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Novel and emerging therapies for the treatment of obesity and related disorders

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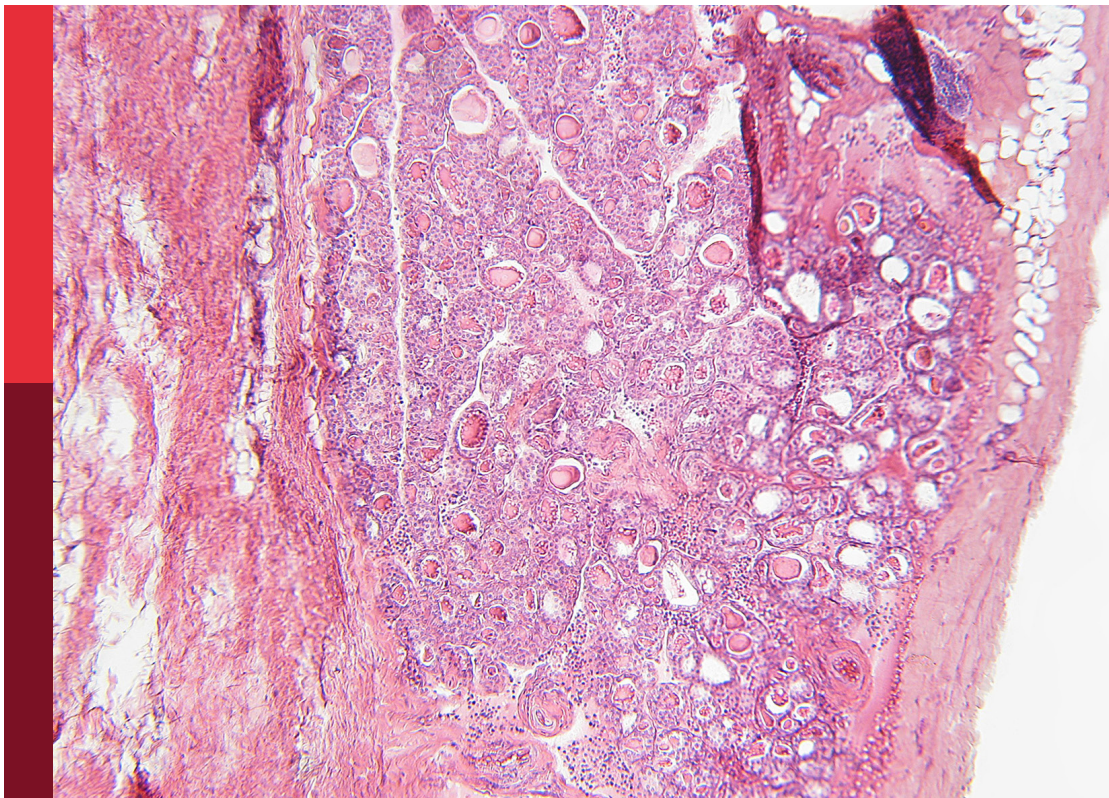

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Novel and emerging therapies for the treatment of obesity and related disorders

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Editorial: Novel and emerging therapies for the treatment of obesity and related disorders

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Editorial on the Research Topic
Novel and emerging therapies for the treatment of obesity and related disorders

Obesity and its associated comorbidities represent a significant burden for healthcare systems worldwide. The current Research Topic includes studies dealing with various topics relevant to obesity, from novel and revolutionary therapies to the diagnosis and physiological characterization of the disease; which contribute to improve our understanding of this important medical challenge.

Assessment of body composition in obesity

Obesity is defined by excessive adipose tissue mass, and people are classified as obese when their body mass index (BMI) is over 30 kg/m^2. However, using BMI as a diagnostic criterion has limitations, as it does not provide information about the subjects' body composition. Computed tomography (CT) at the lumbar area can assess body composition with great accuracy; however, it is an expensive and time-consuming process that requires highly trained individuals. Dual-energy X-ray absorptiometry is less laborious but tends to underestimate fat mass in obese subjects. Palmas et al. eveloped a model able to analyze CT images in an automated way, based on artificial intelligence. Although similar approaches have been utilized to evaluate body composition in cancer patients, it is the first time a tool of this kind was designed for obese subjects as the target population. As a result, the new model was more precise at assessing fat and fat-free mass in obese individuals. These findings will simplify the analysis of body composition in obesity, while still maintaining the inherent advantages of CT, including accurate evaluation of visceral and subcutaneous fat as well as sarcopenia.

Obesity and insulin resistance

Obesity results from an imbalance between energy consumption and expenditure, and it is commonly associated with increased insulin resistance. However, how insulin resistance develops in the context of obesity remains to be clarified. Cooper et al. evaluated glucose fluctuations in obese subjects with normoglycemia by continuous glucose monitoring (CGM). Despite the consumption of a high carbohydrate diet, the daily glucose profile of those individuals was comparable to healthy controls. However, the obese subjects showed significantly higher insulin levels and HOMA-IR; suggesting that enhanced insulin release might be secondary to alterations induced by excess adiposity rather than a direct consequence of acute changes in glycemia, although the potential role of incretins could not be discounted. Moreover, the study indicates that fasting insulin and HOMA-IR values are more valuable than CGM in managing obesity from a clinical perspective.

Other pathologic consequences of obesity

Nonalcoholic fatty liver disease (NAFLD) represents one of the most frequent comorbidities associated with obesity. Jiang et al. explored the role of extracellular vesicles (EVs), released by different tissues, as signaling factors in NAFLD; revealing their significant impact on the pathology of the disease. The authors found that EV abundance and content change dynamically through the different stages of NAFLD, indicating their potential as stage-specific markers. The study also highlights EVs as promising drug delivery systems with low immunogenicity and high biocompatibility. Overall, it suggests that research on EVs in NAFLD shows potential for future diagnostic and therapeutic applications. Obesity also increases the risk of venous thromboembolism (VTE). Low-molecular-weight heparin (LMWH) is commonly used for the treatment of VTE. However, LMWH dosage is the same across different weight groups, including obese subjects, although a low distribution into adipose tissue and total body clearance has been reported in these patients as compared to normal-weight individuals. Liu et al. conducted a systematic review and meta-analysis to explore the appropriate dosage of LMWH for preventing and treating VTE in patients with obesity. They concluded that a higher dosage of LMWH in patients with obesity can reduce the incidence of VTE without increasing the risk of bleeding.

Life-style intervention mimics in the treatment of obesity

Life-style interventions are the first line of therapy for the treatment of obesity; however, this approach often fails to maintain health benefits in the long-term due to low adherence. Herein, Yi et al. reviewed the current clinical and preclinical data on the multiple metabolic benefits promoted by β-aminoisobutyric acid (BAIBA), a novel myokine released by skeletal muscle in response to physical activity. Such benefits include stimulating glucose uptake by peripheral tissues, improving glucose homeostasis, regulating hepatic lipid metabolism, reducing steatosis, and increasing browning and diminishing white adipose tissue depots, all of them highly desirable in the context of obesity. Accordingly, BAIBA is presented as an exercise mimic that could be used as therapy in obese subjects who cannot stick to a consistent exercise regime.

Intestinal inflammation, gut microbiota and obesity

Bariatric surgery can achieve remarkable weight loss. However, it is a highly invasive intervention only recommended for severely obese patients who fail to lose weight after trying other forms of therapy. Active research is going on to elucidate how bariatric surgery elicits its metabolic benefits. In this regard, Cao et al. investigated the effects of sleeve gastrectomy (SG) on colonic inflammation in a model of diet-induced obesity. The authors described substantial reductions in inflammatory markers and increments in markers of intestinal barrier integrity in response to SG.

Moreover, they demonstrated that depletion of gut microbiota highly attenuated these changes, emphasizing the relevant role of the microbiome in the metabolic consequences of bariatric surgery. In addition, Wang et al. studied the hypoglycemic, antioxidant, and anti-inflammatory activity of polysaccharides from the fungus *Cordyceps cicadae* in diabetic mice, also focusing on their effect on the intestinal microbiota. Mice treated with these polysaccharides showed reduced blood glucose levels and antioxidant and anti-inflammatory responses, as confirmed by alleviated tissue damage compared to the control. The microbiota of treated mice had reduced Firmicutes/Bacteroidetes ratio and significantly decreased *Helicobacter* and *Lactobacillus* compared to the diabetic group. Interestingly, the hypoglycemic effects of these polysaccharides were linked to metabolites produced by certain microbiota species increased in the gut of the treated group, highlighting the interest in these natural compounds for treating diabetes. Related to this, Zhang et al. reviewed the evidence linking intestinal barrier dysfunction with the development of systemic inflammation and metabolic alterations. The authors summarized the different strategies available to improve the integrity of the intestinal barrier and how these interventions may affect obesity and other metabolic diseases. Overall, these three studies highlight the importance of preserving intestinal barrier integrity and reducing intestinal inflammation in the context of obesity. Although certain drugs and natural compounds such as metformin, berberine and butyrate have shown promising effects in preclinical studies, more clinical investigation is needed. Importantly, interventions to regulate the composition of intestinal flora, such as oral supplementation with prebiotics, probiotics and postbiotics are currently under consideration for the treatment of obesity.

Brown adipose tissue as target for the treatment of obesity

Since its identification in humans, activation of BAT and induction of thermogenesis have received much attention as potential strategies for maintaining body weight and improving the metabolic alterations often associated with obesity. However, it remains controversial whether activation of the limited amount of BAT in humans could be sufficient to trigger metabolic effects. Zhu et al. explored the provocative idea of BAT or engineered thermogenic cell transplantation to improve metabolic abnormalities. The authors reviewed the preclinical data on brown fat transplants and the *in vitro* generation and subsequent implantation of thermogenic cells. They also assessed the current severe limitations of this technique that preclude the adoption of such intervention at this point, including the progressive loss of the thermogenic phenotype in the implanted cells. More research on this area is warranted to improve the viability of this strategy, while some ideas on how to advance in this direction are already presented in the manuscript.

Conclusion

Historically, pharmacotherapy of obesity has enjoyed limited success because of modest weight-loss effects as well as important side effects such as addiction, cardiovascular events and gastrointestinal complications. Remarkably, novel and revolutionary therapies based on GLP-1 derivative drugs can induce body weight reduction by up to 24% (1), highlighting the importance of innovation for the generation of effective anti-obesity medications. Despite the dramatic effects of these drugs, they are not well-tolerated by a considerable fraction of the patients, and discontinuation of the treatment is associated with a remarkable rebound in body weight. Thus, the search for novel medications is still highly relevant. This Research Topic covers a wide range of potential anti-obesity therapies including exercise mimics, BAT transplantation, microbiota-based strategies, drugs to reduce intestinal inflammation as well as the use of extracellular vesicles as delivery method.

Author contributions

IA: Writing – original draft, Writing – review & editing. MP: Writing – original draft, Writing – review & editing. PF: Writing – original draft, Writing – review & editing. MG: Writing – original draft, Writing – review & editing.

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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Continuous glucose monitoring reveals similar glycemic variability in individuals with obesity despite increased HOMA-IR

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Background/aims: Continuous glucose monitoring is a well-tolerated and versatile tool for management of diabetes and metabolic disease. While its use appears to be feasible to monitor glycemic profiles in diabetics, there is a paucity of data in individuals with obesity and normal glucose tolerance. The aim of this study is to investigate glucose fluctuations and insulin resistance patterns in normoglycemic participants with obesity vs. without obesity and contextualize these results against leading models for obesity.

