid uptake↓ Levels of ICAM-1, VCAM-1, and MCP-1↓	(202)
Si-Miao-Yong-An decoction	Rendong, Xuanshen, Danggui, and Gancao in a ratio of 3:3:2:1	HFD-induced ApoE$^{-/-}$ mice	lipid accumulation↓and Autophagy↑ NF-κB pathway↓	(203)
Tao Hong decoction	Taoren, Honghua, Chuanxiong, Danggui, and Weilingxian in a ratio of 9:9:9:9:9	HFD-induced ApoE$^{-/-}$ mice	Plaque area and Levels of inflammatory cytokines↓ PI3K/Akt/p38 pathway↓	(204)
Bunao-Fuyuan decoction	Huangqi, Baizhi, Chishao, Chuanxiong, Honghua, and Taoren in a ratio of 120:6:5:3:3:3	ox-LDL-induced VMSCs	α-SMA protein and cell proliferation↓ Cell invasion and migration↓ RHOA/ROCK pathway↓	(205)
Huanglian Jiedu decoction	Huanglian, Huangqi, Huangbo, and Zhizi in a ratio of 9:6:6:9	HFD-induced ApoE$^{-/-}$ mice	Levels of TC, TG, LDL-c↓and HDL-c↑ Expression of CRP, IL-6, TNF-α↓	(206)
Liuwei Dihuang formula	Dihuang, Shanzhuyu, Chinese Yam, Zexie, Diaozhilan, and Fuling in a ratio of 32:16:16:12:12:12	HFD-induced ApoE$^{-/-}$ mice Hcy-induced HUVECs	HUVEC apoptosis↓ The ratio of SAM/SAH and plaque formation↓	(207)
Liuwei Dihuang soft capsule	Dihuang, Shanzhuyu, Chinese Yam, Zexie, Diaozhilan, and Fuling in a ratio of 32:16:16:12:12:12	HFD-induced ApoE$^{-/-}$ mice PDGF-BB-induced VSMCs	Lipid deposition and levels of TG, TC, LDL-c↓ Expression of ERα, ERβ, SRC3↑ CyclinD expression and cell migration↓	(208)
Danggui Buxue decoction	Danggui and Huangqi in a ratio of 1:5	hyperplasia/neointima mice model	Levels of IL-1β, TNF-α, MCP-1↓ PI3K/Akt pathway↓	(209)

(Continued)

TABLE 1 Continued

Prescription	Composition (In Chinese)	Evaluation model	Effects and action mechanism	Ref.
Atherosclerosis				
Qingre Huoxue decoction	Huangqin, Chishao, Chuanxiong, Maodongqing, Honghua, Jiangxiang, and Danshen in a ratio of 3:3:2:6:2:2:6	HFD-induced ApoE$^{-/-}$ mice LPS-induced RAW264.7 cells	Body weight and levels of TC, TG, LDL-c↓ Plaque area↓and M2 polarization↑ NF-κB pathway↓	(210)
Liuwei Dihuang formula	Shudihuang, Shanzhuyu, Shanyao, Zexie, Mudanpi, and Fuling in a ratio of 8:4:4:3:3:3	Ang II-induced VSMCs	VSMC proliferation and migration↓ Expression of α-SMA and OPN↓	(211)
Chaihu-Shugan-San formula	Chaihu, Chenpi, Chuanxiong, Baishao, Xiangfu, Zhike, and Gancao in a ratio of 4:4:3:3:3:3:1.	HFD-induced ApoE$^{-/-}$ mice LPS-induced HUVECs	Atherosclerotic plaque areas↓ Levels of TC, TG, LDL-c, TNF-α, IL-1β, IL-6↓ Expression of BDNF and TrkB↑	(212)
Guanmaitong granule	Huangqi, Danshen, Gualou, Huanglian, Sanqi, Xuanshen, Zhebeimu, Huzhang, Shuizhi, and Muli in a ratio of 6:3:3:1.5:3:4.5:3:2:1:0.5	HFD-induced ApoE$^{-/-}$ mice	Levels of TG, TC, LDL-c, TNF-α, IL-6, IL-1β↓ Plaque lipid deposition↓ Plaque collagen content↓ TLR4/MyD88/NF-κB pathway↓	(213)
Myocardial ischemia-reperfusion injury				
Tongmai Yangxin pill	Dihuang, Jixueteng, Maidong, Zhiheshouwu, Ejiao, Gancao, Wuweizi, Dangshen, Cuguijia, Dazao, and Guizhi in a ratio of 10:10:6:6:6:6:6:6:4:4:2	I/R-induced myocardial injury	LVEF and LVFS↑and CK and CK-MB levels↓ MDA content and inflammatory cell infiltration↓ Cardiomyocyte apoptosis↓and PI3K/Akt pathway↑	(214)
Tongmai Yangxin pill	Dihuang, Jixueteng, Maidong, Zhiheshouwu, Ejiao, Gancao, Wuweizi, Dangshen, Cuguijia, Dazao, and Guizhi in a ratio of 10:10:6:6:6:6:6:6:4:4:2	I/R-induced myocardial injury	LVDd and LVDs↓ Inflammatory cell number↓ Activities of CK, LDH, MDA↓and NO activity↑ cAMP/PKA and NO/cGMP pathways↑	(215)
QishenYiqi dripping pill	Huangqi, Danshen, Sanqi, and Jiangxiang in a ratio of 20:65:1:33	I/R-induced myocardial injury	Myocardial infarct size, LVDd, NLRP3 expression↓ LVEF and LVFS↑and PI3K/Akt-mTOR pathway↑	(216)
Yiqi Huoxue formula	Huangqi, Danshen, Sanqi, Chuanxiong, Danggui, Yiyiren, Baizhu, Fuling, Banxia, Juhong, Dilong, and Shuizhi in a ratio of 30:15:10:10:10:15:15:15:15:10:10:3	I/R-induced myocardial injury H/R-induced H9c2 cell injury	Myocardial infarct size↓ Levels of CK and LDH↓ MDA content↓and SOD level↑ H9c2 cell proliferation↑	(217)
Huoxue Jiedu formula	Shaoyao, Chuanxiong, and Huanglian in a ratio of 1:1:1	I/R-induced myocardial injury H/R-induced H9c2 cell injury	Infarcted area, CK-MB and cTnT levels↓ Beclin-1 and LC3-II↓and Bcl-2, p62↑ PI3K/AKT/mTOR pathway↑	(218)
Dried ginger-aconite decoction	Wutou and Ginger in a ratio of 1:1	I/R-induced myocardial injury H/R-induced H9c2 cell injury	SOD level↑and MDA content↓ H9c2 cell apoptosis and myocardial infarct size↓ PI3K/AKT/GSK-3β pathway↑	(219)
Tongmai formula	Danshen, Gegen, and Chuanxiong in a ratio of 1:1:1	I/R-induced myocardial injury H/R-induced neonatal rat ventricular myocyte injury	Myocardial infarct size and cell apoptosis↓ cTnT, CK, LDH levels, and MDA content↓ GSH and SOD activities↑and ROS content↓	(220)
Xin-Ji-Er-Kang formula	Renshen, Yuzhu, Sanqi, Xiebai, Danggui, Maidong, Wuweizi, Danshen, Kushen, Gancao, Huangqi, Yinyanghuo, Jinsilian, and Bingpian in a ratio of 11.71:7.03:3.09:7.80:7.80:7.80:3.93:7.80:7.80:7.80:11.69:7.80:7.8:0.15	I/R-induced myocardial injury H/R-induced cardiomyocyte-like cell injury	Myocardial infarct size and LVDd↓ LVEF and LVFS↑ Apoptosis of cardiomyocytes↓ JAK2/STAT3 pathway↑	(221)

(Continued)

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TABLE 1 Continued

Prescription	Composition (In Chinese)	Evaluation model	Effects and action mechanism	Ref.
Myocardial ischemia-reperfusion injury				
Si-Miao-Yong-An decoction	Jinyinhua, Xuanshen, Danggui, and Gancao in a ratio of 5:5:3:3	I/R-induced myocardial injury	Myocardial infarct size↓and LVEF, LVFS↑ Levels of CK, LDH, TNF-α, IL-6, IL-1β↓ TLR4/NF-κB pathway↓	(222)
Heart failure				
Qishen granule	Huangqi, Danshen, Jinyinhua, Xuanshen, Fuzi, and Gancao in a ratio of 30:15:10:10:9:6	TAC-induced heart failure model TGF-β-stimulated cardiac fibroblasts	LVDd and LVDs↓and LVEF and LVFS↑ Collagen deposition↓ TGF-β/SMADs and PI3K/GSK-3β pathways↓	(223)
Si-Miao-Yong-An decoction	Rendong, Xuanshen, Danggui, and Gancao in ratio of 3:3:2:1	ISO-induced heart failure model ISO-induced H9c2 cell injury	LVEF and LVFS↑and LVDd and LVDs↓ Expression of fibronectin, collagen I, α-SMA↓ PDE5A-Akt and TLR4-NOX4 pathways↓	(224)
Lingguizhugan decoction	Fuling, Guizhi, Baizhu, and Gancao in a ratio of 4:3:3:3	TAC-induced heart failure model	LVEF and LVFS↑and LVDd and LVDs↓ Heart weight, ANP, BNP, α-MHC, cardiac fibrosis↓ Akt-GSK3β/mTOR/P70S6K pathway↓	(225)
XinLi formula	Cheqiancao, Huangqi, Hongshen, Ezhu, and Shanzhuyu in a ratio of 30:40:10:9:12	LAD-induced heart failure model Ang II-induced H9c2 cell injury	LVEF↑and levels of NT-proBNP, cTnT, CK-MB↓ Content of ALD, AGTR1, TGF-β1, HYP↓ Expression of NLRP3, caspase-1, IL-1β, IL-18↓	(226)
Zhenwu decoction	Wutou, Shaoyao, Baishu, Fuling, and Ginger in a ratio of 3:3:2:3:3	DOX-induced heart failure model	LVDd and LVDs↓and LVFS and LVEF↑ Levels of CK-MB, BNP, and NT-proBNP↓ Fibrosis area, collagen I↓and SOD activity↑ Expression of IL-1β, TNF-α, IL-6↓ NF-κB pathway↓and PI3K/Akt pathway↑	(227)
Linggui Zhugan decoction	Fuling, Guizhi, Baizhu, and Gancao in a ratio of 4:3:3:2	LAD-induced heart failure model	LVEF and LVFS↑and LVDs and LVDd↓ MDA production and NT-proBNP levels↓ SOD activity and SIRT1/AMPK/PGC1α pathway↑	(228)
Shenqi Lixin decoction	Renshen, Huangqi, Rougui, Yinyanghuo, Luhui, Shuweicao, Fuling, Baishu, Longyacao, Yimucao, and Gancao in a ratio of 4:4:2:4:3:3:4:3:6:3:2	Adriamycin-induced heart failure model	LVEF and LVFS↑and LVDs and LVDd↓ Myocardial fibrosis↓ NT-proBNP level↓and ATP level↑ Expression of Bax and caspase-3↓	(229)
Jijiu Huiyang decoction	Fuzi, Ginger, Danshen, Baizhu, Taoren, Honghua, and Zhigancao in a ratio of 5:3:9:9:6:6:5	DOX-induced heart failure model	LVEF and LVFS↑ LVDs and LVDd↓ PPARα pathway↓	(230)
Xinfuli granule	Huangqi, Renshen, Danshen, Fuling, and Maidong in a ratio of 9:6:3:3:2	LAD-induced heart failure model Hypoxia/ischemia-induced H9c2 cell injury	LVEF and LVFS↑and LVDs and LVDd↓ Levels of ADP, AMP, LA, LDH, FFA↓ RHOA/ROCK pathway↓	(231)
Qishen granule	Huangqi, Danshen, Rendong, Xuanshen, Wutou, and Gancao in a ratio of 30:15:10:10:9:6	LAD-induced heart failure model LPS-induced RAW264.7 cells	LVEF and LVFS↑and LVDs and LVDd↓ Levels of CK-MB and LDH↓ TLR4/MyD88/NF-κB pathway↓	(232)
BAOXIN granule	Huangqi, Danshen, Zelan, Gancao, Maidong, Fuling, Danggui, Zhike, Dihuang, Jiegeng, Dahuang, and Mahuang in a ratio of 20:13:10:10:10:10:7:7:7:4:4:4	TAC-induced heart failure model	Heart weight and cardiac fibrosis↓ LVEF and LVFS↑and LVDs and LVDd↓ Expression of ANP, BNP, β-MHC, IL-1β, IL-6↓ Expression of TGF-β and collagen I/III↓	(233)
Guanxining injection	Danshen and Chuanxiong in a ratio of 1:1	TAC-induced heart failure model	LVEF and pro-BNP level↑ Collagen volume fraction↓ Expression of SLC7A11, GPX4↑and FTH1↓	(234)