Materials and methods: We designed a prospective, observational pilot study of two cohorts including 14 normoglycemic participants with obesity and 14 normoglycemic participants without obesity. Participants were monitored with continuous glucose monitoring (CGM) for five consecutive days. Insulin resistance levels were measured and glucometric data were extracted from CGM for all participants.

Results: Fasting serum insulin and homeostasis model assessment of insulin resistance (HOMA-IR) were significantly higher in the group with obesity ($P < 0.05$). While the group with obesity had a higher mean blood glucose (MBG), mean amplitude of glycemic excursions (MAGE), and continuous overall glycemic action-1 h (CONGA-1), these differences were not significant. On univariate linear regression, insulin resistance (HOMA-IR) was associated with body mass index (BMI), waist circumference (WC), cohort with obesity, cohort consuming a high glycemic diet, hemoglobin A1c (HbA1c), and fasting insulin levels. WC and fasting insulin levels remained predictors of HOMA-IR in our multivariable model.

Conclusion: While there is much excitement surrounding the use of commercial CGM products in obesity management, our results suggest that fasting insulin and HOMA-IR values may be more clinically useful than CGM data alone.

KEYWORDS

continuous glucose monitoring, glycemic variability, insulin resistance, obesity, obesity management, HOMA-IR

Introduction

The obesity epidemic is a significant public health challenge as the proportion of American adults who are overweight or obese continues to increase (1). The exact etiology of the obesity epidemic remains a matter of debate. An improved understanding and identification of key triggers could result in better prevention and treatment strategies.

Fundamentally, obesity corresponds to excess adiposity. As lipids are anhydrous, they are an efficient means of storing excess energy in a relatively small area. At its core, obesity is the result of excess energy storage and reduced fat oxidation for cellular energy use. This storage process is tightly monitored by the central nervous system, regulated by the hypothalamus, the most important region involved in energy homeostasis (2, 3). Studies have shown either ablation or stimulation of different areas within the hypothalamus can result in hyperphagia or hypophagia due to disruption of the hypothalamic-adipose axis (4–6).

The idea of caloric intake exceeding energy expenditure represents the Energy Balance model for obesity, whereby the brain acts like a thermostat receiving input from numerous sensory pathways and reflexively up-regulates or down-regulates energy intake and expenditure to achieve homeostasis (7, 8).

While weight loss programs have targeted the century-old concept of reducing total caloric intake and increasing expenditure, weight management is not as simple as balancing a checkbook. When intake declines, the body responds by becoming more efficient and utilizing fewer calories; when activity is increased, appetite is increased to consume a greater amount of food (9, 10).

A second model for obesity, the Carbohydrate-Insulin model, posits that a high-carbohydrate diet drives post-prandial hyperinsulinemia, leading to increased fat storage. This occurs instead of oxidation by metabolically active tissues and predisposes to weight gain through increased hunger and a slowed metabolic rate (7, 8). Stated simply, this model prioritizes *what* is eaten, not simply total caloric value, in precipitating hyperinsulinemia.

Increased consumption of carbohydrates with high glycemic indices increases insulin secretion. The increased insulin drives nutrients into fat cells, leaving fewer nutrients for other tissues and stimulating increased food intake. Rather than a central model, the development of obesity occurs through peripheral mechanisms (11).

In line with this theory, modern diets including increased processed, high glycemic-load foods cause hormonal changes that lead to insulin production and promote adiposity (12). Animal studies have shown dietary composition is demonstrated to affect metabolism independent of caloric intake. Rodents fed high vs. low glycemic load diets controlled for macronutrients (carbohydrate, fat, and protein) produce a sequential series of endocrine dysfunction involving hyperinsulinemia, increased adipocyte diameter and other anabolic changes, including greater adiposity, lower energy expenditure, and increased hunger (7, 13–15).

Continuous Glucose Monitoring (CGM), which measures users' glucose concentrations in the interstitial fluid, has made a profound impact on the management of diabetes (16, 17). CGM has been shown to improve quality of glycemic control and quality of life by avoiding multiple finger-sticks, reduce risk of hypoglycemia, obtain far more accurate readings, and enable lower target levels for mean glucose and hemoglobin A1c (HbA1c) for patients with diabetes (18–21).

CGM technology is increasingly cost-effective as factory-calibrated, disposable monitors are now publicly available (22). As a result, many have hypothesized that CGM could function as a dietary Fitbit^{TM} and potentially alter behavior and intake of high glycemic foods.

For advocates of the Carbohydrate Insulin model, the argument is simple: as glucose spikes drive insulin, reducing glucose levels will lower insulin secretion and result in better weight control. Thus preventing glucose spikes and using real-time glycemic biofeedback may offer a more optimized, personalized, and effective weight loss program.

While the thought process is compelling, there is currently little evidence to support CGM usage in individuals with obesity and normal glucose tolerance. This study was undertaken to investigate the clinical utility of CGM—using indicators such

as mean amplitude of glycemic excursions (MAGE), standard deviation of blood glucose (SDBG), mean of daily differences (MODD), and continuous overlapping net glycemic action over 1 h (CONGA-1) as indices for glycemic variability—in normoglycemic adults with obesity (BMI > 30) as compared to a group of normoglycemic adults without obesity (BMI < 30).

Using CGM data from these two groups may offer insight into the plausibility of the Energy Balance model for obesity versus the Carbohydrate-Insulin model. If the Carbohydrate-Insulin model best reflects the etiology of obesity, individuals with obesity should have increased glucose variability with higher amplitude glucose spikes and lower nadirs. But if rising insulin resistance is a consequence of excess adiposity, nutrient overload, and caloric imbalance, we would expect there to be a difference in fasting insulin and insulin resistance, but little difference in glucose variability. Rising insulin would be a compensatory response to regulate glucose. Further, if there is no significant difference in glycemic variability between the two groups, the use of CGM for the treatment of obesity may be less clinically beneficial in this study population than those marketing CGM technology have suggested.

We present a pilot study of 28 participants in which we compare (i) MAGE as a proxy for glycemic variability and (ii) levels of insulin, insulin resistance, and glucometric data extracted from CGM in a cohort of adults with obesity vs. without obesity.

Materials and methods

Study design

We performed a prospective, single institution, observational study from June 2020 to May 2021 of 14 adults with obesity (BMI > 30) and 14 adults without obesity (BMI < 30) who were all between 18 and 50 years of age. Participants were healthy adults, primarily recruited from family, neighbors, and patients seen in the bariatric surgery clinic. The inclusion criteria consisted of the following: (i) participants were clinically stable with no known chronic illness that might affect glucose metabolism including history of hypertension, dyslipidemia, coronary artery disease, or cerebral stroke; (ii) participants had point-of-care HbA1c values <5.7% (39 mmol/mol). Exclusion criteria included (i) previous history of bariatric surgery, and use of antihypertensive, anti-diabetic, thiazide diuretic, or cholesterol-lowering medications; (ii) female participants pregnant at the time of study enrollment; (iii) participants with known hepatic or renal dysfunction. The study was conducted within the Northwell Health Department of Bariatric Surgery after approval by institutional review board. Study participants provided written informed consent prior to study participation.

Anthropometric measurements

Anthropometric measurements, including height, weight, and waist circumference (WC) were obtained as patients were enrolled. BMI was calculated by dividing weight (kg) by height squared (m^2). The average BMI in the group with obesity was 38.4 kg/m^2 and the average BMI in the group without obesity was 23.7 kg/m^2.

Laboratory examinations

Prior to implantation, fasting glucose and fasting insulin were measured to render a HOMA-IR score for each participant. HOMA-IR scores were calculated by [fasting glucose (mg/dl) × fasting insulin (μU/ml)]/405. HbA1c values were also measured.

Continuous glucose monitoring

All participants were equipped with *i*Pro2 continuous glucose recorder (Medtronic, Northridge, CA, USA). On day 0, a CGM sensor (Enlite Sensor) was inserted into the subcutaneous abdominal fat tissue and calibrated according to standard Medtronic operating guidelines. The *i*Pro2 continuous glucose recorder measures subcutaneous tissue interstitial glucose levels continuously, recording values every 5 min, within a range of 40–400 mg/dl. With the *i*Pro2 CGM inserted, patients checked their blood glucose levels three times daily with a OneTouch® Verio™ Flex meter (LifeScan, Malvern, PA, USA) to calibrate the *i*Pro2 continuous glucose sensor. On day 5, the participants returned to the research site, and the monitors were removed. The recorded data, including range, SD, glycemic variability indexes, and mean blood glucose were downloaded with Medtronic's CareLink System and stored in a secure Northwell REDCap database for further analysis. **Figure 1** shows an example of aggregate data from a 24-h period of CGM.

Diet assessments

Participants were asked to keep detailed food logs by entering all foods consumed during the study period into the *i*Pro2 myLog cellphone app, as well as capture corresponding photos of all foods consumed. Participants followed an *ad libitum* diet. Total net carbs were estimated by two Registered Dietitians for each participant, and diets were then classified as being high or low in total net carbs. Carbohydrate information for each meal submission was obtained from the Carb Manager app (Wombat Apps LLC, Redmond, WA, USA). Diets classified as "high" contained the highest proportion of carbohydrate

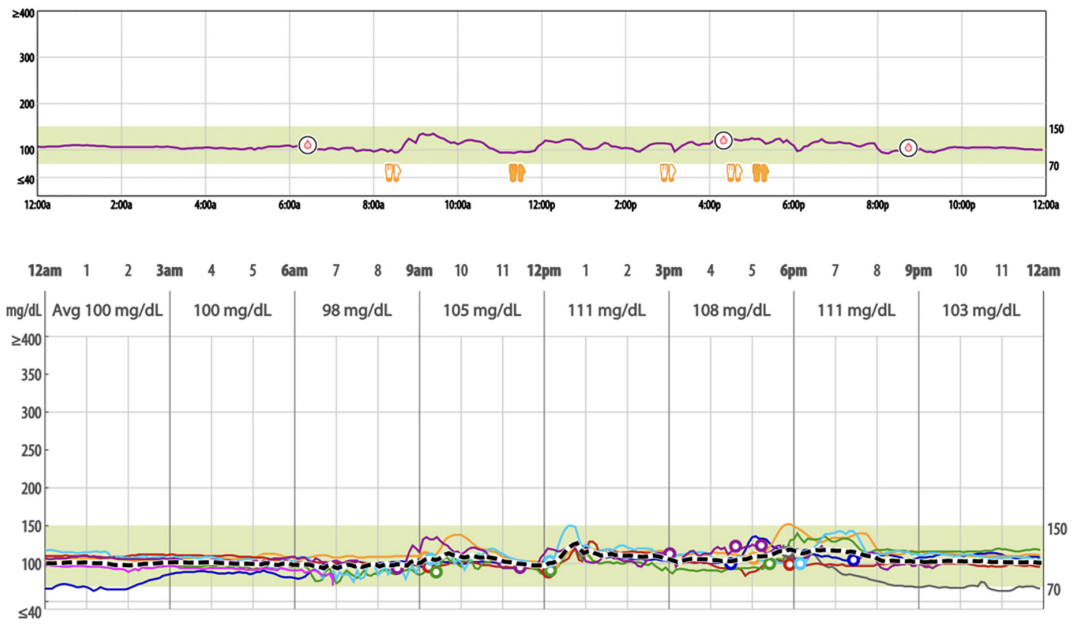

FIGURE 1
Example of a 24-hour period of continuous glucose monitoring with iPro2 and overlayed weeklong sensor data (mg/dl).

intake from refined sources. Diets classified as "low" included the highest proportion of fiber from vegetables and whole foods. Post-prandial peaks for each meal were recorded and average peaks for each participant with obesity vs. without obesity were analyzed to determine if meals of certain glycemic load were associated with blood sugar peaks.

Assessment of glycemic variability

Glycemic variability was calculated using EasyGV version 9.0.R2 (© University of Oxford). The metrics generated from EasyGV in this analysis are mean of daily differences (MODD), mean amplitude of glycemic excursion (MAGE), continuous overlapping net glycemic action over 1 h (CONGA1), and the standard deviation of the glucose values (SDBG).

Statistical analyses

All analyses were performed using the SPSS 16.0 statistical software for Windows (SPSS, Chicago, IL, USA). Values are shown as mean with standard deviation. Fasting glucose, fasting insulin, HbA1c, and HOMA-IR scores were imported into the REDCap database. Calculations for range of glucose values and MAGE from the CGM data were performed using the nadir-to-peak excursions. The two groups were compared using the Student *t*-test. A univariate linear regression analysis was performed to explore predictors of both glycemic variability (MAGE) and insulin resistance (HOMA-IR) for the following set of covariates: age, female gender, member of adult with obesity cohort, high glycemic load diet during study, fasting plasma glucose and serum insulin, and specific CGM metrics. Parameters statistically significant on univariate analysis were considered for a multivariable linear regression analysis. $P < 0.05$ was considered statistically significant.

Results

We enrolled 30 participants for this pilot study: two were excluded from analysis due to missing CGM values. Data were analyzed from 28 participants, ranging from 21 to 49 years, 14 of whom were adults with obesity (BMI $>$ 30 kg/m^2) and 14 were adults without obesity (BMI $<$ 30 kg/m^2). The baseline characteristics for the two cohorts were similar with respect to age, gender, and race/ethnicity, as shown in Table 1. BMI, WC, fasting serum insulin, and HOMA-IR were all significantly higher in the group with obesity than in the group without obesity ($P < 0.05$). No significant differences between the two groups were observed in HbA1c and fasting plasma glucose levels, confirming normoglycemia.

All participants included in the study successfully calibrated their CGM monitors on the day of implantation. Once calibrated, CGM data were generated for an average of 5.09 days [range 4–6]. Table 2 shows the data extracted from CGM. While the group with obesity had a higher mean blood glucose (MBG), mean amplitude of glycemic

TABLE 1 Baseline demographic and clinical characteristics of the participants in each group.

	Group without obesity	Group with obesity	Statistics
Age (years) mean	30.1 ± 7.49	32.4 ± 8.54	$t = 0.753$, $p = 0.458$
Gender			$\chi^2 = 0.164$, $p = 0.686$
Male	4 (28.6%)	5 (35.7%)	
Race/Ethnicity			$\chi^2 = 0.634$, $p = 0.889$
Caucasian	5 (35.7%)	6 (42.9%)	
Black	2 (14.3%)	3 (21.4%)	
Hispanic	3 (21.4%)	2 (14.3%)	
Other	4 (28.6%)	3 (21.4%)	
BMI (kg/m^2) mean	23.7 ± 2.2	38.4 ± 5.9	$t = 8.73$, $p = 0.000$*
Waist circumference (cm) mean	87.0 ± 1.27	108.4 ± 5.90	$t = 13.27$, $p = 0.000$*
Male	78.4 ± 5.12	95.0 ± 6.50	$t = 7.51$, $p = 0.000$*
Female			
HbA1c mean	5.27 ± 0.31	5.39 ± 0.22	$t = 1.18$, $p = 0.248$
Fasting plasma glucose (mg/dl) mean	85.4 ± 6.69	89.1 ± 4.40	$t = 1.17$, $p = 0.096$
Fasting serum insulin (μU/ml) mean	6.19 ± 2.12	17.32 ± 7.61	$t = 5.27$, $p = 0.000$*
HOMA-IR mean	1.30 ± 0.47	3.8 ± 1.64	$t = 5.48$, $p = 0.000$*

Statistics are presented using *t*-test for the continuous variables (all denoted by means with standard deviations) and a chi-square test for categorical variables.
*Significant difference between the two groups.

TABLE 2 Results extracted from continuous glucose monitoring and dietary logs in each group.

	Group without obesity	Group with obesity	Statistics
Mean blood glucose (mmol/l)	5.50 ± 0.39	5.69 ± 0.35	$t = 1.36$, $p = 0.187$
Mean amplitude of glycemic excursions (mmol/l)	1.26 ± 0.56	1.46 ± 0.37	t = 1.11, $p = 0.275$
Standard deviation of blood glucose (mmol/l)	0.92 ± 0.32	0.80 ± 0.18	$t = 1.22$, $p = 0.232$
Continuous overall net glycemic action-1 h (mmol/l)	5.11 ± 0.40	5.34 ± 0.27	$t = 1.78$, $p = 0.090$
Mean of daily differences (mmol/l)	0.91 ± 0.25	0.80 ± 0.13	$t = 1.46$, $p = 0.156$
% High glycemic load diet	18.18%	66.67%	$\chi^2 = 4.85$, $p = 0.028$*

Statistics are presented using *t*-test for the continuous variables (all denoted by means with standard deviations) and a chi-square test for categorical variables.
*Significant difference between the two groups.

excursions (MAGE), and continuous overall glycemic action-1 h (CONGA-1) than the group without obesity, these differences were not found to be significant. The standard deviation of blood glucose (SDBG) and mean of daily differences (MODD) were found to be higher, although not significantly, in the group without obesity than in the group with obesity.

For the diet assessment, only 20 of the 28 participants maintained detailed food logs with corresponding photo submissions. Based on the limited data, two Registered Dietitians focused the analysis on carbohydrate quality and quantity—estimating the proportion of refined carbohydrate versus fiber, as well as the total carbohydrate content of the meal. Of the 20 diets that could be analyzed, 12 were low glycemic load and 8 were high glycemic load. Chi-square analysis showed that of the 20 adults who kept dietary logs, there was a difference in glycemic load between the two cohorts, as seen in Table 2.

Tables 3 and 4 show the covariates analyzed through linear regression analysis. When assessing glycemic variability and insulin resistance (using MAGE and HOMA-IR as proxies respectively), only HOMA-IR demonstrated significant results. BMI, WC, adults with obesity, high glycemic diet, HbA1c, and fasting insulin levels maintained an independent association with HOMA-IR. The multivariable model was significant [$F(6,19) = 367.80$, $p < 0.001$] accounting for 99% of the variance (adjusted R^2). WC [β = 0.019 (0.005–0.033), $p = 0.010$] and fasting insulin levels [β = 0.212 (0.193–0.232), $p < 0.001$] were found to be predictors of HOMA-IR.

Discussion

This pilot study, which recruited normoglycemic men and women of diverse backgrounds, showed that glucometric data measuring glucose variability was similar in groups with obesity and without obesity. In contrast, even in this small sample, fasting insulin and HOMA-IR were significantly higher in participants with obesity. Surprisingly, while not statistically significant, the standard deviation of blood glucose was higher in participants without obesity.

On linear regression analysis, we found associations with HOMA-IR—and not MAGE—for BMI, WC, HbA1c, and fasting insulin levels. On multivariable analysis, WC and fasting insulin levels remained significantly associated with HOMA-IR. Taken together, these preliminary results suggest that the rise in insulin may be secondary to the development of insulin resistance and a compensatory mechanism in the glucose regulation process.

The results of this study lend support to the Energy Balance model for obesity. If a high glycemic load were the impetus for development of obesity, then greater glycemic variability would be seen in our cohort with obesity, which was not the case. To obtain further insight, we had patients keep dietary logs that were reviewed by Registered Dietitians, which showed that although our cohort with obesity on average ate meals with a higher glycemic load, their glycemic variability was not different from that of the cohort without obesity. However, we acknowledge that our small sample size and the difficulty in obtaining accurate dietary logs limits interpretation of these results.

Consistent with this are studies that demonstrate that excess nutrients within the skeletal muscle cells signal the cell membrane to block insulin-dependent glucose uptake by muscle cells. Petersen KF et al. demonstrated that insulin-resistant individuals have marked defects in muscle glycogen synthesis

TABLE 3 Univariate linear regression analysis of factors associated with mean amplitude of glycemic excursions (MAGE).

	Univariate analysis	
Covariate	***β [95% CI]***	***P*-value**
Age (years)	0.005 [−0.019–0.029]	0.672
Gender		0.803
Male	Ref	
Female	0.049 [−0.352–0.450]	
BMI (kg/m^2)	0.011 [−0.011–0.032]	0.310
WC (cm)	0.009 [−0.006–0.025]	0.231
Adult with obesity cohort	0.198 [−0.168–0.565]	0.275
High glycemic load diet	0.310 [−0.144–0.765]	0.169
Fasting plasma glucose (mmol/l)	0.019 [−0.013–0.050]	0.231
HbA1c	0.268 [−0.414–0.950]	0.427
Fasting insulin (μU/ml)	0.010 [−0.014–0.034]	0.863
HOMA-IR	0.046 [−0.061–0.153]	0.880
Standard deviation of blood glucose (mmol/l)	0.047 [−0.686–0.781]	0.895
Continuous overall net glycemic action-1 h (mmol/l)	0.404 [−0.108–0.916]	0.117
Mean of daily differences (mmol/l)	−0.004 [−0.919–0.910]	0.992

and divert their ingested energy into hepatic *de novo* lipogenesis. When insulin-resistant individuals are challenged with a high glycemic meal challenge, their post-prandial plasma glucose concentrations were similar to insulin-sensitive individuals (23).

For CGM to be effective, it must be assumed that glucose values are reflective of insulin secretion. However, this assumption has not been proven to be true in normoglycemic populations. Furthermore, excess fructose has been implicated in the development of obesity, diabetes, cardiovascular disease, non-alcoholic fatty liver disease, and cancer (24–28). Fructokinase C, present in the liver, converts fructose into metabolites such as citrate and uric acid that result in the net breakdown of ATP and endothelial dysfunction (29). Interestingly, fructose ingestion does not markedly raise glucose values as both dextrose or sucrose do and thus its intake would not be sharply detected by CGM (30, 31).

The data for the use of CGM devices in normoglycemic individuals with obesity are not robust (32). Salkind et al. (33) performed an observational study using CGM that compared contestants on the Biggest Loser reality show who were morbidly obese and were either normoglycemic or pre-diabetic. While there was no difference in glycemic variability between the two groups, Salkind et al. state that both groups had greater glycemic variability as compared with historical controls. However, one limitation of this study, and many other CGM studies in the literature, is that there is no contemporaneously studied normal weight control group, which our study indeed has (34).