(Continued)

TABLE 1 Continued

Prescription	Composition (In Chinese)	Evaluation model	Effects and action mechanism	Ref.
Heart failure				
YiQiFuMai powder	Renshen, Maidong, and Wuweizi in a ratio of 1:3:1.5	LAD-induced heart failure model	LVEF and LVFS↑and LVDs and LVDd↓ Cardiac fibrosis and p38 MAPK/ERK$_{1/2}$ pathway↓	(235)
Guanxinning injection	Danshen and Chuanxiong	TAC-induced heart failure model	SBP, DBP, LVDs, LVDd↓ LVEF and LVFS↑and p38/c-Fos/Mmp1 pathway↓	(236)
Qiangxin recipe	Huangqi, Chuanxiong, Fuzi, Fuling, Cheqianzi, Dangshen, Guizhi, Nvzhenzi, Tinglizi, Taoren, Taizishen, and Zhuling in a ratio of 10:5:5:5:5:5:3:5:10:5:5:5	DOX-induced heart failure model DOX-induced H9c2 cell injury	Cell viability and glucose metabolism↑ Levels of BNP and cTnI↓ LVEF↑	(237)
Xinshuitong capsule	Huangqi, Danshen, Guizhi, Zexie, and Yumixu in a ratio of 6:4:4:3:3	DOX-induced heart failure model	LVEF and LVFS↑and LVDs and LVDd↓ Levels of BNP, BUN, AST, ALT↓	(238)
WuShen decoction	Renshen, Danshen, Xuanshen, Beishashen, and Kushen in a ratio of 1:3:2:2:1	LAD-induced heart failure model	LVEF and LVFS↑and LVDs and LVDd↓ Cardiac fibrosis and infarct size↓ TGF-β1/Smad2/3 pathway↓	(239)
Hypertension				
Qingda granule	Tianma, Gouteng, Huangqin, and Lianzixin in a ratio of 12:10:6:5	Spontaneously hypertensive rats Ang II-stimulated cardiac fibroblasts	SBP, DBP, MAP↓and LVEF and LVFS↑ α-SMA, collagen III, cardia fibrosis↓ TGF-β1/Smad$_{2/3}$ pathway↓	(240)
Danzhi Xiaoyao powder	Chaihu, Baishao, Danggui, Fuling, Baizhu, Mudanpi, Zhizi, and Gancao ina ratio of 2:2:2:2:2:1:1:1	Spontaneously hypertensive rats	SBP, DBP, MAP↓ Anxiety-like behavior↓	(241)
Guizhi decoction	Guizhi, Baishao, and Gancao in a ratio of 3:2:2	HFD-induced hypertension model	Blood pressure and collagen content↓ Expression of IL-6, IL-1β, MMP2, MMP9↓	(242)
Qingda granule	Tianma, Gouteng, Huangqin, and Lianzixin in a ratio of 12:10:5:6	Ang II-hypertension model Ang II-stimulated VSMCs	SBP, DBP, MAP, Cell viability↓ MAPK and PI3K/Akt pathways↓	(243)
Gedan Jiangya decoction	Gouteng, Danshen, Gegen, Duzhong, Xiakucao, and Niuxi in a ratio of 2:5:6:3:3:4	Spontaneously hypertensive rats	SBP and DBP↓ Expression of collagen I/III, α-SMA, IL-1β, IL-6↓NF-κB pathway↓	(244)
Zhengganxifeng decoction	Niuxi, Ludou, Longgu, Mulike, Guike, Baishao, Xuanshen, Tiandong, Chuanxiong, Maiya, Yinchenhao, and Gancao in a ratio of 30:30:15:15:15:15:15:15:6:6:6:4.5	Spontaneously hypertensive rats	SBP, DBP, MAP↓ Firmicutes to Bacteroidetes ratio↓ SCFA production↑	(245)
Qing Gan Zi Shen Tang formula	Guizhencao, Weimao, Huanglian, Nvzhen, Shanzhuyu, and Xuanshen in a ratio of 10:5:1:4:4:5	HFD-induced hypertension model	SBP, DBP, MAP↓ Levels of TG, LDL-c↓and HDL-c↑	(246)
Zi Shen Huo Luo formula	Xuanshen, Niuxi, Huanglian, Mudan, Yimucao, and Rougui in a ratio of 20:15:12:12:20:3	Spontaneously hypertensive rats Aldosterone-induced H9c2 cells and cardiac fibroblasts	SBP, DBP, MAP↓and LVSP, ± dp/dt max↑ Cardiac fibrosis↓and cell proliferation↑ EGFR/ERK pathway↓	(247)
Myocardial infarction				
Buyang Huanwu decoction	Huangqi, Danggui, Chisao, Chuanxiong, Taoren, Honghua, and Dilong in a ratio of 120:10:10:10:10:10:4.5	Ligature-induced myocardial infarction model	Angiogenesis↑ PI3K/Akt/GSK3β pathway↑	(248)
Taohong siwu decoction	Shudihuang, Chuanxiong, Chishao, Danggui, Honghua, and Taoren in a ratio of 3:2:2:3:3:4	Ligature-induced myocardial infarction model TGF-β1-induced cardiac fibroblasts	Myocardial fibrosis↓ Cell proliferation and collagen expression↓ TGFBR1/Smad2/3 pathway↓	(249)
Xuefu Zhuyu decoction	Danggui, Dihuang, Taoren, Honghua, Chisao, Zhiqiao, Gancao, Chaihu,	Ligature-induced myocardial infarction model	Mitochondria damage↓ Number of autophagosomes and	(250)

(Continued)

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TABLE 1 Continued

Prescription	Composition (In Chinese)	Evaluation model	Effects and action mechanism	Ref.
Myocardial infarction				
	Chuanxiong, Jiegeng, and Niuxi in a ratio of 9:9:12:9:6:6:6:3:4.5:4.5:9		lysosomes↓ Expression of LC3-B and P62↓	
Yiqihuoxue decoction	Huangqi, Danggui, Renshen, Chuanxiong, and Sanqi	Ligature-induced myocardial infarction model	LVEF and LVFS↑and levels of LDH, CK-MB↓ JNK/MAPK pathway↑	(251)
Qingre Huoxue decoction	Huangqin, Shaoyao, Chuanxiong, Maodongqing, Honghua, Jiangxiang, and Danshen in a ratio of 3:3:2:6:2:2:6	Ligature-induced myocardial infarction model	LVEF and LVFS↑ MCP-1, IL-17A, TNF-α and IL-1β levels↓ LC3B, Beclin-1, ATG5, ATG7↑and p62 level↓ PI3K/Akt pathway↓	(252)
Qingyi decoction	Dahuang, Baishao, Chaihu, Zhizi, Yanhusuo, Muxiang, and Huangqin, in a ratio of 3:3:3:3:2:2:2	Severe acute pancreatitis-induced myocardial infarction model	LVEF and LVFS↑ Levels of IL-1β, IL-6, TNF-α↓ STIM1/Orai1-SOCE pathway↓	(253)
Shuangxinfang	Danshen, Chuanxiong, Baihe, and Dazao in a ratio of 20:12:30:30	Ligature-induced myocardial infarction model	LVEF and LVFS↑and LVDs and LVDd↓ Myocardial fibrosis and levels of IL-1β, TNF-α↓ TLR4/NF-κB pathway↓	(254)
Qishen granule	Huangqi, Danshen, Rendong, Xuanshen, Wutou, and Gancao in a ratio of 30:15:10:10:9:6	Ligature-induced myocardial infarction model OGD/R, ISO, Ang II and LPS-ATP-induced H9c2 cell injury	LVEF and LVFS↑and LVDs and LVDd↓ Levels of LDH, CK-MB, NLRP3, IL-1β, IL-18↓ Cell apoptosis, ROS level, NF-κB pathway↓	(101)
Others				
Jia-Wei-Si-Miao-Yong-An decoction	Jinyinhua, Lianqiao, Xuanshen, Rougui, Danggui, Danshen, Gancao, and Huzhang in a ratio of 15:15:15:9:15:15:15:9	Acute coronary syndrome model (acute coronary syndrome)	Levels of CK-MB, cTnI, IL-2, TNF-α↓ The abundance of *Bacteroides* and *Rikenellaceae RC9 gut group*↑ The abundance of *Clostridium sensu stricto 1*, *Prevotella*, *unclassified o Bacteroidales*, and *Ruminococcus gauvreauii group*↓	(255)
Zhen-Wu decoction	Fuzi, Shaoyao, Fuling, Baizhu, and Shengjiang in a ratio of 3:3:3:2:3	Uremia-induced cardiac endothelial injury Npx-induced cardiovascular endothelial injury (uremic cardiomyopathy)	LVEF↑and fibrosis area, MDA level↓ Expression of IL-1β and IL-6↓ Cell death and ROS level↓ Nrf2/keap1 pathway↑	(256)
Qingda granule	Tianma, Gouteng, Huangqin, Hehua in a ratio of 12:10:6:5	Obesity-induced hypertension and cardiac dysfunction (hypertension and cardiac dysfunction)	SBP, DBP, MAP↓and LVEF, LVFS↑ Levels of TG, TC↓and HDL-c, Akt pathway↓	(257)
Si-Miao-Yong-An decoction	Jinyinhua, Xuanshen, Danggui, and Gancao in a ratio of 3:3:2:1	TAC-induced heart failure model (heart failure)	LVEF↑and fibrosis area and collagen content↓ TGFβ1/TAK1/p38/Smad pathway↓	(258)
Huoxin pill	Lingzhi, Linshe, Xiongzhang, Niudanfen, Zhenzhufen, Renshen, Ganchan, Chuanwutou, Bingpian, and Honghua in a ratio of 20:1.2:2.4:1.2:2.4:18:1.8:9:1.2:2	ISO-induced cardiac fibrosis model (myocardial fibrosis)	Expression of α-SMA and collagen I/III↓ Cell viability and migration↓ TGF-β1/Smad pathway↓	(259)
Yunpi-Huoxue-Sanjie formula	Baizhu, Zhiqiao, Tianhuafen, Muli, and Tubiechong in a ratio of 5:2:3:10:2	HFD/streptozotocin-induced diabetic cardiomyopathy High glucose-induced H9c2 cells (diabetic cardiomyopathy)	Levels of FFA, TG, MDA↓and CAT activity↑ LVDs and LVDd↑and LVEF and LVFS↓ Expression of Atg7, Beclin1, LC3 II/LC3 I↑	(260)
Fufang Xueshuantong formula	Sanqi, Danshen, Huangqi, and Xuanshen in a ratio of 25:8:5:8	Streptozotocin-induced diabetic cardiomyopathy (diabetic cardiomyopathy)	LVEF and LVFS↑and collagen I/III and TGF-β1↓ Wnt/β-Catenin pathway↓	(261)