In another study, Ma et al. (35) demonstrate that MBG levels and glycemic variability were increased in abdominally obese men with normal glucose tolerance who were of Han ethnicity. In contrast to our study, there was a difference in baseline mean blood glucose within their two cohorts, a smaller difference in BMI, and they only analyzed males with central obesity. Within Asian populations, the prevalence of diabetes with lower body mass index levels is well-documented (36), and we speculate that many of the patients in this cohort may have been pre-diabetic as baseline HbA1c was not documented.

More consistent with our findings is a recent study using CGM in adolescents with obesity (37). Investigators used CGM to compare whether glucose variability is altered during time-restricted eating (TRE). They found no difference in variability when TRE was compared to a diet that was not time limited. Theoretically, time restriction lowers insulin levels. The absence of any difference could be that TRE as used in this study does not result in the anticipated reduction in insulin levels, or that glucose value is not a sensitive method of measuring insulin.

While it is known that dietary interventions for normalizing glycemic levels are associated with changes in health markers, including fasting blood glucose level and HbA1c, the role of CGM in weight management is not established (38, 39). Although the idea of CGM translating to improved health outcomes is compelling, the commercialization of this technology has begun without clinical, peer-reviewed evidence of efficacy for weight loss.

Advocates of CGM have suggested that nutrition can be personalized by identifying specific foods that cause spikes in glucose levels. However, the standard American diet involves consumption of a variety of food groups at the same time and furthermore, failure to raise blood sugar levels is not synonymous with healthy eating. When a sugary dessert is eaten after a heavy meal, it causes less of a rise in blood sugar than when eaten on an empty stomach (40). The temporal sequence of carbohydrate ingestion during a meal has a significant impact on post-prandial glucose excursions.

Thus, there are many factors that contribute to glucose excursions or the lack thereof; and while CGM can identify glucose excursions, its role in weight loss for the modern consumer is not established. Our pilot study does not eliminate the possibility that if a participant wore a glucose monitor and tailored one's diet to lower glucose levels, that there would be weight loss. However, our results point toward metrics for insulin resistance, such as HOMA-IR, as potentially stronger clinical markers for dysglycemia and metabolic syndrome.

Furthermore, there may be harm related to the use of CGM in its capacity to raise false alarms and lead to unnecessary healthcare use, including excess clinical visits and inappropriate medication administration. Users may detect glycemic drops or spikes—prompting health changes such as increased snacking—when in reality, these values are biologically insignificant.

One major limitation of our pilot study is that it occurred during the COVID-19 pandemic, which restricted recruitment. Our preliminary findings need to be further explored in a larger cohort, and we cannot eliminate the possibility that with a greater sample there would not be a subtle difference in glycemic

TABLE 4 Univariate and multivariable linear regression analysis of factors associated with HOMA-IR.

Covariate	Univariate analysis		Multivariable analysis	
	β *[95% CI]*	*P*-value	β *[95% CI]*	*P*-value
Age (years)	−0.019 [−0.108–0.069]	0.660		
Gender		0.285		
Male	Ref			
Female	−0.773 [−0.227–0.682]			
BMI (kg/m^2)	0.155 [0.103–0.207]	0.000*	0.004 [−0.017–0.025]	0.666
WC (cm)	0.107 [0.065–0.148]	0.000*	0.019 [0.005–0.033]	0.010*
Adult in obesity cohort	2.493 [1.534–3.452]	0.000*	−0.276 [−0.616–0.064]	0.103
High glycemic load diet	0.092 [−0.023–0.206]	0.000*	0.026 [−0.199–0.250]	0.808
Fasting plasma glucose (mmol/l)	−0.083 [−0.180–0.014]	0.112		
HbA1c	2.737 [0.427–5.047]	0.022*	−0.051 [−0.404–0.301]	0.758
Fasting insulin (μU/ml)	0.222 [0.214–0.230]	0.000*	0.212 [0.193–0.232]	0.000*
MAGE	0.046 [−0.061–0.153]	0.880		
Standard deviation of blood glucose (mmol/l)	−1.542 [−4.188–1.1.05]	0.242		
Continuous overall net glycemic action-1 h (mmol/l)	1.274 [−0.650–3.198]	0.185		
Mean of daily differences (mmol/l)	−2.222 [−5.492–1.048]	0.174		

*Denotes statistical significance.

variability and other measured parameters. However, despite the small sample size, there remained a difference in fasting insulin and HOMA-IR between the two groups, suggesting rising insulin levels being a compensatory process rather than merely the result of a high glycemic load. This was further demonstrated through linear regression analysis, which showed HOMA-IR to be associated with BMI, WC, HbA1c, and fasting insulin levels, while MAGE was not found to be associated with these factors.

Additionally, the best measures of glycemic variability and insulin response remain unknown. MAGE, as extracted from CGM, is viewed as the most comprehensive index for assessment of intraday glycemic variability, but it does not account for insulin responsiveness and other processes of post-prandial cellular metabolism. Future studies should recruit a larger cohort of normoglycemic adults to assess the utility of CGM in predicting dysglycemia and aiding weight loss efforts.

Conclusion

While there is much excitement surrounding the use of commercial CGM products in management of obesity, our preliminary results suggest that fasting insulin and HOMA-IR values may be more clinically useful than CGM data alone. The absence of increased glycemic variability in normoglycemic individuals is suggestive that the Energy Balance model may represent a more accurate conceptual framework for obesity. Finally, the application of CGM in weight loss should await further trials.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Northwell Health Institutional Review Board (no. 19-1069). The patients/participants provided their written informed consent to participate in this study.

Author contributions

DC and MR contributed in conception, design, statistical analysis, and supervised the study. DC, SZ, BF, and AB contributed to data collection, data analysis, and manuscript drafting. All authors had final approval of the submitted and published versions of the manuscript.

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Conflict of interest

MR and SZ disclose that they are teaching consultants for Medtronic and Johnson & Johnson.

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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Sleeve gastrectomy decreases high-fat diet induced colonic pro-inflammatory status through the gut microbiota alterations

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Background: High-fat diet (HFD) induced obesity is characterized with chronic low-grade inflammation in various tissues and organs among which colon is the first to display pro-inflammatory features associated with alterations of the gut microbiota. Sleeve gastrectomy (SG) is currently one of the most effective treatments for obesity. Although studies reveal that SG results in decreased levels of inflammation in multiple tissues such as liver and adipose tissues, the effects of surgery on obesity related pro-inflammatory status in the colon and its relation to the microbial changes remain unknown.

Methods: To determine the effects of SG on the colonic pro-inflammatory condition and the gut microbiota, SG was performed on HFD-induced obese mice. To probe the causal relationship between alterations of the gut microbiota and improvements of pro-inflammatory status in the colon following SG, we applied broad-spectrum antibiotics cocktails on mice that received SG to disturb the gut microbial changes. The pro-inflammatory shifts in the colon were assessed based on morphology, macrophage infiltration and expressions of a variety of cytokine genes and tight junction protein genes. The gut microbiota alterations were analyzed using 16s rRNA sequencing. RNA sequencing of colon was conducted to further explore the role of the gut microbiota in amelioration of colonic pro-inflammation following SG at a transcriptional level.

Results: Although SG did not lead to pronounced changes of colonic morphology and macrophage infiltration in the colon, there were significant decreases in the expressions of several pro-inflammatory cytokines including interleukin-1β (IL-1β), IL-6, IL-18, and IL-23 as well as increased expressions of some tight junction proteins in the colon following SG, suggesting an improvement of pro-inflammatory status. This was accompanied by changing populations of the gut microbiota such as increased richness of *Lactobacillus* subspecies following SG. Importantly, oral administrations of broad-spectrum antibiotics to delete most intestinal bacteria abrogated surgical effects to relieve colonic pro-inflammation. This was further confirmed by transcriptional analysis

of colon indicating that SG regulated inflammation related pathways in a manner that was gut microbiota relevant.

Conclusion: These results support that SG decreases obesity related colonic pro-inflammatory status through the gut microbial alterations.

KEYWORDS

bariatric surgery, sleeve gastrectomy, colonic pro-inflammation, obesity, gut microbiota

Introduction

Obesity is characterized with chronic low-grade inflammation in various tissues and organs (1). Growing evidence has proposed colon as a critical site that displays pro-inflammatory features in response to high-fat diet (HFD) intake earlier than other metabolic tissues (2). In rodents, exposure to HFD leads to morphological changes such as shortened colon length and histological changes such as increased macrophage infiltration in the epithelium and lamina propria (2). This is accompanied by alterations to the inflammation cytokines in the colon including increased levels of pro-inflammatory cytokines tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), IL-6, IL-18, IL-23, and interferon-γ (IFN-γ), coupled with reductions in anti-inflammatory cytokines TNF-β, IL-10, and IL-33 (3, 4). In human, several studies have indicated that individuals with excessive HFD consumption have a higher inflammatory tone, which is closely related to the disruption of intestinal immune homeostasis and higher incidence of inflammatory bowel diseases (5, 6). These results point to an increased inflammation in the colon associated with HFD intake and obesity.

The HFD-induced obesity is not only linked with pro-inflammatory changes in the colon but also alterations of the gut microbiota. Colon is the location where the largest population of bacteria reside. A wide range of data have demonstrated significant alterations of the gut microbiota following HFD intake, which is highly associated with pro-inflammatory status in the colon (7, 8). For instance, patients with obesity display a rise in the abundance of certain microbes in the gut such as *Enterobacter* and *Desulfovibrio* which have a property of promoting inflammation (9–11), while they also tend to have a decline in the richness of some inflammation-protective bacteria such as *Akkermansia muciniphila* and *Lactobacillus* (12). This is corroborated by evidence from animal experiments showing similar imbalance within the gut microbial community in relation to inflammation changes in the colon following HFD feeding (13). Importantly, inoculation of the intestinal bacteria from HFD-fed mice promotes inflammation in the large bowel (14), pointing toward gut microbiota being a potential causal mediator linking HFD intake to colonic pro-inflammation.

The importance of colonic pro-inflammation associated with the gut microbiota changes following HFD intake is highlighted by its detrimental effects on the gut physiology. One important aspect of colonic physiology affected by local inflammation is the gut barrier function. Literature has shown a strong correlation between colonic pro-inflammation and decreased gut barrier integrity in the context of HFD feeding (4). Increased inflammatory cytokines such as IL-1β and IL-18 in the colon disrupt expressions of a variety of tight junction proteins that play important roles in the maintenance of barrier function, leading to a defect in gut barrier and an increase in intestinal permeability (15–17). Consequently, the increased colonic permeability allows translocation of aberrant microbes with pro-inflammatory activity into other metabolic tissues, causing systemic low-grade inflammation and worsened metabolic disorders (18).

Given the vital role of colonic pro-inflammation following HFD intake in the disruption of gut barrier and induction of more inflammation in the distant tissues (6), manipulations on the gastrointestinal tract with anti-inflammation potential represent promising and effective therapeutic strategies. Among the possible interventions on the gastrointestinal tract is bariatric surgery such as sleeve gastrectomy (SG). SG is currently one of the most effective treatments for obesity (19). Both human and rodent studies have recently indicated that SG can restore the disrupted gut microbiota resulting from HFD intake (20, 21), with substantial rises in the abundance of multiple anti-inflammation bacteria such as *Lactobacillus* (22, 23). Correspondingly, alterations of the intestinal bacteria following bariatric surgery are associated with improvements in the low-grade inflammation within different tissues such as liver and adipose tissue (24, 25). However, the effects of SG on colonic pro-inflammation and its relation to the gut microbiota remain unclear.