(Continued)

TABLE 1 Continued

Prescription	Composition (In Chinese)	Evaluation model	Effects and action mechanism	Ref.
Others				
Danzhi Jiangtang capsule	Taizishen, Dihuang, Mudanpi, Xieze, Tusizi, and Shuizhi in a ratio of 6:5:4:4:3:3	HFD/streptozotocin-induced diabetic cardiomyopathy High glucose-induced H9c2 cells (diabetic cardiomyopathy)	LVEF and LVFS↑ Cell apoptosis and levels of IL-1β and IL-6↓ TLR4/MyD88/NF-κB pathway↓	(262)

ABCA1, ATP-binding cassette transporter A1; ACSL4, Acyl-CoA synthetase long-chain family member 4; ApoE$^{-/-}$, Apolipoprotein-E deficient; BA, Bile acid; CK-MB, Creatine kinase MB; COX2, Cyclooxygenase-2; cTnT, Cardiac troponin T; DBP, Diastolic blood pressure; FTH1, Ferritin heavy chain 1; GPX4, Glutathione peroxidase 4; GSH, Glutathione; HDL-c, High-density lipoprotein-cholesterol; HFD, High-fat diet; H/R, Hypoxia/reoxygenation; HUVECs, Human umbilical vein endothelial cells; ICAM-1, Intercellular adhesion molecule-1; ISO, Isoproterenol; I/R, Ischemia/reperfusion; iNOS, Inducible nitric oxide synthase; LAD, left anterior descending ligation; LDH, Lactate dehydrogenase; LDLR$^{-/-}$, LDL receptor deficient; LDL-c, Low-density lipoprotein cholesterol; LOX-1, Lectin-like oxidized low-density lipoprotein receptor-1; LVDd, Left ventricular diastolic diameter; LVDs, Left ventricular systolic diameter; LVDP, Left ventricular diastolic pressure; LVEF, Left ventricular ejection fraction; LVFS, Left ventricular shortening fraction; LVSP, Left ventricular systolic pressure; LV Vol, Left ventricle volume; MAP, Mean arterial pressure; MCP-1, Monocyte chemoattractant protein-1; MDA, Malondialdehyde; OGD/R, Oxygen-glucose deprivation/reoxygenation; PDGF, Platelet-derived growth factor; PDE5A, Phosphodiesterase 5A; PKG I, cGMP-dependent protein kinase 1; PPARγ, Peroxisome proliferator-activated receptor gamma; SAM, S-Adenosyl methionine; SAH, S-Adenosyl homocysteine; SBP, Systolic blood pressure; SRA1, scavenger receptor A1; TAC, Transverse abdominal aortic constriction; TC, Total cholesterol; TG, Triglyceride; VCAM-1, Vascular cell adhesion molecule-1; VSMCs, Vascular smooth muscle cell.
↑ upregulated, ↓ downregulated.

pharmacology study showed that Schisandra extracts have the potential for therapeutic effects on atherosclerosis by regulating immune inflammation and oxidative stress (104). Recently, the key mechanisms of TCM extracts in CVD may be associated with immunomodulation, antioxidant, anti-cell death, anti-inflammatory, and gut microbiota regulation. For example, Quince extract exhibited hypolipidemic, antioxidant, anti-inflammatory, anti-thrombotic, and vascular endothelium protective effects on HFD-induced atherosclerosis (105). *Plantago asiatica* L. seeds extracts prevented isoproterenol-induced cardiac hypertrophy by restoration of autophagy and inhibition of cardiomyocyte apoptosis (106). The ethyl acetate extracts of *Cinnamomi Ramulus* protect rats from myocardial ischemia-reperfusion injury by suppression of NLRP3 inflammasome activation and pyroptosis (107). In doxorubicin-induced chronic heart failure, the combination of aqueous extracts of *Aconiti Lateralis Radix Praeparata* and *Zingiberis Rhizoma* has a better therapeutic effect than their single aqueous extracts, which may be associated with improving left ventricular function and promoting mitochondrial energy metabolism through activation of the PPARα/PGC-1α/Sirt3 pathway (108). Treatment with bay leaf extracts exhibited an anti-inflammatory effect in the rat model of myocardial infarction (109), reflected by reducing the levels of C-reactive protein and myeloperoxidase. Another study showed that aqueous extracts of *Ligustrum robustum* attenuated atherosclerosis development by modulating gut microbiota composition and metabolism, as evidenced by increased relative abundance of genus *Bifidobacterium*, and reduced serum TMAO and bile acid, as well as decreased cholesterol absorption (110). In addition, single TCM extracts used for the treatment of CVD have been shown to regulate mitochondrial homeostasis and maintain normal autophagy function, as well as have anti-ERS and anti-contractile effects. For instance, Vilella et al. (111) reported that green tea extracts ameliorated cardiomyopathy progression by improving mitochondrial function. In streptozotocin-induced diabetic atherosclerosis, Ginkgo biloba leaf extracts reduced plaque lipid deposition and serum inflammatory cytokines secretion via inhibiting ERS and mTOR-mediated autophagy (112). Granado et al. (113) proved that Marjoram extracts prevented inflammatory response, apoptosis, and oxidative stress of cardiomyocytes induced by coronary ischemia-reperfusion, as well as possessed anti-contractile effects in aorta segments. Taken together, the cardioprotective effects of single TCM extracts on various CVDs were confirmed, but its underlying mechanisms and safety need to be further explored before clinical practice.

3.3 Compounds isolated from TCM for CVD

With the development of pharmaceutical chemistry and pharmacology, many scholars have conducted studies on the bioactive components of TCM in recent years. It has been found that a large number of effective compounds extracted from TCM, such as phenolic acids, flavonoids, stilbenes, anthraquinones, saponins, terpenoids, alkaloids, polysaccharides, etc., all of which possessed therapeutic effects on various CVDs (Table 3).

3.3.1 Phenolic acids

Phenolic acids are a subclass of plant phenolics that can be isolated and extracted from many traditional Chinese herbs such as *Angelica sinensis*, *Salvia miltiorrhiza*, *Cinnamomi ramulus*, *Lonicera japonica*, *Radix Paeoniae Rubra*, *Ligusticum wallichii*, *etc.* Modern pharmacological studies have confirmed that phenolic acids have a variety of biological activities, including antioxidant, anti-inflammation, anti-coagulant, and hypolipidemic (114). Of note, numerous studies have demonstrated that phenolic acids have been shown to have a therapeutic effect on CVD (115, 116). Vanillic acid, a phenolic compound extracted from *Angelica sinensis*, could alleviate hypoxia/reoxygenation-induced H9c2 cardiomyocyte injury by inhibiting cell apoptosis and oxidative stress (117). Cinnamic acid is an active phenolic acid extracted from *Cinnamomi ramulus* that has a cardioprotective effect against myocardial ischemia-reperfusion injury by inhibiting NLRP3 inflammasome-mediated inflammation and cardiomyocyte pyroptosis (118). Shen et al. (119) showed that Salvianolic acid B can effectively inhibit ferroptosis and mitochondrial oxidative stress by activation of the Nrf2 pathway, thereby attenuating myocardial

TABLE 2 Summary of traditional Chinese medicine extracts in the prevention and treatment of various cardiovascular diseases from 2018-2023.

Extracts	Evaluation model	Effects and action mechanism	Ref.
Atherosclerosis			
Aqueous extracts of *Tribulus terrestris*	HFD-induced ApoE$^{-/-}$ mice ox-LDL/FBS-induced VSMCs	Liver weight and atherosclerotic plaque size↓ VSMC proliferation and migration↓ Akt/MEK/ERK pathway↓	(263)
Aqueous extracts of *Dendrobium catenatum*	High-cholesterol diet-induced zebrafish atherosclerosis model Low shear stress-induced endothelial cell dysfunction model	Atherosclerotic plaque size and macrophage infiltration↓ Levels of TC and TG↓ MDA content↓and SOD activity↑	(264)
Ethanol extracts of *Psoralea corylifolia*	HFD-induced LDLR$^{-/-}$ mice ox-LDL-induced HUVEC injury	Atherosclerotic lesion size and macrophage infiltration↓ Expression of VCAM-1 and ICAM-1↓and cholesterol efflux↑ PARγ-ABCA1/ABCG1 pathway↑and NF-κB pathway↓	(265)
Ethyl acetate extracts of *Caesalpinia sappan*	HFD-induced ApoE$^{-/-}$ mice	Macrophage infiltration and atherosclerotic lesion size↓	(266)
Methanol extracts of Ophiopogonis Radix	ox-LDL-induced mouse peritoneal macrophage cells	Levels of TG and TC↓ SOD, GSH-Px activities, and ABCA1 expression↑	(267)
Ethanol extracts of *Arctium lappa*	TNF-α-induced HUVEC injury	Cell viability and expression of IL-1β, TNF-α, IL-6↓ NF-κB pathway↓	(268)
Aqueous extracts of *Eucommia ulmoides*	HFD-induced ApoE$^{-/-}$ mice	Atherosclerotic lesion sizes and total cholesterol↓ Expression of TNF-α, IL-1β, MIF↓	(269)
Ethanol extracts of *Usnea diffracta*	HFD- and vitamin D3-induced atherosclerotic rat model	Atherosclerotic lesion sizes↓ Levels of TC, TG, LDL-c↓and HDL-c↑ AST and ALT activities and levels of TNF-α, IL-1β, MCP-1↓ TLR5/MyD88/NF-κB pathway↓	(270)
Ethanol extracts of *Ganoderma lucidum* spore	HFD-induced atherosclerotic rabbit model ox-LDL-induced THP-1 cells	Levels of TC, TG, LDL-c↓and HDL-c↑ Atherosclerotic lesion sizes and foam cell formation↓ Expression of LXRα, ABCA1 and ABCG1↑	(271)
Aqueous extracts of *Salvia miltiorrhiza*	HFD-induced ApoE$^{-/-}$ mice ox-LDL-induced HUVECs ox-LDL-induced RAW264.7 cells	Atherosclerotic lesion sizes and levels of TG and IL-6↓ Expression of p62↓and LC3B II↑ Foam cell formation↓	(272)
Ethanol extracts of *Salvia miltiorrhiza*	HFD-induced atherosclerotic rat model	Levels of TC, TG, LDL-c↓and HDL-c↑ Abundance of *Actinobacteriota* and *Proteobacteria*↑ Growth of *Firmicutes* and *Desulfobacterita*↓	(273)
Butanol extracts of *Acanthopanax senticosus*	HFD-induced ApoE$^{-/-}$ mice	Atherosclerotic lesion sizes↓ Levels of TC, TG, LDL-c↓and HDL-c↑ Levels of TNF-α, IL-1β, IL-6↓and NF-κB pathway↓	(274)
Ethanol extracts of *Edgeworthia gardneri*	HFD-induced ApoE$^{-/-}$ mice ox-LDL-induced macrophages and RAW264.7 cells	Atherosclerotic lesion sizes↓ Macrophage content in atherosclerotic plaque↓ Macrophage foam cell formation↓and CYP7A11 expression↑	(275)
Ethanol extract of *Schisandrae chinensis*	HFD-induced atherosclerotic rat model	Atherosclerotic lesion sizes↓ Levels of TG, LDL-c↓and HDL-c↑and Nrf2/HO-1 pathway↑	(276)
Myocardial ischemia-reperfusion injury			
Ethyl acetate extracts of *Cinnamomi Ramulus*	I/R-induced myocardial injury	LVEF and LVFS↑and expression of IL-1β, IL-6, TNF-α↓ NLRP3/Caspase-1 pathway↓	(107)
Ethanol extracts of *Origanum majorana*	I/R-induced myocardial injury LPS-treated aorta segments	Cardiac contractility (noradrenaline and endothelin-1)↓ Expression of IL-1β, IL-6↓and SOD-1↑	(113)