In the present study, using a mouse model of SG, we determined the effects of SG on HFD feeding related colonic pro-inflammation. We found a decrease in the expressions of pro-inflammatory cytokines genes and an upregulation of the genes encoding tight junction proteins in the colon of SG-treated mice, suggesting improvements of colonic pro-inflammation following SG. This was accompanied with significant alterations of the gut microbiota. Further studies using broad-spectrum antibiotics to perturb the gut microbiota changes following SG showed diminished effects of SG to improve pro-inflammation in the colon. Additional colonic transcriptome analysis supported that SG resulted in modifications of inflammatory pathways in a manner that was gut microbiota relevant. Altogether, these results demonstrate that SG leads to improvements in the HFD-induced colonic pro-inflammation, associated with alterations of the gut microbiota.

Method

Animal studies

To investigate the effects of SG on HFD-feeding induced colonic pro-inflammation, twenty 4-week-old male C57BL/6J mice were purchased from Charles River (Beijing, China) and allowed to acclimate in the laboratory for two weeks prior to the start of HFD intake. Before surgery, all mice were fed 60% HFD (Research Diets D12492, New Brunswick, New Jersey, USA.) for twelve weeks. Post that, the HFD-induced obese mice were randomized based on body weight to receive either SG (n=11) or sham surgery (SHAM, n=9). All mice were maintained on the same HFD for eight weeks following surgery until euthanasia. One mouse died and three mice suffered abdominal abscess after SG and were excluded.

To study the role of the gut microbiota in the improvements of HFD-feeding related colonic pro-inflammation following SG, another twenty 4-week-old male C57BL/6J mice (Charles River, Beijing, China) were used. All mice were fed 60% HFD (Research Diets D12492, New Brunswick, New Jersey, USA.) for twelve weeks starting at 6 weeks of age. Two days prior to surgery, all mice were provided with broad-spectrum antibiotics cocktails added in the drinking water to delete most of the intestinal bacteria (26). Then, these mice were randomized based on body weight to undergo either SG (SG-ABX, n=11) or sham surgery (SHAM-ABX, n=9). All mice were maintained on the same HFD and antibiotics cocktails for eight weeks following surgery until euthanasia. One mouse died and two mice suffered abdominal abscess after SG and were excluded.

All mice were housed under specific pathogen-free conditions at an ambient temperature with a 12-12 light-dark cycle and had ad libitum access to food and water. All animal experiments were conducted in accordance with National Research Council Guide for Care and Use of Laboratory Animals and approved by the Department of Laboratory Animal Science Fudan University.

Antibiotics treatment

The broad-spectrum antibiotics cocktails comprises four types of antibiotics purchased from Sigma Aldrich (Shanghai, China), namely neomycin trisulfate (#N6386), metronidazole (#M1547), vancomycin hydrochloride (#V2002) and ampicillin (#A9518). The antibiotics cocktails were freshly prepared by adding the antibiotics powders into drinking water, reaching a concentration of 1 g/L for neomycin trisulfate, 0.25 g/L for metronidazole, 0.5 g/L for vancomycin hydrochloride and 1 g/L for ampicillin (26). The antibiotics-containing drinking water was stored in the light-protected bottles and changed three times weekly.

Surgical procedures

SG and sham procedures were performed as previously described (21). Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg; Sigma, Shanghai, China). The stomach was gently exposed after dissection of the gastrosplenic ligaments. For SG, the glandular stomach was closed at 4 mm proximal of the pylorus toward the fundus using a 5-mm titanium clip (Ethicon, Somerville, NJ). After that, 80% glandular stomach and entire non-glandular stomach were excised along the outside of the clip, leaving a tubular gastric remnant. The gastric remnant with the clip was then enhanced using interrupted 8-0 Prolene sutures. For sham surgery, gentle pressure was applied on the stomach with nontoothed blunt forceps. Immediately after surgery, mice were placed on a heat mat and subcutaneously administered 1 ml warm 5% Glucose and Sodium Chloride Injection. Mice were fasted for food on the day of surgery and resumed HFD one day after surgery. Body weight following surgery was monitored daily for the first week and then weekly.

Mixed-meal tolerance test

Mixed-meal tolerance test (MMTT) was performed 6 weeks following surgery. All mice were fasted for 4 hours before oral gavage of liquid meal (volume 200 ml Ensure Plus spiked with a 25 mg dextrose). After that, tail vein blood glucose levels were measured using glucometers (Contour TS, Shanghai, China) at 0, 15, 30, 45, 60, 90, and 120 minutes.

Serum lipids measurement

Blood samples were collected when mice were euthanized after 4-hour fasting. Serum levels of triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) were measured using automatic biochemical analyser (Siemens Healthcare Diagnostics Inc, ADVIA XPT, USA) according to the manufacturer's instructions.

Quantitative real-time PCR

Total RNA was extracted from colon tissues using TRIzol (BioTNT, Shanghai, China) and then reversed into Complementary DNA by PrimeScript RT kit (Takara RR047, Beijing, China). PCR was performed using the TB Green Premix (Takara RR420, Beijing, China) on a QuantStudio 6 (ThermoFisher) system. Primers of target genes were purchased from Integrated DNA Technologies (Sangon Biotech, Shanghai, China) and verified by melting curve analysis. The expression levels of target genes were normalized to β-actin gene and calculated using the $2^{-\Delta\Delta CT}$ method.

Histology and immunohistochemistry

The colon segments were fixed in 4% buffered formalin for 48 hours prior to paraffin embedding and hematoxylin and eosin (H&E) staining. Crypt depth of colon of each mouse was measured in three different fields under x 400 high power field (HPF) by the software (K-Viewer 1.5.5.2, China). For evaluation of the macrophage infiltration in the colon, CD68 staining was performed using a rabbit anti-mouse CD68 primary antibody (Abcam ab283654, Shanghai, China) and

secondary antibody (Jackson, Philadelphia, USA), and further counted in three different sections under HPF by two blinded observers.

Isolation of colonic lamina propria and flow cytometric analysis

The macrophages from colonic lamina propria were isolated by lamina propria dissociation kit (Miltenyi Biotec mouse130-097-410, Shanghai, China) according to the manufacturer's instructions. Briefly, intestinal fat was removed, and colon was cut open and washed slowly in PBS (Ca^{2+} and Mg^{2+} free). The colon segments were cut into 1 mm pieces and added into Enzyme D, Enzyme R, and Enzyme A for incubation at 37°C for 45 minutes. The supernatant was then filtered through 75 um nylon mesh and centrifuged at 500g for with 10 minutes. After centrifuge, the pellets were collected and re-suspended in 40% Percoll (Solarbio, Beijing, China). The 40% Percoll solution with suspended cells was transferred into 80% Percoll and re-centrifuged at 2000 rpm for 20 minutes at room temperature. The white interphase was collected after centrifuge and washed twice with PBS. Before flow cytometric analysis, single-cell suspensions isolated from the colon were further stained for 30 minutes on ice with fluorophore-conjugated commercial antibodies to F4/80 (Invitrogen 11-4801-82, USA), CD11b (Invitrogen 12-0112-82, USA) and CD11c (Invitrogen 17-0114-81, USA). After preparation, those cells were re-suspended in PBS with 0.5% FBS and analyzed using FACSAriaIII (BD Bioscience, USA). The data were analyzed by FlowJo software (Becton, Dickinson and Company, USA).

16s rRNA sequencing

Fecal and cecal samples were collected when mice were euthanized, and immediately frozen at -80°C upon collection. Total genomic DNA was extracted from samples using the OMEGA Soil DNA Kit (M5635-02) (Omega Bio-Tek, Norcross, GA, USA), following manufacturer's instructions. Then the DNA was stored at -20°C prior to further analysis. PCR amplicons were purified with Vazyme VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China) and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). After quantification, amplicons were pooled in equal amounts. Pair-end 2 x 250 bp sequencing was performed using the Illlumina MiSeq platform with MiSeq Reagent Kit v3 at Shanghai Personal Biotechnology Co., Ltd (Shanghai, China). Sequencing data analyses were performed using QIIME2. Briefly, raw sequences after trimming were analyzed by the cutadapt plugin and the dada2 plugin. After that, non-singleton amplicon sequence variants (ASVs, 100% operational taxonomic units (OTUs)) were generated. Microbial taxonomic classification was performed to ASVs according to the classify-sklearn alignment algorithm (27) against the Greengenes database (Release 13.8) of 99% OTUs reference sequences (28). Alpha diversity metrics including Chao1 and Shannon calculated by the diversity plugin were performed to estimate richness and diversity respectively. Beta diversity metrics including unweighted UniFrac distance matrix were scaled and visualized through principal coordinates analysis (PCoA), and significance of the clustering between groups was determined *via* permutational multivariate analysis of variance (PERMANOVA). Random Forest Classifier with 10-fold cross-validations, MetagenomeSeq analysis, and Linear discriminant analysis (LDA) effect size (LEfSe) with default parameters were computed to identify significantly different microbes in abundance between groups at different taxonomic levels.

RNA sequencing and analysis

Total RNA was isolated from colon tissues using Trizol Reagent (Invitrogen Life Technologies, USA) and sequenced on NovaSeq 6000 platform (Illumina, USA) by Shanghai Personal Biotechnology Cp. Ltd. R language Pheatmap (1.0.8, China) software package was used to perform bi-directional clustering analysis to identify all differentially expressed genes (DEGs) between two surgical groups. The top Gene Ontology (GO) was used to perform GO enrichment analysis based on the DEGs. P-value was calculated by hypergeometric distribution method (the standard of significant enrichment is $P < 0.05$). Cluster Profiler (3.4.4) software was used to carry out the enrichment analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway of DEGs, focusing on the significant enrichment pathway with $P < 0.05$.

Statistical analyses

Data were presented as mean ± SEM. Differences in body weight and blood glucose levels during MMTT between two surgical groups were evaluated using two-way analysis of variance (ANOVA) with *post-hoc* Sidak test for multiple comparisons (29). Other simple comparisons between two surgical groups were assessed with Student's t-test or non-parametric Mann–Whitney U tests. In addition, comparisons among four surgical groups were assessed using two-way analysis of variance (ANOVA) with *post-hoc* Tukey's test for multiple comparisons. All statistical analyses were conducted using GraphPad Prism 8 software (La Jolla, CA). Data were considered statistically significant when $P < 0.05$ (2-sided significance testing).

Results

SG leads to improvements in the HFD-feeding induced colonic pro-inflammatory status

Twenty 4-week-old male C57BL/6J mice were fed 60% HFD for 12 weeks and then randomized based on body weight to receiving either SG or SHAM operation. Mice were kept on the same 60% HFD following surgery until euthanasia (Figure 1A). SG led to significant weight loss as compared to SHAM operation (Figure 1B). SG mice also displayed significantly lower glucose levels during mixed meal tolerance test (MMTT) (Figure 1C), suggesting an improved glucose tolerance following SG. Besides, SG-treated mice had significantly decreased levels of TC, LDL-C and HDL-C (Figure 1D).

In terms of colonic morphology, there was no difference in the length of colon and weight of cecum between SG and SHAM groups (Figures 1E, F). However, H & E staining indicated a decreased depth of colonic crypts of SG mice as compared to SHAM mice (Figure 1G). To determine the effects of SG on HFD feeding associated colonic pro-inflammation, we measured mRNA expression levels of different cytokines in the colon using qPCR. The mRNA expression levels of pro-inflammatory cytokines such as IL-6, IL-1β, IL-18, and IL-23 were downregulated in the colon of SG mice relative to SHAM mice, while those anti-inflammatory factors such as IL-10, TNF-β, IL-33 were not impacted by SG (Figure 1H). Given that HFD feeding associated colonic pro-inflammation induces gut barrier defects (30), we measured mRNA expression levels of two common intestinal tight junction proteins zonula occludens 1 (ZO-1) and Occludin. SG increased relative expression levels of ZO-1 and Occludin in the colon as compared to SHAM operation (Figure 1I), implicating a possible improvement in the colonic barrier following SG. Collectively, these data demonstrate that SG results in improvements in the HFD feeding related colonic pro-inflammation.

HFD-feeding induced macrophage infiltration in the colon is not affected by SG

Literature has recently proposed an important role of macrophage infiltration in the HFD feeding associated colonic pro-inflammation (31). We therefore measured mRNA expression levels of macrophage-related chemokine genes including CCL2, CCL7 and CCL12 and marker genes such as F4/80, CD68, CD11b and CD11c in the colon. All these genes except CD11c showed comparable expression levels between SG and SHAM groups (Figures 2A, B). Likewise, immunohistochemistry tests revealed no difference in the numbers of $CD68^+$ macrophages infiltrated in the colon epithelium between SG and SHAM mice (Figure 2C). This was further confirmed by flow cytometry analysis of colonic lamina propria where SG mice had similar numbers of $F4/80^+CD11b^+CD11c^-$ sub-population to SHAM mice (Figure 2D). These results indicate that SG has no effect on the HFD-feeding induced macrophage infiltration in the colon.

SG results in alterations of the gut microbiota

Given the association of colonic pro-inflammation upon HFD intake with alterations to the gut microbiota (32), we next characterized the intestinal bacterial changes following SG. The fecal microbiota of SG mice displayed higher richness than that of SHAM mice, as estimated by higher levels of Chao 1 index (Figures 3A, B). For the overall composition of the gut microbiota, unweighted UniFrac PCoA revealed a differential

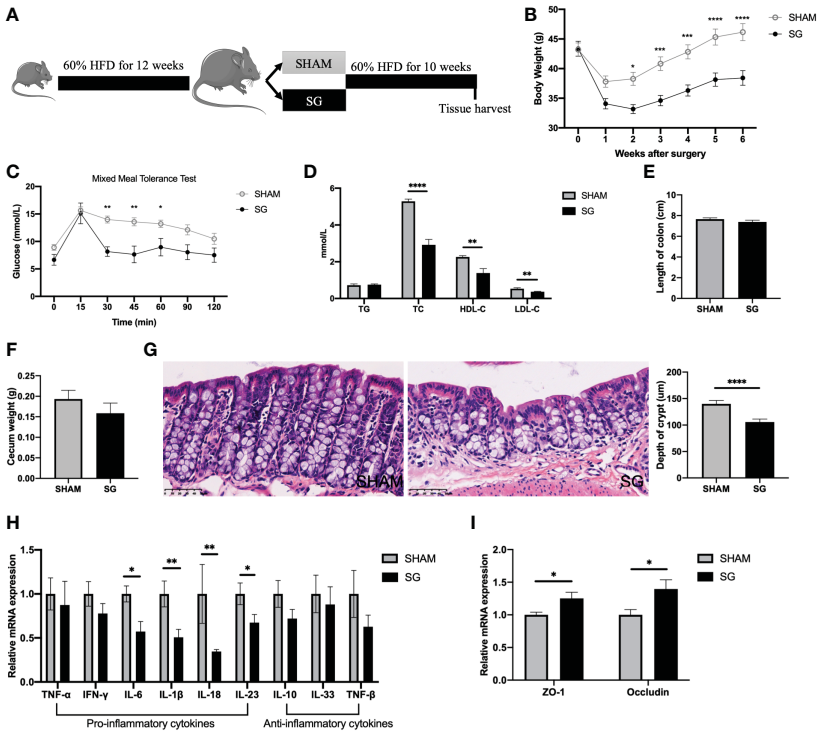

FIGURE 1
SG led to improvements in the HFD-feeding induced colonic pro-inflammatory status. **(A)** Experimental design and timeline (SG n=7; SHAM n=9). **(B)** Body weight. **(C)** Mixed meal tolerance test (MMTT). **(D)** Serum lipids. **(E)** Length of colon. **(F)** Cecum weight. **(G)** Representative H&E-staining images of colon (400x; scale bar, 50 um) and quantification of depth of crypt. **(H)** Gene expressions of inflammatory cytokines in the colon. **(I)** Gene expressions of tight junction proteins in the colon. n = 7–9/group; Data are presented as means ± SEM. Two-way ANOVA with *post hoc* Sidak test for multiple comparisons **(B, C)** and Student's t-test **(D, I)** were used for significance assessments. ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05. SHAM, sham surgery; SG, sleeve gastrectomy.

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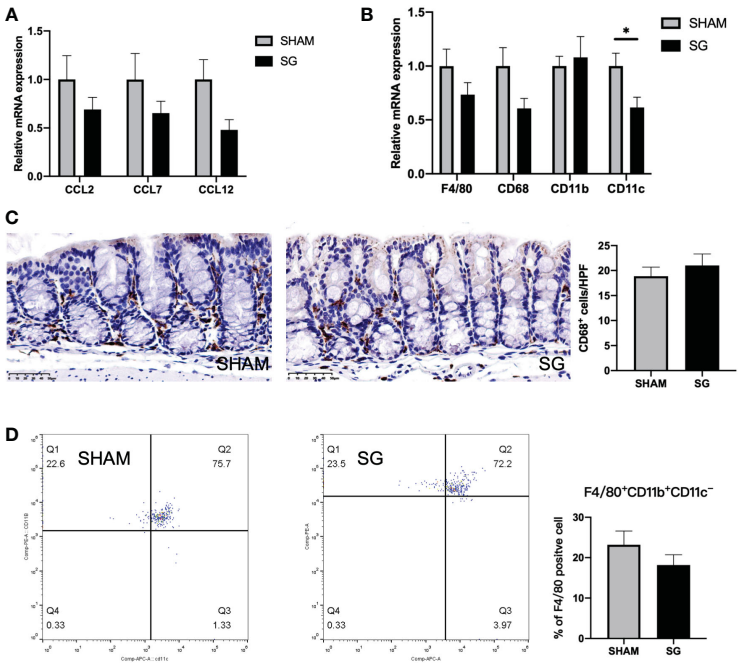

FIGURE 2
HFD feeding induced macrophage infiltrations in the colon are not affected by SG. **(A)** Gene expressions of macrophage-related chemokines. **(B)** Gene expressions of macrophage-related markers. **(C)** Representative histological images of colon stained with anti-CD68 antibody (400x; scale bar, 50 um) and quantification of CD68 positive cells. **(D)** Representative FACS analysis of F4/80$^+$CD11b$^+$CD11c$^-$ cells in colonic lamina propria. The right panel indicates the percentage of F4/80$^+$CD11b$^+$CD11c$^-$ cells among F4/80+ cells in colonic lamina propria. n = 7–9/group; Data are presented as means ± SEM. Student's t-test was used for significance assessments. *P < 0.05. SHAM, sham surgery; SG, sleeve gastrectomy.

clustering of fecal samples between SG and SHAM mice (Figure 3C). This was confirmed by PERMANOVA which showed significance in unweighted UniFrac distances of samples between SG and SHAM groups (F value = 1.85, P = 0.003), suggesting an alteration in the overall composition of the gut microbiota following SG. In terms of the detailed compositions of the gut microbiota, *Firmicutes* was the major phylum in the fecal microbial communities of SHAM and SG mice, while *Desulfovibrio* subspecies showed a decreasing trend in the abundance following SG (Supplementary Figure 1A). Random Forest classifier was used to identify differentially enriched microbes between SHAM and SG groups. It indicated that bacterial subspecies belonging to *Lactobacillus* genus were the main discriminators for SG compared to SHAM gut microbiota, with substantial rises in the relative richness following SG (Figure 3D). This was consistent with results generated from MetagenomeSeq analysis showing that multiple bacteria under *Lactobacillales* order were abundant following SG relative to SHAM operation (Figure 3E). Additional LDA effect size (LEfSe) analysis revealed more taxonomic differences in the microbial composition between SHAM and SG groups (Supplementary Figure 1D). For instance, SG microbiota were enriched for *Blautia* subspecies, whereas SHAM microbiota were enriched for *Desulfovibrio* subspecies. Taken together, these data indicate that SG leads to significant alterations of the gut microbiota, featured by decreases of pro-inflammatory microbes such as *Desulfovibrio* and increases of anti-inflammatory microbes like *Lactobacillus*. This suggests a potential association of changed gut microbiota with decreased colonic pro-inflammation following SG.

Administration of broad-spectrum antibiotics compromises SG's ability to improve HFD-feeding induced colonic pro-inflammation

We next sought to determine whether the improvements of colonic pro-inflammation following SG were dependent on the alterations of the gut microbiota. Another cohort of twenty 4-week-old male C57BL/6 mice were fed 60% HFD for 12 weeks and then randomized based on body weight to receiving either SG (SG-ABX) or SHAM (SHAM-ABX) operation. All mice were kept on the same 60% HFD following surgery until euthanasia. They were also provided with broad-spectrum antibiotics in the drinking water to delete most intestinal bacteria (26) starting from 2 days before surgery till the end of the study (Figure 4A).

To confirm the efficacy of antibiotics cocktails in the gut microbiota deletion, 16s rRNA sequencing was used to characterize the bacterial changes in the cecum contents of SG and SHAM mice that received antibiotics treatment. Chao1 and Shannon index indicated extremely low levels of richness and diversity of the gut microbiota in the antibiotics-treated groups (Supplementary Figures 2A, B). This was consistent with compositions of the microbial community where *Proteobacteria* was the main phylum left in the gut, accounting for 90% of the entire bacterial abundance following antibiotics administration (Supplementary Figure 2D). At species level, there were fewer microbes that had much lower richness in the cecum contents (Supplementary Figure 2E). These data suggest that the broad-spectrum antibiotics treatment successfully deleted most microbes in the cecum. In this situation, no differences were