(Continued)

TABLE 2 Continued

Extracts	Evaluation model	Effects and action mechanism	Ref.
Myocardial ischemia-reperfusion injury			
Ethanol extracts of *Melissa officinalis*	I/R-induced myocardial injury	dp/dt max and dp/dt min values↑ Coronary venous effluent, collagen content, oxidative stress↓	(277)
Methanol extracts of *Galium verum*	I/R-induced myocardial injury	dp/dt max values and dp/dt min↑ Levels of TBARS, O^{2-}, H_2O_2↓and SOD, CAT activities↑	(278)
Methanol extracts of *Allium ursinum*	I/R-induced myocardial injury	dp/dt max values, dp/dt min, SLVP, SOD, CAT activities↑ Levels of TBARS, O^{2-}, H_2O_2↓	(279)
Ethanol extracts of *Cinnamomum zeylanicum*	I/R-induced myocardial injury	Myocardial infarct size and levels of cTnl, LDH, MDA↓ SOD, GSH, and CAT activities↑	(280)
n-butanol extract of *Potentilla anserina*	I/R-induced myocardial injury	Activities of GSH, SOD, CAT↑and MDA content↓ Apoptosis of cardiomyocyte↓	(281)
Methanol extracts of *Dunaliella salina*	I/R-induced myocardial injury	Myocardial infarct size, LDH level, number of neutrophils↓ dp/dt max, SLVP↑and TLR4/NF-κB pathway↓	(282)
Methanol extracts of *Taraxacum officinale*	I/R-induced myocardial injury	LDH and CK levels, myocardial infarct size↓ Activities of GSH and CAT↑	(283)
Aqueous extracts of *Crataegus persica*	I/R-induced myocardial injury in diabetic rats	Expression of Nrf2, DJ-1↑ Activities of GSH, SOD, CAT↑and MDA content↓	(284)
Ethanol extracts of *Melissa Officinalis*	I/R-induced myocardial injury	Myocardial infarct size, MDA content, LDH level↓ SOD activity↑	(285)
Ethanol extracts of *Pueraria lobata* and *Salvia miltiorrhiza*	I/R-induced myocardial injury	Myocardial infarct size and levels of CK and LDH↓ VEGFR2/ERK pathway↑	(286)
Ethanol extracts of *Salvia miltiorrhiza* and *Andrographis paniculata*	I/R-induced myocardial injury	Levels of IL-6, TNF-α, IL-1β, MCP-1, IL-33↓ NLRP3/ASC/Caspase-1 pathway↓	(287)
Heart failure			
Ethanol extracts of *Crataegus pinnatifida*	DOX-induced heart failure model	LVDs and LVDd↓and dp/dt max↑ Levels of BNP, CK-MB, IL-6, IL-1β, TNF-α↓ GSH-Px and CAT activity↑and MDA content↓	(288)
Ethanol extracts of *Ginkgo biloba*	LAD-induced heart failure model	Expression of IL-1β and TNF-α↓ LVEF and LVFS↑	(289)
Ethanol extracts of *Ophiopogon japonicus*	DOX-induced heart failure model	dp/dt max, LVEF, LVFS↑and LVDs, LVDd↓ Levels of CK-MB, LDH, AST, IL-6, IL-1β, TNF-α↓ Activities of SOD, GSH-Px, CAT↑and MDA content↓ p38 MAPK pathway↓	(290)
Alkaloid extracts of *Aconitum carmichaeli*	AAC-induced heart failure model	LVEF and LVFS↑and LVDs and LVDd↓ Levels of ANP, NT-proBNP, TNF-α↓ Expression of α-SMA and collagen I/III↓	(291)
Myocardial infarction			
Aqueous extracts of *Salvia miltiorrhiza*	LAD-induced myocardial infarction model	LVEF and LVFS↑and LVDs and LVDd↓ Levels of BNP, TNF-α, IL-1β↓ TLR4/TRAF6/NF-κB pathway↓	(292)
Ethanol extracts of *Schisandra chinensis*	ISO-induced myocardial infarction model	LDH, CK levels↓and SOD, GSH-Px, CAT activities↑ Nrf2/HO-1 pathway↑	(293)
Aqueous extracts of *Spinacia oleracea*	ISO-induced myocardial infarction model	Levels of LDH, CK-MB, IL-6, TNF-α, TC, TG↓ Activities of SOD, CAT, GSH-Px and GR↑	(294)

(Continued)

TABLE 2 Continued

Extracts	Evaluation model	Effects and action mechanism	Ref.
Myocardial infarction			
Aqueous extracts of *Gentianella acuta*	ISO-induced myocardial infarction model	Levels of LDH, CK, IL-6, TNF-α↓ TLR4/MyD88/NF-κB pathway↓	(295)
Methanol extracts of *Agrimonia pilosa*	ISO-induced myocardial infarction model	Levels of CK-MB, LDH, CK↓ ROS generation and MDA levels↓and SOD activity↑ PI3K/Akt pathway↑	(296)
Ethanol extracts of *Syringa pinnatifolia*	LAD-induced myocardial infarction model Hypoxia-induced H9c2 cell injury	Levels of CK-MB, LDH, and inflammatory cell infiltration↓ p53-mediated apoptotic pathway↓	(297)
Ethanol extracts of *Anchusa italica*	LAD-induced acute myocardial infarction model	LVEF and LVFS↑and LVDs and LVDd↓ Myocardial infarct size and levels of TNF-α, IL-1β, IL-6↓ PI3K/Akt/mTOR pathway↓	(298)
Hypertension			
Aqueous extracts of *Whitmania pigra*	Spontaneously hypertensive rats Ang II-induced H9c2 cells	LVEF and LVFS↑and LVDs and LVDd↓ Blood pressure↓and expression of collagen I/III, TGF-β↓ H9c2 cell viability↑and p38/JNK pathway↓	(299)
Aqueous extracts of *Momordica charantia*	High salt-induced hypertension	MAP, SBP, MDA content↓and activities of CAT and SOD↑	(300)
Ethanol extracts of *Plantago asiatica*	Spontaneously hypertensive rats	MAP, SBP, collagen deposition↓ LVEF and LVFS↑and LVDs and LVDd↓	(301)
Aqueous extracts of *Eriobotrya japonica*	Spontaneously hypertensive rats Ang II-induced H9c2 cells	LVEF and LVFS↑ GATA4-NFATc3 pathway↓	(302)
Aqueous extracts of *Chimonanthus salicifolius*	Spontaneously hypertensive rats	LDL-c, TC, TG levels↓and HDL-c level↑and ERS↓	(303)
Others			
Aqueous extracts of *Salvia miltiorrhiza*	HFD-fed db/db mice High glucose-induced VSMCs	Plaque area and ROS generation↓ Expression of KLF10 and HO-1↓and cell viability↓	(304)
Ethanol extracts of *Plantago asiatica*	ISO-cardiac hypertrophy ISO-induced H9c2 cells	Collagen deposition and expression of BNP, ANP, β-MHC↓ Cardiomyocyte apoptosis↓	(106)
Ethanol extracts of *Lycium chinense*	HFD/streptozotocin-induced diabetic cardiomyopathy	Blood glucose and levels of TG, AST, LDH, CK-MB↓ Expression of IL-6, IL-1β, TNF-α↓ MDA content↓and activities of CAT, GSH-Px, SOD↑ p53-mediated apoptotic pathway and NF-κB pathway↓	(305)
Aqueous extracts of *Arnebiae Radix*	Acetylcholine and $CaCl_2$-induced atrial fibrillation	AF duration↓and induction time of AF↑ Atrial fibrosis, α-SMA, and collagen I expression↓ LVFS↑and atrial enlargement (LAD, LA area)↓	(306)
Aqueous extracts of *Dendrobium candidum*	ISO-induced cardiac hypertrophy model ISO-induced H9c2 cells	LVSP, Heart body/body weight ratio, LV/TL ratio↓ Serum levels of ANP and BNP↓ Collagen deposition and ERK pathway↓	(307)
Ethanol extracts of *Smilax glabra*	TAC-induced cardiac hypertrophy model ISO-induced H9c2 cells	Myocardial fibrosis and collagen content↓ Expression of ANP, BNP, β-MHC, NT-proBNP↓ Raf/MEK/ERK pathway↓	(308)
Ethanol extracts of *Centella asiatica*	ISO-induced cardiac hypertrophy model ISO-induced atrial cardiomyocytes	Heart/body weight ratio↓and levels of AST, BNP, ANP↓ Collagen content, cardiac fibrosis, expression of TNF-α, IL-6↓ MDA content↓and SOD expression↑ PI3K/Akt pathway↑and NF-κB pathway↓	(309)

(Continued)

TABLE 2 Continued

Extracts	Evaluation model	Effects and action mechanism	Ref.
Others			
Aqueous extracts of *Angelica sinensis* and *Hedysarum polybotrys*	X-irradiation-induced myocardial fibrosis X-irradiation-induced cardiac fibroblasts	Myocardial fibrosis↓and TGF-β1 expression↓ Cardiac fibroblast apoptosis↓ Expression of miR-21, collagen 1α, c-Jun, OPN↓	(310)
Aqueous extracts of *Salvia miltiorrhiza* and *Carthamus tinctorius*	HFD/streptozotocin-induced diabetic cardiomyopathy Sodium palmitate-treated H9c2 cells	Glucose level↓and insulin level↑ Cardiomyocyte cross-sectional↓and LVFS↑ Levels of BNP and cell apoptosis↓	(311)

AAC, Abdominal aortic coarctation surgery; ANP, Atrial natriuretic peptide; BNP, Brain natriuretic peptide; dp/dt min, Minimum rate of left ventricular pressure development; dp/dt max, Maximum rate of left ventricular pressure development; GSH, glutathione; LA, left atrium; LAD, Left atrial diameter; LVEDP, Left ventricular end-diastolic pressure; LV/TL, Left ventricular weight/tibia length; LVSP, Left ventricular systolic pressure; SLVP, Systolic left ventricular pressure.
↑ upregulated, ↓ downregulated.

infarction. Another study reported that ferulic acid ameliorated atherosclerotic injury by modulating gut microbiota and lipid metabolism (120), as evidenced by reducing the relative abundance of *Erysipelotrichaceae* and *Firmicutes* and increasing the relative abundance of *Ruminococcaceae*, as well as downregulating serum levels of total cholesterol, triglyceride, and low-density lipoprotein cholesterol and atherogenic index in HFD-fed ApoE$^{-/-}$ mice. In addition, we summarized many phenolic acids such as caffeic acid, protocatechuic acid, chlorogenic acid, gallic acid, benzoic acid, and erucic acid for the treatment and prevention of CVD, which are listed in Table 3.

3.3.2 Flavonoids

Flavonoids are secondary metabolites widely found in TCM and have various pharmacological activities that are beneficial to human health (121), such as antioxidant, anti-apoptosis, anti-inflammation, antitumor, *etc.* Of note, many studies have found that flavonoid compounds can play an effective protective role in the treatment of CVD (122). Functionally, scutellarin, a flavonoid compound extracted from *Erigeron breviscapus*, possessed protective effects against cardiac hypertrophy (123), diabetic cardiomyopathy (124), atherosclerosis (125), myocardial ischemia-reperfusion injury (126), and myocardial infarction (127) via inhibition of inflammation, oxidative stress, and apoptosis. Baicalein extracted from *Scutellaria baicalensis* inhibited Ang II/oxidized low-density lipoprotein-induced inflammation via inactivation of the AMPK/NF-κB pathway, thus showing anti-atherosclerotic activity (128). Wogonin, one of the main flavonoid compounds of *Scutellaria radix*, ameliorated isoproterenol-induced myocardial infarction via suppression of inflammation and oxidative stress (129). Naringenin was the main flavonoid that existed in various citrus fruits, bergamots, and tomatoes. Naringenin treatment inhibited myocardial ischemia-reperfusion-induced inflammation, lipid peroxidation, and ferroptosis by activating the Nrf2/GPX4 pathway (130). Naringenin suppressed blood pressure, cholesterol triglycerides, LDL, serum malondialdehyde (MDA), and nitric oxide, as well as increased serum superoxide dismutase and glutathione via blocking the STAT3 pathway in obesity-associated hypertension (131). Abukhalil et al. (132) reported that galangin, a natural flavonoid found in lesser galangal and honey, exerted a protective effect on diabetic cardiomyopathy by reduction of oxidative stress, inflammation, and hyperglycemia. Last but not least, pinocembrin belongs to this series of flavonoids and exerts an antioxidant effect on heart failure by activating the Nrf2/HO-1 pathway, evidenced by reducing ROS level in heart tissue and serum MDA level and improving cardiac function (133). Taken together, flavonoids possess a range of biological activities that prevent the development and progression of CVD, and their potential mechanisms are summarized in Table 3.

3.3.3 Stilbenes

Stilbenes are compounds with a stilbene parent structure connected by a vinyl group between two benzene rings and have a typical conjugated structure. Stilbenes are widely found in TCM, including *Polygonum cuspidatum* and *Polygonum multiflorum*, and have beneficial effects on human health. Resveratrol, a main compound extracted from *Polygonum cuspidatum*, can prevent myocardial ischemia-reperfusion injury by inhibition of oxidative stress and ferroptosis (134). Maayah et al. (135) found that resveratrol treatment inhibited cardiac NLRP3 inflammasome activation and reduced inflammatory responses, and thus alleviated doxorubicin-induced cardiomyopathy. Another study showed that resveratrol protects against atherosclerosis by reducing TMAO levels and enhancing hepatic bile acid biosynthesis through the remodeling of intestinal flora (136). Polydatin, an active component in *Polygonum cuspidatum*, can ameliorate acute myocardial infarction-induced cardiac damage by inhibition of oxidative stress and cell apoptosis via activation of the Nrf2/HO-1 pathway (137). Zhang and colleagues (138) confirmed that polydatin can inhibit inflammation and pyroptosis by blocking the NLRP3/caspase-1 pathway and triggering mTOR-mediated autophagy, thereby exerting an anti-atherosclerosis effect. 2,3,4',5-tetrahydroxystilbene 2-O-β-D-glucoside (TSG) is extracted and purified from *Polygonum multiflorum*, which can prevent the development and progression of atherosclerosis by reducing lipid accumulation and inflammation in ApoE$^{-/-}$ mice fed with HFD (139). These results suggested that stilbenes exhibited therapeutic effects on CVD via different mechanisms (Table 3).

3.3.4 Anthraquinones

Anthraquinones are compounds with unsaturated cyclic diketone structures and are widely found in some Chinese herbal medicines (140). Accumulating studies have shown that anthraquinones

TABLE 3 Summary of traditional Chinese medicine compounds in the prevention and treatment of various cardiovascular diseases from 2018-2023.

Compound	cardiovascular diseases (model)	Biological activity	Ref.
Phenolic acids			
Salvianolic acid A	Atherosclerosis (animal and cellular models)	Anti-pyroptosis and anti-inflammation	(312)
	Myocardial infarction (animal and cellular models)	Anti-apoptosis	(313)
	Diabetic cardiomyopathy (animal model)	Improving mitochondrial function and anti-apoptosis	(314)
	Hypertension (animal and cellular models)	Anti-apoptosis	(315)
Salvianolic acid B	Atherosclerosis (cellular model)	Anti-inflammation, anti-pyroptosis, and anti-ERS	(316)
	Myocardial ischemia-reperfusion injury (animal and cellular models)	Anti-ferroptosis, anti-apoptosis, antioxidant, and anti-inflammation	(317, 318)
	Myocardial infarction (animal model)	Anti-ferroptosis	(119)
	Uremic cardiomyopathy (animal model)	Anti-inflammation and anti-fibrosis	(319)
	Diabetic cardiomyopathy (animal and cellular models)	Angiogenesis	(320)
Chlorogenic acid	Heart failure (animal model)	Anti-inflammation, antioxidant, and anti-apoptosis	(321)
	Myocardial infarction (animal model)	Anti-inflammation and anti-oxidative stress	(322)
	Hypertension (animal model)	Modulation of gut microbiota	(323)
	Diabetic cardiomyopathy (animal and cellular models)	Anti-ERS and anti-apoptosis	(324)
Gallic acid	Atherosclerosis (animal model)	Modulation of gut microbiota	(325)
	Heart failure (animal and cellular models)	Activation of autophagy and anti-fibrosis	(326, 327)
	Atrial fibrillation (animal model)	Inhibiting immunoproteasome	(328)
	Hypertension (animal model)	Antioxidant	(329)
	Cardiac hypertrophy (animal model)	Antioxidant	(330)
Syringic acid	Myocardial ischemia-reperfusion injury (animal model)	Anti-apoptosis	(331)
	Cardiac hypertrophy (animal model)	Anti-fibrosis	(332)
	Diabetic cardiomyopathy (animal model)	Antioxidant	(333)
Caffeic acid	Atherosclerosis (animal model)	Anti-inflammation	(334)
	Hypertension (animal model)	Antioxidant	(335)
	Cardiac remodeling (animal and cellular models)	Anti-fibrosis	(336)
Punicalagin	Atherosclerosis (cellular model)	Anti-inflammation	(337)
	Myocardial ischemia-reperfusion injury (animal model)	Antioxidant and anti-apoptosis	(338)
	Diabetic cardiomyopathy (animal and cellular models)	Improving mitochondrial function	(339)
Ferulic acid	Atherosclerosis (animal model)	Modulation of gut microbiota	(120)
	Myocardial ischemia-reperfusion injury (animal model)	Anti-ferroptosis and antioxidant	(340)
	Heart failure (animal model)	Antioxidant and anti-apoptosis	(341)
	Myocardial infarction (cellular model)	Activation of autophagy	(342)
	Diabetic cardiomyopathy (animal model)	Modulation of gut microbiota and anti-apoptosis	(343)
Cinnamic acid	Atherosclerosis (animal model)	Antioxidant	(344)
	Myocardial ischemia-reperfusion injury (animal model)	Anti-inflammation and anti-pyroptosis	(118)
	Cardiomyopathy (animal and cellular models)	Antioxidant, anti-inflammation, and anti-dyslipidemia	(345, 346)

(Continued)

TABLE 3 Continued

Compound	cardiovascular diseases (model)	Biological activity	Ref.
Flavonoids			
Formononetin	Atherosclerosis (cellular model)	Anti-inflammation and antioxidant	(347)
	Myocardial ischemia-reperfusion injury (animal model)	Anti-inflammation and antioxidant	(348)
	Myocardial infarction (animal model)	Anti-inflammation	(349)
	Hypertension (animal model)	Anti-inflammation	(350)
Baicalein	Atherosclerosis (cellular model)	Anti-inflammation	(128)
	Myocardial ischemia-reperfusion injury (cellular model)	Antioxidant	(351)
	Hypertension (cellular model)	Anti-fibrosis and anti-inflammation	(352)
	Cardiac hypertrophy (animal model)	Antioxidant and activation of autophagy	(353)
	Diabetic cardiomyopathy (animal model)	Antioxidant and anti-inflammation	(354)
Baicalin	Atherosclerosis (animal model)	Anti-inflammation	(355)
	Myocardial ischemia-reperfusion injury (animal and cellular models)	Anti-ferroptosis and anti-inflammation	(356, 357)
	Cardiac hypertrophy (animal model)	Activation of the SIRT3 pathway	(358)
	Cardiomyopathy (animal model)	Anti-inflammation	(359)
	Hypertension (animal model)	Modulation of gut microbiota	(360)
Hesperidin	Atherosclerosis (animal model)	Anti-inflammation	(361)
	Myocardial ischemia-reperfusion injury (animal model)	Inhibition of autophagy	(362)
	Cardiac hypertrophy (animal model)	Anti-inflammation, anti-apoptosis, and antioxidant	(363)
Hyperoside	Atherosclerosis (cellular model)	Anti-inflammation	(364)
	Myocardial ischemia-reperfusion injury (animal model)	Antioxidant	(365)
	Myocardial infarction (animal model)	Anti-inflammation	(366)
	Heart failure (animal model)	Anti-apoptosis and activation of autophagy	(367)
Puerarin	Atherosclerosis (cellular model)	Anti-inflammation and antioxidant	(368)
	Myocardial ischemia-reperfusion injury (animal and cellular models)	Anti-ferroptosis and anti-inflammation	(369)
	Heart failure (animal and cellular models)	Anti-apoptosis and anti-inflammation	(370)
	Cardiac hypertrophy (animal model)	Activation of PPARα/PGC-1 pathway	(371)
	Hypertension (animal model)	Antioxidant	(372)
	Myocardial infarction (animal model)	Anti-apoptosis	(373)
	Diabetic cardiomyopathy (animal and cellular models)	Anti-inflammation	(374)
Quercetin	Atherosclerosis (cellular model)	Anti-inflammation and activation of autophagy	(375)
	Myocardial ischemia-reperfusion injury (animal and cellular models)	Anti-apoptosis	(376)
	Diabetic cardiomyopathy (animal model)	Anti-inflammation	(377)
	Myocardial infarction (animal model)	Anti-fibrosis	(378)
	Atrial fibrillation (animal and cellular models)	Anti-fibrosis	(379)
Kaempferol	Atherosclerosis (animal model)	Antioxidant	(380)

(Continued)

TABLE 3 Continued

Compound	cardiovascular diseases (model)	Biological activity	Ref.
Flavonoids			
	Heart failure (animal model)	Antioxidant and anti-inflammation	(381)
	Diabetic cardiomyopathy (animal model)	Antioxidant	(382)
Naringenin	Atherosclerosis (animal model)	Anti-inflammation, activation of autophagy, and anti-ERS	(383, 384)
	Myocardial ischemia-reperfusion injury (animal and cellular models)	Anti-ferroptosis, antioxidant, and anti-inflammation	(130, 385)
	Hypertension (animal model)	Antioxidant	(131)
	Cardiac hypertrophy (animal and cellular models)	Antioxidant	(386)
	Diabetic cardiomyopathy (animal model)	Antioxidant, anti-inflammation, and anti-apoptosis	(387)
Tilianin	Atherosclerosis (cellular model)	Anti-inflammation	(388)
	Myocardial ischemia-reperfusion injury (animal model)	Antioxidant, anti-apoptosis, and anti-inflammation	(389, 390)
	Diabetic cardiomyopathy (animal and cellular models)	Antioxidant and anti-inflammation	(391)
Biochanin A	Atherosclerosis (animal and cellular models)	Anti-inflammation	(392)
	Myocardial ischemia-reperfusion injury (animal model)	Anti-inflammation	(393)
	Diabetic cardiomyopathy (animal model)	Antioxidant	(394)
	Myocardial infarction (animal model)	Anti-inflammation	(395)
Hydroxysafflor Yellow A	Atherosclerosis (animal model)	Anti-inflammation	(396)
	Myocardial ischemia-reperfusion injury (animal model)	Activation of autophagy and anti-inflammation	(397)
	Diabetic cardiomyopathy (animal model)	Antioxidant	(398)
	Cardiac hypertrophy (animal model)	Antioxidant	(399)
Xanthohumol	Atherosclerosis (cellular model)	Modulation lipid metabolism	(400)
	Myocardial ischemia-reperfusion injury (animal model)	Anti-ferroptosis	(401)
	Cardiac hypertrophy (animal model)	Anti-fibrosis	(402)
Dihydromyricetin	Atherosclerosis (animal model)	Anti-inflammation	(403)
	Myocardial ischemia-reperfusion injury (animal and cellular models)	Improving mitochondrial function and antioxidant	(404)
	Cardiomyopathy (animal model)	Anti-inflammation and antioxidant	(405)
	Cardiac hypertrophy (animal model)	Antioxidant	(406)
Acacetin	Atherosclerosis (animal model)	Antioxidant and anti-inflammation	(407)
	Myocardial ischemia-reperfusion injury (animal model)	Antioxidant, anti-inflammation, and anti-apoptosis	(408)
	Cardiac hypertrophy (animal model)	Anti-inflammation, antioxidant, and anti-apoptosis	(409)
	Diabetic cardiomyopathy (animal and cellular models)	Antioxidant	(410)
	Hypertension (animal model)	Improving mitochondrial function	(411)
Icariin	Atherosclerosis (animal and cellular models)	Anti-apoptosis	(412)
	Myocardial ischemia-reperfusion injury (cellular model)	Antioxidant and anti-ferroptosis	(413)
	Myocardial infarction (animal model)	Immunomodulatory	(414)
	Atrial fibrillation (animal model)	Improving mitochondrial function	(415)
	Hypertension (animal model)	Antioxidant	(416)
	Cardiac hypertrophy (cellular model)	Activation of autophagy	(417)
	Diabetic cardiomyopathy (animal model)	Improving mitochondrial function and anti-fibrosis	(418)