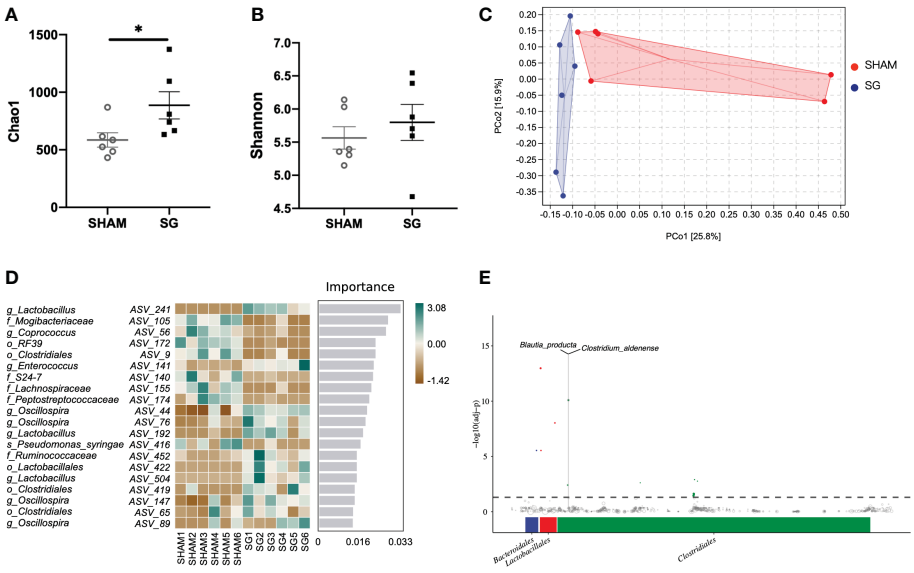

FIGURE 3
SG resulted in alterations of the gut microbiota. **(A, B)**. Chao1 and Shannon index of the gut microbiota in the fecal samples. Student's t-test was used for significance assessments. *P < 0.05. **(C)** Unweighted UniFrac principle coordinates analysis (PCoA). **(D)** Discriminatory importance scores of top-ranked ASVs identified by the Random Forest analysis. A comparison of the relative abundance of top-ranked ASVs between SG and SHAM gut microbiota. Green and brown indicate the degree of relative abundance. **(E)** MetagenomeSeq analysis showing significantly enriched microbes following SG relative to SHAM operation. The X and Y axis represent taxonomic order and the -log10(adj-Pvalue) value, respectively. Dots of blue, red, and green represent abundant microbes under *Bacteroidales*, *Lactobacillales*, and *Clostridiales*, respectively. n = 6/group; Data are means ± SEM. SHAM, sham surgery; SG, sleeve gastrectomy.

observed between SG and SHAM mice in the microbial richness and diversity, as estimated by Chao1 and Shannon index, respectively (Supplementary Figures 2A, B). Likewise, no bacterial taxa were identified as varying significantly in the relative abundance between SG-treated and sham-operated mice using Random Forest classifier, MetagenomeSeq analysis or LEfSe (data not shown given the extremely low richness of the microbes detected). For the overall composition of the gut microbiota, unweighted UniFrac PCoA did not show pronouncedly differential clustering of samples between SG-ABX and SHAM-ABX groups (PERMANOVA F value =0.84, P =0.614) (Supplementary Figure 2C). Together, these data reveal no marked alterations of the gut microbiota following SG relative to SHAM operation in the context of antibiotics treatment.

Although antibiotics cocktails eliminated most bacteria in the gut, SG still led to significant weight loss, improved glucose tolerance and decreased serum levels of TC and LDL-C (Figures 4B–D).

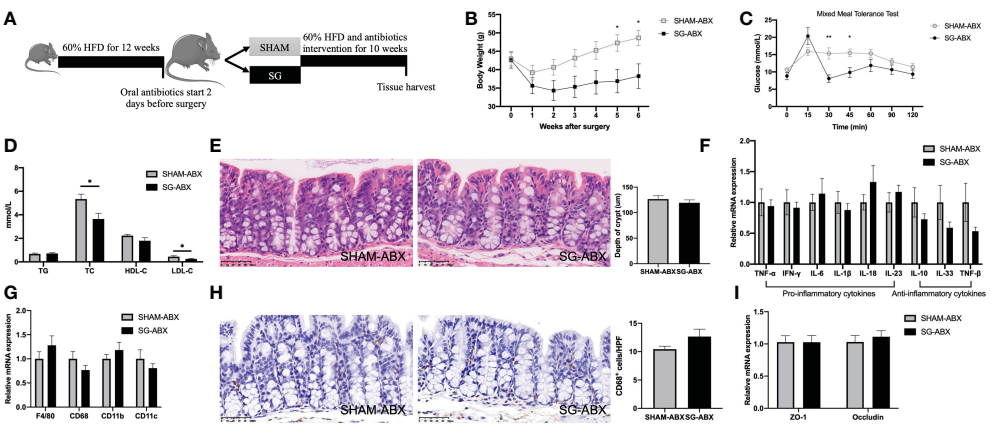

FIGURE 4
Administration of broad-spectrum antibiotics comprises SG's ability to improve HFD-feeding induced colonic pro-inflammation. **(A)** Experimental design and timeline (SG-ABX n=8, SHAM-ABX n=9). **(B)** Body weight. **(C)** Mixed meal tolerance test (MMTT). **(D)** Serum lipids. **(E)** Representative H&E-staining images of colon (400x; scale bar, 50 um) and quantification of depth of crypt. **(F)** Genes expressions of inflammatory cytokines in the colon. **(G)** Gene expressions of macrophages markers in the colon. **(H)** Representative histological images of colon stained with anti-CD68 antibody (400x; scale bar, 50 um) and quantification of CD68 positive cells. **(I)** Gene expressions of tight junction proteins in the colon. n = 8/group; Data are presented as means ± SEM. Two-way ANOVA with *post hoc* Sidak test for multiple comparisons **(B, C)** and Student's t-test **(D–H)** were used for significance assessments. **P < 0.01, *P < 0.05. SHAM, sham surgery; SG, sleeve gastrectomy; ABX, antibiotics.

Interestingly, weight loss, glucose tolerance and serum lipid levels were all comparable between SG and SG-ABX groups (Supplementary Figures 3A–F). For the histological features of HFD feeding associated colonic pro-inflammation, no difference was observed in the crypt depth between the two surgical groups under antibiotics treatment (Figure 4E). However, unlike what was shown in the Figure 1, SG had no effect on the expressions of various inflammation cytokines in the colon in the absence of the gut microbiota changes, as the pro-inflammatory cytokines including IL-6, IL-1β, IL-18, and IL-23 displayed comparable mRNA expression levels between antibiotics-treated SG and SHAM groups (Figure 4F; Supplementary Figure 4A). Likewise, there were no differences in the relative expression levels of ZO-1 and Occludin genes in the colon between antibiotics-treated SG and SHAM mice (Figure 4I; Supplementary Figure 4B), implicating no improvement in the gut barrier integrity following SG when there were no intestinal bacterial changes resulting from oral administration of antibiotics. On the other hand, whereas antibiotics-treated animals had reduced colonic macrophage infiltration, SG-ABX and SHAM-ABX groups showed comparable levels of macrophage infiltration and related genes expressions in the colon (Figures 4G, H, Supplementary Figures 4C, D). Altogether, these results indicate an important role of the gut microbiota in the improvements of HFD related colonic pro-inflammation following SG.

SG significantly modifies the colonic transcriptome, including pathways linked to inflammation regulation

To further probe the relationship between improvements of colonic pro-inflammation and the gut microbiota changes following SG at transcriptional level, we conducted RNA sequencing analysis of colon tissues obtained from both SG and SHAM mice with and without antibiotics treatment. For mice that did not receive antibiotics cocktails, volcano plots illustrated 179 differentially expressed genes (DEGs) in the colon between SG and SHAM groups (Figure 5A). Both Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of these DEGs showed a significant enrichment of multiple pathways that were related to inflammation regulation in the colon including *inflammatory response*, *leukocyte migration* and *chemotaxis* as well as *PPAR signaling pathway* (Figure 5B; Supplementary Figure 5A). On the other hand, for mice that received antibiotics cocktails, volcano plots identified only 73 DEGs between SG and SHAM groups. Neither GO nor KEGG pathway analyses of these DEGs showed an enrichment of inflammation-associated pathways (Figures 5C, D; Supplementary Figure 5B). Taken together, these findings demonstrate that SG exerts transcriptional modifications of inflammatory pathways in the colon in a manner that was gut microbiota relevant.

Discussion

Obesity is characterized with chronic low-grade inflammation in various tissues and organs associated with changing compositions of the gut microbiota (9). Colon has recently emerged as a critical site not only because it is the first place to display inflammatory features in response to HFD intake (2) but also because it is where the bulk of the intestinal bacteria are located. HFD intake induces gut dysbiosis which may in turn initiate pro-inflammatory activities in the colon (3). The colonic pro-inflammation further damages intestinal barrier which allows bacteria to translocate into distant tissues to cause more inflammation and dysfunction (3, 33). Bariatric surgery such as SG is currently the most efficacious treatment for obesity (34). Although SG

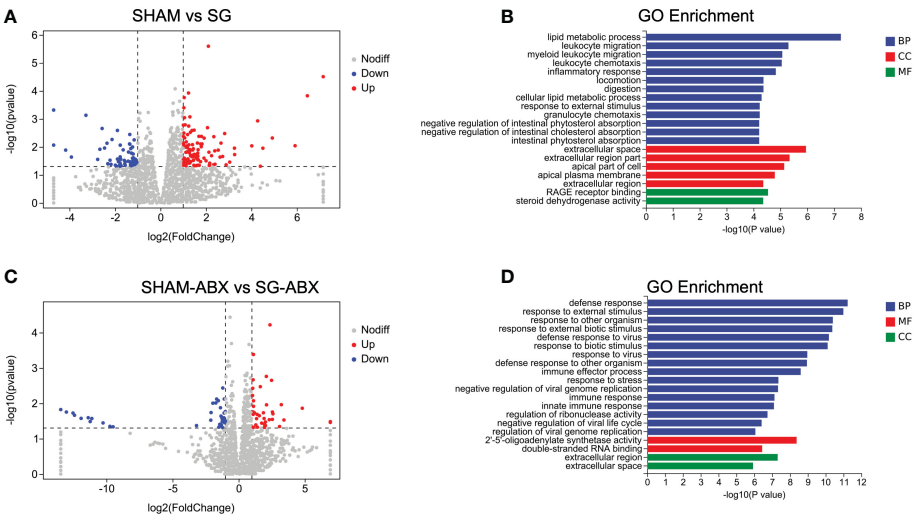

FIGURE 5
SG significantly modulated the colonic transcriptome. **(A)** Volcano plots shows differentially expressed genes (DEGs) of colon between SHAM and SG groups. Blue dots and red dots represent significantly down-regulated and up-regulated genes, respectively (Log2 Fold change >1, Bonferroni-adjusted $P < 0.05$). **(B)** Enrichment analysis of Gene Ontology (GO) including Biological Process (BP), Molecular Function (MF), and Cell Component (CC) based on the DEGs between SHAM vs SG. **(C)** Volcano plots shows DEGs of colon between antibiotics-treated SG and SHAM groups. Blue dots and red dots represent significantly down-regulated and up-regulated genes, respectively (Log2 Fold change >1, Bonferroni-adjusted $P < 0.05$). **(D)** Enrichment analysis of GO based on the DEGs between SHAM-ABX vs SG-ABX. n = 6–8/group. SHAM, sham surgery; SG, sleeve gastrectomy; ABX, antibiotics.

does not involve anatomical alterations of the intestinal tract, this surgical approach does lead to a wide range of alterations in gut physiology in the distal bowel such as changing bacterial populations (35). In the present study, using a mouse model of SG, we found that the surgical effects extend to the improvements of pro-inflammatory status in the colon which are gut microbiota relevant.