(Continued)

TABLE 3 Continued

Compound	cardiovascular diseases (model)	Biological activity	Ref.
Flavonoids			
Scutellarin	Atherosclerosis (animal model)	Anti-apoptosis	(125)
	Myocardial ischemia-reperfusion injury (animal and cellular models)	Anti-inflammation and anti-apoptosis	(126)
	Cardiac hypertrophy (cellular model)	Anti-inflammation	(123)
	Diabetic cardiomyopathy (animal model)	Anti-apoptosis, anti-inflammation, and antioxidant	(124, 419)
	Myocardial infarction (animal model)	Antioxidant, anti-apoptosis, and anti-inflammation	(127)
Morin	Atherosclerosis (cellular model)	Anti-inflammation and activation of autophagy	(420)
	Myocardial ischemia-reperfusion injury (animal model)	Antioxidant	(421)
Epigallocatechin-3-gallate	Myocardial ischemia-reperfusion injury (animal model)	Antioxidant and anti-inflammation	(422)
	Heart failure (animal model)	Antioxidant	(423)
	Myocardial infarction (animal model)	Anti-apoptosis and anti-inflammation	(424)
	Hypertension (animal model)	Antioxidant	(425)
	Cardiac hypertrophy (cellular model)	Improving mitochondrial function and anti-fibrosis	(426, 427)
	Diabetic cardiomyopathy (animal model)	Anti-fibrosis	(428)
	Atrial fibrillation (animal model)	Anti-fibrosis	(429)
Stilbenes			
Resveratrol	Atherosclerosis (cellular model)	Anti-inflammation	(430)
	Myocardial ischemia-reperfusion injury (cellular model)	Anti-ferroptosis, improving mitochondrial function, and antioxidant	(134, 431)
	Heart failure (patients with heart failure)	Anti-inflammation	(432)
	Myocardial infarction (animal model)	Antioxidant, anti-inflammation, and anti-ferroptosis	(433, 434)
	Hypertension (animal model)	Antioxidant, anti-inflammation, and modulation of gut microbiota	(435, 436)
	Cardiac hypertrophy (animal model)	Antioxidant and activation of autophagy	(437)
	Diabetic cardiomyopathy (animal model)	Antioxidant	(438)
	Atrial fibrillation (animal model)	Anti-apoptosis and anti-fibrosis	(439)
Polydatin	Atherosclerosis (animal model)	Anti-inflammation, antioxidant, and activation of autophagy	(138, 440)
	Myocardial infarction (cellular model)	Antioxidant	(137)
	Cardiomyopathy (animal model)	Improving mitochondrial function and antioxidant	(441)
Raloxifene	Atherosclerosis (animal model)	Anti-inflammation	(442)
	Heart failure (animal model)	Anti-inflammation and antioxidant	(443)
Anthraquinones			
Emodin	Myocardial ischemia-reperfusion injury (cellular model)	Anti-inflammation and anti-pyroptosis	(444)
	Heart failure (animal model)	Anti-apoptosis	(445)
	Cardiac hypertrophy (animal model)	Anti-fibrosis	(446)
Aloe-emodin	Atherosclerosis (animal model)	Activation of autophagy	(150)
	Myocardial infarction (animal model)	Anti-apoptosis and anti-fibrosis	(151)
	Hypertension (animal and cellular models)	Anti-inflammation	(152)
Kanglexin	Atherosclerosis (animal and cellular models)	Hypolipidemic	(447)

(Continued)

TABLE 3 Continued

Compound	cardiovascular diseases (model)	Biological activity	Ref.
Anthraquinones			
	Myocardial ischemia-reperfusion injury (animal model)	Anti-inflammation and anti-pyroptosis	(448)
Saponins			
Astragaloside IV	Atherosclerosis (cellular model)	Anti-inflammation, antioxidant, and anti-apoptosis	(157, 449)
	Myocardial ischemia-reperfusion injury (animal model)	Antioxidant and anti-apoptosis	(450)
	Heart failure (animal model)	Angiogenesis	(451)
	Myocardial infarction (animal and cellular models)	Anti-inflammation, angiogenesis, and anti-pyroptosis	(155, 452)
	Hypertension (animal model)	Anti-inflammatory and antioxidant	(453)
	Diabetic cardiomyopathy (animal model)	Anti-ferroptosis, antioxidant, and activation of autophagy	(454, 455)
Ginsenoside Rb1	Atherosclerosis (cellular model)	Antioxidant and anti-inflammation	(456)
	Myocardial ischemia-reperfusion injury (animal and cellular models)	Antioxidant and improving mitochondrial function	(457)
	Heart failure (animal model)	Improving mitochondrial function	(458)
	Diabetic cardiomyopathy (animal model)	Antioxidant, anti-apoptosis, anti-fibrosis, and anti-inflammation	(459)
Ginsenoside Rb2	Atherosclerosis (animal and cellular models)	Anti-inflammation	(460)
	Myocardial ischemia-reperfusion injury (animal model)	Anti-inflammation and antioxidant	(461)
Notoginsenoside R1	Atherosclerosis (cellular model)	Anti-inflammation, anti-apoptosis, and antioxidant	(462)
	Myocardial ischemia-reperfusion injury (animal model)	Anti-apoptosis	(463)
	Cardiomyopathy (animal and cellular models)	Anti-apoptosis, antioxidant, and anti-fibrosis	(464)
	Cardiac hypertrophy (animal model)	Anti-inflammation	(465)
Terpenoids			
Tanshinone IIA	Atherosclerosis (animal model)	Anti-inflammation and anti-pyroptosis	(466)
	Myocardial ischemia-reperfusion injury (animal model)	Antioxidant, anti-inflammation, and anti-apoptosis	(467)
	Myocardial infarction (animal model)	Antioxidant	(468)
	Diabetic cardiomyopathy (cellular model)	Anti-ERS and anti-oxidative stress	(469)
	Cardiac fibrosis (animal model)	Anti-fibrosis and antioxidant	(470)
Paeoniflorin	Atherosclerosis (cellular model)	Anti-apoptosis and activation of autophagy	(163)
	Myocardial ischemia-reperfusion injury (animal model)	Antioxidant and anti-apoptosis	(471)
	Heart failure (animal model)	Anti-fibrosis	(472)
	Hypertension (animal model)	Anti-inflammation and antioxidant	(473)
Catalpol	Atherosclerosis (cellular model)	Anti-inflammation, antioxidant, and anti-ERS	(474)
	Myocardial ischemia-reperfusion injury (animal and cellular models)	Antioxidant and anti-inflammation	(475)
	Hypertension (cellular model)	Anti-inflammation	(476)
	Diabetic cardiomyopathy (animal model)	Anti-apoptosis	(477)
Crocin	Atherosclerosis (animal model)	Anti-inflammation	(478)
	Myocardial ischemia-reperfusion injury (animal and cellular models)	Anti-ERS	(479)
	Myocardial infarction (animal model)	Anti-inflammation	(480)

(Continued)

TABLE 3 Continued

Compound	cardiovascular diseases (model)	Biological activity	Ref.
Terpenoids			
	Hypertension (animal model)	Antioxidant	(481)
	Diabetic cardiomyopathy (animal model)	Activation of autophagy and anti-apoptosis	(482)
Ginkgolide B	Atherosclerosis (animal model)	Modulation of gut microbiota, anti-inflammation, and antioxidant	(483, 484)
	Myocardial ischemia-reperfusion injury (cellular model)	Anti-inflammation and anti-apoptosis	(485, 486)
	Myocardial infarction (animal model)	Anti-inflammation	(487)
	Cardiac hypertrophy (cellular model)	Activation of autophagy	(488)
	Diabetic cardiomyopathy (animal model)	Antioxidant and anti-fibrosis	(489)
Lycopene	Atherosclerosis (animal model)	Inhibition of cholesterol and antioxidant	(490)
	Myocardial ischemia-reperfusion injury (cellular model)	Improving mitochondrial function, anti-apoptosis, and anti-ERS	(491, 492)
	Cardiac hypertrophy (animal and cellular models)	Antioxidant and improving mitochondrial function	(493)
Artemisinin	Atherosclerosis (animal model)	Anti-inflammation and antioxidant	(494, 495)
	Myocardial ischemia-reperfusion injury (animal model)	Anti-inflammation	(165)
	Hypertension (animal model)	Antioxidant	(496)
	Diabetic cardiomyopathy (animal model)	Anti-inflammation and anti-fibrosis	(497)
Oridonin	Atherosclerosis (animal model)	Anti-inflammation and antioxidant	(498)
	Myocardial ischemia-reperfusion injury (animal model)	Anti-inflammation and anti-pyroptosis	(499)
	Myocardial infarction (animal model)	Anti-inflammation and anti-fibrosis	(500)
	Cardiac hypertrophy (animal and cellular models)	Activation of autophagy	(501)
Alkaloids			
Berberine	Atherosclerosis (animal model)	Modulation of gut microbiota	(502)
	Myocardial ischemia-reperfusion injury (animal and cellular models)	Anti-inflammation, antioxidant, and anti-apoptosis	(503, 504)
	Heart failure (animal model)	Improving mitochondrial function	(505)
	Myocardial infarction (animal model)	Anti-inflammation	(506)
	Hypertension (animal model)	Modulation of gut microbiota	(507)
	Cardiac hypertrophy (animal and cellular models)	Activation of autophagy	(508)
	Diabetic cardiomyopathy (cellular model)	Anti-inflammation	(509)
Colchicine	Atherosclerosis (cellular model)	Anti-inflammation and anti-pyroptosis	(510)
	Heart failure (animal model)	Anti-inflammation	(511)
	Cardiomyopathy (animal and cellular models)	Anti-inflammation	(512)
	Myocardial infarction (animal model)	Anti-inflammation	(513)
Sinomenine	Atherosclerosis (animal model)	Anti-inflammation and antioxidant	(514)
	Myocardial ischemia-reperfusion injury (animal model)	Anti-apoptosis, anti-inflammation, antioxidant	(515)
	Heart failure (animal model)	Anti-fibrosis and anti-inflammation	(516)
	Cardiac hypertrophy (animal and cellular models)	Antioxidant and anti-inflammation	(517)
Nuciferine	Atherosclerosis (animal model)	Anti-apoptosis and activation of MMP12/Akt pathway	(518)
	Myocardial ischemia-reperfusion injury (animal model)	Anti-apoptosis and activation of PPAR-γ	(519)

(Continued)

TABLE 3 Continued

Compound	cardiovascular diseases (model)	Biological activity	Ref.
Alkaloids			
	Myocardial infarction (animal model)	Anti-inflammation	(520)
Polysaccharides			
Dendrobium huoshanense	Atherosclerosis (zebrafish model)	Antioxidant and anti-inflammation	(521)
Laminaria japonica	Atherosclerosis (animal model)	Modulation of gut microbiota	(522)
Cordyceps militaris	Atherosclerosis (animal model)	Improving hyperlipidemia	(523)
Undaria pinnatifida	Atherosclerosis (animal model)	Anti-inflammation	(524)
Cipangopaludina chinensis	Atherosclerosis (animal model)	Modulation of gut microbiota	(525)
Poria cocos	Atherosclerosis (animal model)	Anti-inflammation	(526)
Lycium barbarum	Atherosclerosis (animal model)	Modulation of gut microbiota	(527)
	Myocardial ischemia-reperfusion injury (animal model)	Improving mitochondrial function and antioxidant	(528)
	Cardiac hypertrophy (animal model)	Anti-inflammation	(529)
Schisandra chinensis	Cardiac hypertrophy (animal model)	Antioxidant	(530)
Chuanminshen violaceum	Myocardial ischemia-reperfusion injury (animal model)	Anti-ferroptosis	(531)
Polygonatum sibiricum	Heart failure (animal model)	Antioxidant, anti-inflammation, and anti-apoptosis	(532)
Astragalus membranaceus	Heart failure (animal model)	Anti-inflammation	(533)

↑ upregulated, ↓ downregulated.

havevarious biological activities, including antitumor, antioxidant, and anti-inflammation (141), *etc.* Emodin (1,3,8-trihydroxy-6-methylanthraquinone), a natural anthraquinone derivative, can be extracted and purified from natural plants such as *Rhei* radix et rhizoma, *Polygoni Cuspidat*, *Polygoni multiflori*, which protects against various CVDs (142). Previous studies have demonstrated that emodin exhibited a therapeutic effect on atherosclerosis via inhibition of inflammatory response (143), suppression of PPAR-γ-mediated lipid metabolism (144) and endothelial cell apoptosis (145), reducing oxidative stress (146). Other studies found that emodin can prevent cardiac hypertrophy (147), restrict vasodilation by activation of K^+-ATP channels (148), and inhibition of myocardial fibrosis (149). Aloe-emodin is an active ingredient in *Rheum palmatum* and *Aloe vera*, which prevents the progression of various CVDs. For example, Tang et al. (150) reported that aloe-emodin exerted an anti-atherosclerosis effect by reducing atherosclerotic plaque in the aorta and lipid accumulation and promoting endothelial autophagy. Yu et al. (151) showed that aloe-emodin inhibited the development of cardiac fibrosis and hypertrophy in rats with chronic myocardial infarction by suppressing cardiac apoptosis and oxidative stress via the inactivation of the TGF-β/Smad pathway. Another study found that aloe-emodin exhibited specific therapeutic value in hypertension-related CVD by inhibiting NLRP3 inflammasome activation (152). Moreover, other anthraquinone compounds have protective effects against CVD, which is summarized in Table 3.

3.3.5 Saponins

Saponins are a class of glycosides with triterpenoids or steranes, which are widely found in natural plants and have been reported to have many pharmacological activities, including antitumor, anti-inflammation, anti-oxidative stress, *etc.* Importantly, previous studies have shown that saponins were shown to be effective in treating CVD (Table 3) (153), such as atherosclerosis, myocardial infarction, myocardial ischemia-reperfusion injury, heart failure, cardiomyopathy, and hypertension. Astragaloside IV (AS-IV) is the main active ingredient purified from *Astragalus membranaceus* and serves as an effective therapeutic agent for the treatment of CVD (154). For example, AS-IV could markedly reduce myocardial infarction-induced myocardial fibrosis, cardiac hypertrophy, and macrophage pyroptosis by inhibition of the ROS/caspase-1/GSDMD pathway (155). Yin et al. (156) showed that AS-IV protects against myocardial ischemia-reperfusion injury by suppressing cardiomyocyte apoptosis and serum cardiac troponin levels via blocking CaSR/$ERK_{1/2}$ and the related apoptotic pathways. Another study found that AS-IV treatment suppressed inflammation, plaque area, and serum lipids in HFD-induced atherosclerosis by blocking the MAPK/NF-κB pathway (157). Other studies proved that AS-IV can attenuate the progression of myocardial fibrosis (158), heart failure (159), and cardiac hypertrophy (160) by inhibiting Nrf2-mediated oxidative stress. Ginsenosides (mainly including the ginsenosides Rb1, Rb2, Rb3, Rc,

Rd, Re, Rg3, and Rh2 and compound K) serve as the main active constituents of *Panax ginseng* and exert protection against CVD by suppression of oxidative stress, cholesterol accumulation, inflammation, and insulin resistance (161).

3.3.6 Terpenoids

Terpenoids are a large group of organic compounds present in TCM and can be effectively used for treating various diseases. Importantly, the preventive and therapeutic effects of terpenoids on CVD have received increasing attention (Table 3), which was associated with their remarkable biological activities, such as anti-inflammation, antioxidant, and anti-apoptosis. Tanshinone IIA, a fat-soluble component of *Salvia miltiorrhiza*, could protect against heart failure by inhibition of cardiomyocyte apoptosis via activating the AMPK/mTOR-mediated autophagy pathway (162). Paeoniflorin, a bioactive component extracted from *Paeonia lactiflora*, can ameliorate ox-LDL-induced atherosclerosis by inhibiting apoptosis and adhesion molecule expression via autophagy enhancement in human umbilical vein endothelial cells (163). Andrographolide, a bioactive labdane diterpenoid extracted from *Andrographis paniculate*, exhibited anti-oxidative stress capacity against adverse cardiac remodeling after myocardial infarction by activating the Nrf2/HO-1 pathway (164). Artemisinin, a sesquiterpene lactone compound with peroxisome bridging group structure purified from *Artemisia annua*, prevented myocardial ischemia-reperfusion injury by inhibition of cardiac autophagy and NLRP3 inflammasome activation (165). Taken together, terpenoids may serve as an effective therapeutic agent for the treatment of various CVDs by different mechanisms.

3.3.7 Alkaloids

Alkaloids are a class of nitrogen-containing basic organic compounds and widely found in TCM. Of note, alkaloids exert protective effects against CVDs by suppression of inflammation, oxidative stress, and cardiomyocyte apoptosis (Table 3). Berberine, a natural isoquinoline alkaloid isolated from *Rhizoma coptidis*, possessed profound pharmacological activities for the treatment of various CVDs (166), including atherosclerosis, cardiac hypertrophy, heart failure, myocardial infarction, and arrhythmia. Similarly, palmatine was a potential candidate drug for the treatment of cardiac hypertrophy by activating the Nrf2/ARE pathway (167). Matrine, a quinolizidine alkaloid derived from *Sophora flavescens*, could attenuate diabetic cardiomyopathy by reducing inflammatory cytokines levels and oxidative stress (168). Cyclovirobuxine D, a steroidal alkaloid extracted from *Buxus microphylla*, exerted a cytoprotective effect against HFD diet- and streptozotocin-induced rat diabetic cardiomyopathy by activating Nrf2-mediated antioxidant responses (169). Cordycepin is an active ingredient in *Cordyceps sinensis* that can prevent myocardial ischemia-reperfusion injury by activating the AMPK/mTOR-mediated autophagy (170). Colchicine, a botanical alkaloid derived from *Colchicum autumnale*, exerted unique anti-inflammatory effects in the therapy of various CVDs (171), including atherosclerosis, heart failure, atrial fibrillation, and myocardial infarction.

3.3.8 Polysaccharides

Polysaccharides widely exist in natural plants, which are a kind of complex structure of natural polymer compounds (172). Currently, natural polysaccharides are attracting considerable attention worldwide due to their versatile biological activities and few side effects. Of note, numerous studies have shown that bioactive polysaccharides exhibit profound efficiency in controlling the risk factors of CVD (173), such as inflammatory response, oxidative stress, hypertension, and hyperlipidemia. Polysaccharides derived from *Gelidium crinale* reduced oxidative stress and inflammation in oxidized low-density lipoprotein-induced atherosclerosis (174). Huang et al. (175) found that the administration of polysaccharides from *Eriobotrya japonica* effectively reduced oxidative damage and inflammation induced by myocardial ischemia-reperfusion injury. Astragalus polysaccharides could ameliorate diabetic cardiomyopathy progression by improving cardiac function and inhibiting cardiomyocyte apoptosis via the inactivation of the ERS pathway (176). *Lycium barbarum* polysaccharides could reduce the levels of inflammatory cytokines (e.g., IL-6 and TNF-α) and plasma lipid peroxidation in a pressure overload-induced heart failure rat model (177). In addition, polysaccharides extracted from TCM, such as *Polygonatum sibiricum*, *Opuntia dilleniid*, *Plantago asiatica*, *Angelica sinensis*, and *Ganoderma lucidum*, also have therapeutic effects on various CVDs (Table 3).

3.3.9 Others

In addition to the above-mentioned compounds isolated from TCM for the prevention of CVD, other active ingredients in TCM have been reported to have therapeutic effects on various CVDs. Schisandrin B, bioactive dibenzocyclooctadiene derivatives found in *Schisandra chinensis*, could alleviate diabetic cardiomyopathy by reducing cardiac inflammation and damage via blocking MyD88-dependent inflammation (178). Schisandrin B prevented hypoxia/reoxygenation-induced cardiomyocyte injury by inhibiting inflammation and oxidative stress, which was associated with the activation of the AMPK/Nrf2 pathway (179). Morronisid, an iridoid glycoside extracted from *Cornus officinalis*, promoted angiogenesis and improved cardiac function in rats with acute myocardial infarction (180). Sulforaphane is a natural glucosinolate found in *Raphanus sativus*, which inhibited cardiac cell ferroptosis by activating the AMPK/Nrf2 pathway (76). Schisandrol A, a bioactive lignan extracted from *Schisandra chinensis*, could inhibit cardiomyocyte apoptosis induced by myocardial ischemia-reperfusion via increasing 14-3-3θ expression (181). Collectively, natural compounds from TCM exert anti-CVD effects, which may be developed as an effective therapeutic agent for the treatment of CVD in clinical.

4 Clinical study of the TCM for the prevention and treatment of CVD

Accumulating evidence has reported that TCM has a wide range of pharmacological effects in various CVDs and its beneficial

efficacy has been proved *in vitro* cell models or animal experiments. Importantly, several clinical studies are underway to explore the safety and efficacy of TCM decoction and injections for the treatment of various CVDs. For example, several studies provided a reliable evaluation of the efficacy and safety of Xuefu Zhuyu granules (182) and Xuefu Zhuyu granules (183) in the treatment of patients with coronary heart disease. Other randomized controlled trials similarly analyzed the efficacy and safety of Zhuling decoction (184) and Buyang Huanwu decoction (185) in the treatment of heart failure. A multicenter, randomized, double-blind, placebo-controlled clinical trial found that Qing-Xin-Jie-Yu granule reduced inflammation and cardiovascular endpoint in patients with coronary heart disease (186). A phase I clinical trial by Hu et al. (187) showed that Danhong injection promoted endothelial progenitor cell mobilization by increasing the expression of Akt, eNOS, and MMP-9 in patients with coronary heart disease. Lai et al. (97) found that treatment with TCM formula (Songling Xuemaikang capsule) improved blood pressure in patients with mild hypertension and was well tolerated. Another study confirmed that astragalus injection was a safe and effective therapeutic agent in the clinical management of heart failure (188). In addition, several clinical trials have shown that the combination of TCM and standard drugs for CVD treatment was advantageous to simple conventional Western medicine in relieving clinical symptoms (25, 189). Chao et al. (190) reported that TCM formula combined with Western medicine reduced blood lipid levels and inflammatory factors in patients with coronary heart disease. Zhang et al. (191) showed that modified Xiaojianzhong decoction combined with conventional Western medicine alleviated the progression of chronic heart failure by improving heart function and maintaining gastrointestinal hormones. Another study found that treatment with Jianpi Huazhi pill combined with Western medicine (anti-heart failure) led to decreasing the levels of inflammatory cytokines and improving the composition of the gut microbiota (192). Meanwhile, several clinical studies are completed or ongoing to evaluate the safety and efficacy of TCM combined with Western medicine for the treatment of CVD according to Chinese Clinical Trial Registry (Table 4). Many researchers have proved that treatment with TCM based on the standard drug not only prevented CVD progression and improved quality of life but also

TABLE 4 The ongoing clinical trials of traditional Chinese medicine combined with Western medicine for cardiovascular diseases therapy from 2018-2023.

No.	Disease	Interventions	Status	Sponsor	Clinical Trial ID
1	Atherosclerosis	Tongxinluo capsule+CWM	Completed	Qilu Hospital of Shandong University	ChiCTR1900025842
2	Atherosclerosis	Xiaochaihu decoction+CWM	Not recruiting	Shanghai Sixth People's Hospital	ChiCTR2000032470
3	Atherosclerosis	Yanshi Jiangzhi formula+CWM	Not recruiting	Shanghai Tenth People's Hospital	ChiCTR2000036785
4	Atherosclerosis	Yishen Huazhuo decoction+CWM	Not recruiting	Longhua Hospital Shanghai University of Traditional Chinese Medicine	ChiCTR2300071014
5	Atherosclerosis	Huoxue Jiedu formula+CWM	Recruiting	Xiyuan Hospital, Chinese Academy of Traditional Chinese Medicine	ChiCTR2300074283
6	Atherosclerosis	Huazhuo Tiaozhi granule+CWM	Not recruiting	Guang'anmen Hospital, China Academy of Chinese Medical Sciences	ChiCTR2400079454
7	Myocardial ischemia-reperfusion injury	Shenxiang Suhe pill+CWM	Recruiting	Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University	ChiCTR2200055170
8	Heart failure	Yiqihuoxuelishui formula+CWM	Recruiting	Dongfang Hospital Affiliated to Beijing University of Chinese Medicine	ChiCTR1900022036
9	Heart failure	Yangyin Shuxin formula+CWM	Completed	The First Affiliated Hospital of Tianjin University of Traditional Chinese Medicine	ChiCTR2000030921
10	Heart failure	LuHong formula+CWM	Not recruiting	Shuguang Hospital Affiliated to Shanghai University of traditional Chinese Medicine	ChiCTR2000037368
11	Heart failure	Qiangxin formula+CWM	Recruiting	Shanghai Hospital of Traditional Chinese Medicine	ChiCTR2000037254
12	Heart failure	Shenfu Xiangshao decoction+CWM	Not recruiting	Shanghai Putuo District Central Hospital	ChiCTR2000036639
13	Heart failure	Shen'ge formula+CWM	Not recruiting	Longhua Hospital affiliated to Shanghai University of Traditional Chinese Medicine	ChiCTR2000036533
14	Heart failure	Shenshao pill+CWM	Recruiting	The First Teaching Hospital of Tianjin University of Traditional Chinese Medicine	ChiCTR2100042242
15	Heart failure	Shenge powder+CWM	Not recruiting	Nanxiang Hospital	ChiCTR2100049790

(Continued)

TABLE 4 Continued

No.	Disease	Interventions	Status	Sponsor	Clinical Trial ID
16	Heart failure	Yixin formula+CWM	Not recruiting	Yueyang Hospital of Integrated Traditional Chinese and Western Medicine Affiliated to Shanghai University of Traditional Chinese Medicine	ChiCTR2100051882
17	Heart failure	Fangji Huangqi decoction+CWM	Recruiting	The Second Affiliated Hospital of Tianjin University of Traditional Chinese Medicine	ChiCTR2100054580
18	Heart failure	Xin-Li-Fang formula+CWM	Not recruiting	The Second Affiliated Hospital of Guangzhou University of Chinese Medicine (Guangdong Provincial of Chinese Medicine)	ChiCTR2200058649
19	Heart failure	Kangxin formula+CWM	Not recruiting	The First Affiliated Hospital of Guangzhou University of Chinese Medicine	ChiCTR2300069435
20	Heart failure	Yangxinxue granules+CWM	Not recruiting	Qionglai Hospital of Traditional Chinese Medicin	ChiCTR2300074840
21	Heart failure	Shexiang Baoxin pill+CWM	Not recruiting	Sichuan Provincial People's Hospital	ChiCTR2300076014
22	Heart failure	Yiqi Huayu decoction+CWM	Recruiting	Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine	ChiCTR2400082425
23	Heart failure	Qiwei Fangji Huangqi granule+CWM	Not recruiting	Hangzhou Traditional Chinese Medicine Hospital	ChiCTR2400080029
24	Hypertension	Bushen Jiangya granule+CWM	Recruiting	Guang′anmen Hospital, China Academy of Chinese Medical Sciences	ChiCTR1900028572
25	Hypertension	Shugan Wendan decoction+CWM	Not recruiting	Guangzhou University of Chinese Medicine	ChiCTR2000034557
26	Hypertension	Dingxuan Shuyu formula+CWM	Completed	Shuguang Hospital Affiliated to Shanghai University of Chinese Medicine	ChiCTR2000040386
27	Hypertension	Chaigui decoction+CWM	Completed	Wuxi Hospital of Traditional Chinese Medicine	ChiCTR2300076783
28	Hypertension	Huoxue Qiyang Qutan prescription+CWM	Recruiting	Shanghai Yueyang Integrated Traditional Chinese Medicine and Western Medicine Hospital	ChiCTR2400081580
29	Myocardial infarction	Qishen Yiqi drop pill+CWM	Not recruiting	The Second Affiliated Hospital of Tianjin University of Traditional Chinese Medicine	ChiCTR2000029136
30				Peking University First Hospital	ChiCTR2300069035
31	Myocardial infarction	Shexiang Tongxin drop pill+CWM	Recruiting	Beijing University of Chinese Medicine Dongzhimen Hospital	ChiCTR2300075069
32	Septic cardiomyopathy	Fuling Sini decoction+CWM	Recruiting	Beijing University of Chinese Medicine Shenzhen Hospital (Longgang)	ChiCTR2100045549
33	Combined blood stasis with dilated cardiomyopathy	Kuoxinfang granule+CWM	Recruiting	Longhua Hospital, Shanghai University of Traditional Chinese Medicine	ChiCTR2100049536
34	Coronary artery disease	Shexiang Baoxin pill+CWM	Recruiting	Gansu Provincial Hospital	ChiCTR2400080152

CWM, conventional Western medicine.

reduced the incidence of adverse cardiovascular events in patients (193–195). More interestingly, TCM may be an effective alternative method to Western medicine in modern American healthcare, but some barriers prevent its integration into Western health systems, such as the fact that TCM is not accredited by the American Board of Medical Specialties, available TCM therapies may impose an undesired burden for patients, and TCM therapies are individualized. However, no cardiovascular drug or combination of drugs has shown significant efficacy in all patients with CVD, and standard Western medicine can lead to adverse side effects. From an economic point of view, TCM therapies are cheaper than Western medicine and have a better prognosis for patients with CVD. Based on the current situation, TCM may be an attractive alternative for patients with CVD.

5 Conclusion and prospects

As the leading cause of death after malignant tumors, CVD is difficult to treat clinically and imposes a huge economic and health burden on people worldwide. The morbidity and mortality of CVD are continuously increasing, and the treatment is ineffective because

of its complex pathogenesis. In recent years, TCM has been particularly prominent in the treatment of 95 certain diseases, including CVD, offering a new perspective in the modern era for the prevention and treatment of diseases such as COVID-19. In this review, we found that TCM (formulas, extracts, and compounds) can combat CVD through multiple mechanisms, including anti-inflammatory, antioxidant, improving mitochondrial dysfunction, anti-cell death (such as autophagy, apoptosis, ferroptosis, pyroptosis), and regulating gut microbiota. Meanwhile, clinical trials have proven the efficacy and safety of TCM in alleviating the symptoms of CVD. However, there are still some challenges that must be overcome in TCM for CVD treatment. (1) With the rapid advancement of science, there is a need to utilize network pharmacology approaches and multi-omics technologies, such as nutrigenomics, metabolomics, proteomics, gut microbial macrogenomics and immunomics, to reveal the physiological functions and mechanism explanations of TCM in combating CVD; (2) The metabolic, toxicity, and pharmacokinetic profiles of TCM fight against patients with CVD in clinical trials need to be further validated; (3) The construction of TCM resources for common quality standards to ensure active ingredient in TCM; (4) Research on active ingredients of TCM is limited by defects includes unstable chemical structure, low bioavailability and easy oxidation, and liposome embedding or nanoparticle formulation can be considered; (5) Development of CVD models with human disease characteristics for exploring the pharmacological activity of TCM, such as primate animal models that can avoid species barriers leading to ineffectiveness; (6) Designing TCM delivery systems to improve its stability, bioavailability, and efficacy in the gastrointestinal tract.

In conclusion, TCM possesses good anti-CVD effects and is an indispensable active drug for the treatment of CVD. Based on the latest evidence, this review summarized the pathogenesis of CVD and systematically analyzed and discussed the mechanisms of TCM in preventing CVD, as well as its clinical trials. This review aims to provide a scientific and effective comprehensive reference for TCM in CVD therapy and to better utilize and develop the treasures of TCM.

Author contributions

JD: Conceptualization, Investigation, Writing – original draft. LQ: Investigation, Writing – original draft. YL: Writing – review & editing. ML: Funding acquisition, Writing – review & 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.

Publisher's note

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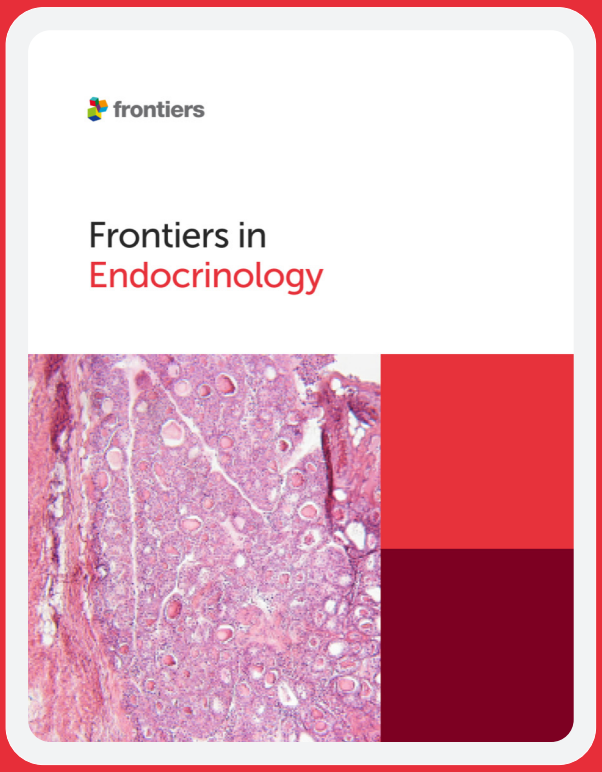

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