The inflammatory shift in the colon following HFD intake is regarded as low-grade since it is not associated with apparent histological features of active inflammation (3). However, it is considered as pro-inflammatory because it is characterized with increased levels of macrophage infiltration and expressions of various inflammation cytokines (2). Here we found that, although mice undergoing SG showed decreased depth of crypt without markedly reduced macrophages infiltration in the colon, they had a significant reduction in the expressions of multiple pro-inflammatory cytokines including IL-1β, IL-6, IL-18, and IL-23. This was supported by transcriptional signatures in colon revealing that SG regulated multiple inflammation-related pathways. Consistent with what we have found, one study in rats recently indicates decreases of inflammatory cytokines IFN-γ, IL-17, and IL-23 in the distal jejunum following SG (36).

The pro-inflammatory cytokines in the colon are associated with gut barrier dysfunction (37). Literature has previously shown that certain inflammatory cytokines such as IL-1β can directly suppress the expressions of a variety of tight junction proteins which are essential for the gut barrier integrity (16). In line with the decreased levels of inflammatory cytokines in the colon following SG, we observed increased expressions of two common tight junction proteins in the colon, implicating a potential improved gut barrier following SG. In parallel, recent studies demonstrate that SG can increase gene expressions of intestinal tight junction proteins and improve gut barrier function in obese mice that receive HFD (38). Given that improved gut barrier function and decreased permeability prevent translocation of inflammation from intestine into circulation and periphery (33), it is therefore possible that the improvements of pro-inflammation and gut barrier function in the colon following SG contribute to an overall relief of inflammatory condition observed in obesity. Interestingly, previous studies have revealed that patients who underwent SG experience decreased levels of pro-inflammatory cytokines in the circulation and liver following surgery (25, 39). Collectively, these data suggest that SG results in improvements in the HFD feeding associated colonic pro-inflammation.

Bariatric surgery has been reported to change the gut microbial populations (22, 23), and alterations in the gut microbiota have been pointed as a potential modulator of the colonic inflammation observed in obesity (40). Therefore, we next characterized the intestinal bacterial changes following SG. We found that SG led to disparate gut microbial compositions, accompanied by increased richness and diversity of the microbial community. Studies in both human and animals propose that a more diverse and abundant bacterial community is beneficial to intestinal health, in connection to decreased inflammation levels and improved local defense (41, 42). More importantly, we identified significant alterations in the abundance of various bacterial populations following SG. Notable in mice that received SG was an expansion of *Lactobacillus* subspecies. We and others have consistently observed that SG led to considerable increases in the richness of *Lactobacillus* in obese rodents (22, 23, 43). These microbes are generally regarded as "healthy" bacteria and can be found in a variety of foods and probiotics which are often used to treat intestinal health issues (44, 45). Previous studies have indicated that administration of probiotics containing multiple *Lactobacillus* strains reduces inflammation and enhances gut barrier function in obese mice (39, 46). Additionally, certain bacteria that promote inflammation such as *Desulfovibrio* (47) were found decreased in abundance following SG. Together, these results suggest a strong association of changed gut microbiota with decreased colonic inflammation following SG.

The key question then becomes whether SG improves pro-inflammatory conditions in the colon through the gut microbial changes. We applied broad-spectrum antibiotics cocktails to eliminate the gut microbiota changes following surgery. We found that, in the absence of the gut microbiota alterations upon antibiotics treatment, SG had no effect on HFD feeding induced colonic pro-inflammation, as manifested by unchanged expression levels of inflammatory cytokines such as IL-1β, IL-6, and IL-23. A caveat here is that antibiotics treatment itself appealed to reduce expressions of certain pro-inflammatory cytokines like IL-18 in the colon, and therefore the window for improvements in these parameters is smaller. Nevertheless, the increases in the gene expressions of tight junction proteins in the colon following SG were also absent when antibiotics were orally administrated. Further transcriptomic analysis of colon showed no inflammation pathway that was regulated by SG in the absence of bacterial changes. Together, these results support that SG decreases HFD feeding related colonic pro-inflammation in a gut microbiota dependent way.

Given that macrophage plays an important role in HFD-induced colonic inflammation (2), we measured macrophage infiltration in the colonic epithelium to reveal its potential relation to the gut microbiota and colonic inflammation following SG. We found that numbers of macrophages infiltrated in the colonic epithelium were profoundly decreased by antibiotics treatment but not affected by SG. These data indicate that macrophage infiltration in the colon is at least partially dependent on the gut microbiota, but it is not associated with specific alterations of the gut microbiota and improvements of colonic pro-inflammation following SG. The gut microbial changes have impacts on composition and function of various intestinal immune cells (48). Future studies will need to assess which immune cells other than macrophage are most relevant to the reduced pro-inflammatory levels in the colon following SG.

Bariatric surgery exerts profound alterations to gut physiology (35). While controversy remains, accumulating evidence indicates that many aspects of the physiological changes taking place following surgery are influenced by the gut microbiota (49). The improvements of pro-inflammation in the colon following SG represent one clear example of changes in gut physiology that involve intestinal bacterial effects. In the present study, we applied broad-spectrum antibiotics for evaluating the potential influence of the gut microbiota following SG. This method induced successful deletion of majority of bacteria in the gut. Surprisingly, metabolic improvements imparted by SG including weight loss, improved glucose tolerance and decreased serum lipid levels were not affected by antibiotics administration. These results suggest that the gut microbiota may be dispensable to improvements in these parameters but still important to other physiological changes resulting from SG such as decreased pro-

inflammation levels in the colon. Nonetheless, gut microbial transfer studies are still needed to address a cause-and-effect relationship between the gut microbiota and metabolic benefits of SG.

One limitation of the current work is that we could not identify all microbes present in samples using 16s rRNA sequencing due to its limited sequencing depth and power. Future work using metagenomic sequencing will be needed to gain more comprehensive information on alterations of the gut microbiota following SG. Another limitation is that we did not measure related microbial metabolites that potentially regulate intestinal inflammation such as bile acids (50, 51). Bile acids and bile acid receptors have been proposed as critical molecular underpinnings for the beneficial effects of SG (52–56). Several lines of evidence have demonstrated a strong association of changed gut microbiota with increased bile acids levels and signaling following SG (22, 53, 57). Importantly, administration of antibiotics cocktails like what we used herein leads to disturbed bile acids metabolism and suppressed bile acids signaling following SG (26). This disrupted gut microbiota-bile acids interaction impairs SG's effects to increase gut hormone secretions which is another pivotal example of gut physiological changes occurring after SG (26). Interestingly, bile acids have been linked with controlling inflammation in mouse models of colitis (58, 59). It is possible to hypothesize that changes in bile acids metabolism and signaling work as a potential mediator linking the gut microbiota to the improvements of colonic inflammation following SG. Investigations on bile acids metabolism will provide a mechanistic insight on how the gut microbiota reduces intestinal inflammation following SG.

In conclusion, our findings demonstrate that SG leads to improvements in the HFD-induced colonic pro-inflammation, associated with alterations of the gut microbiota. Depletion of the gut bacterial changes following SG through administration of broad-spectrum antibiotics compromised SG's effects to relieve pro-inflammation status in the colon. These results point to changes in the intestinal bacteria as important gut adaptation to surgical manipulations on the gastrointestinal tract that mediate alleviations of inflammation in the colon.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA915230.

Ethics statement

The animal study was reviewed and approved by the Department of Laboratory Animal Science Fudan University.

Author contributions

Authors QY and YS conceived, designed, and supervised the study; CC, XT, and HY conducted this study; CC and XT analyzed the results; QY, YS, CC, and XT wrote the manuscript; RH and QS provided guide of surgical procedures. All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

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SUPPLEMENTARY FIGURE 1
SG results in alterations of the gut microbiota. **(A-C)** Microbiota composition at phylum, genus, and species level respectively. **(D)** Cladogram generated by LEfSe indicating differentially enriched microbes at phylum, class, order, family, genus, and species levels between the two groups. n = 6/group. SHAM = sham surgery, SG = sleeve gastrectomy.

SUPPLEMENTARY FIGURE 2
Alternations of the gut microbiota after antibiotics treatment. **(A)** Chao1 index. **(B)** Shannon index. **(C)** Unweighted UniFrac principle coordinates analysis (PCoA). **(D-E)** Major phylum and species of the gut microbiota. n = 5/group; Data are presented as means ± SEM. Two-way ANOVA with *post hoc* Sidak test for multiple comparisons (Panel A and B) *P < 0.05. SHAM = sham surgery, SG = sleeve gastrectomy, ABX = antibiotics.

SUPPLEMENTARY FIGURE 3
Antibiotics treatment does not impair weight loss, improved glucose tolerance and decreased serum lipid levels following SG. **(A-B)**. Body weight. **(C)** Changes in body weight. **(D)** Body weight percentage. **(E)** Mixed meal tolerance test (MMTT). **(F)** Serum lipids. Two-way ANOVA with *post hoc* Sidak test (Panel **B**, **D**, and **E**) and Tukey's test (Panel **A**, **C**, and **F**) for multiple comparisons were used

for significance assessments. ****P < 0.0001, ***P < 0.001, ** P < 0.01, *P < 0.05 SHAM vs SG; ####P < 0.0001, ##P < 0.01, #P < 0.05 SHAM-ABX vs SG-ABX. SHAM = sham surgery, SG = sleeve gastrectomy, ABX = antibiotics.

SUPPLEMENTARY FIGURE 4

Comparisons in mRNA expression levels of inflammatory cytokines and tight junction proteins as well as macrophage infiltration in the colon among four surgical groups. **(A)**. Genes expressions of inflammatory cytokines in the colon. **(B)** Gene expressions of tight junction proteins in the colon. **(C)** Quantification of CD68 positive cells. **(D)** Gene expressions of macrophages markers in the colon. n = 7-9/group; Data are presented as means ± SEM. Two-way ANOVA with *post hoc* Tukey's test for multiple comparisons (Panel **A**, **B**, **C**, and **D**) was used for significance assessments. **P < 0.01, *P < 0.05 SHAM vs SG; ##P < 0.01, SHAM-ABX vs SG-ABX. SHAM = sham surgery, SG = sleeve gastrectomy, ABX = antibiotics.

SUPPLEMENTARY FIGURE 5

SG significantly modulates the colonic transcriptome. **(A-B)** Enrichment analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) based on differentially expressed genes (DEGs) between SHAM vs. SG **(A)** and SHAM-ABX vs. SG-ABX **(B)**. n = 6-8/group.

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Enhancing intestinal barrier efficiency: A novel metabolic diseases therapy

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Physiologically, the intestinal barrier plays a crucial role in homeostasis and nutrient absorption and prevents pathogenic entry, harmful metabolites, and endotoxin absorption. Recent advances have highlighted the association between severely damaged intestinal barriers and diabetes, obesity, fatty liver, and cardiovascular diseases. Evidence indicates that an abated intestinal barrier leads to endotoxemia associated with systemic inflammation, insulin resistance, diabetes, and lipid accumulation, accelerating obesity and fatty liver diseases. Nonetheless, the specific mechanism of intestinal barrier damage and the effective improvement of the intestinal barrier remain to be explored. Here, we discuss the crosstalk between changes in the intestinal barrier and metabolic disease. This paper also highlights how to improve the gut barrier from the perspective of natural medicine, gut microbiota remodeling, lifestyle interventions, and bariatric surgery. Finally, potential challenges and prospects for the regulation of the gut barrier-metabolic disease axis are discussed, which may provide theoretical guidance for the treatment of metabolic diseases.

KEYWORDS

intestinal barrier, metabolic diseases, natural medicine, gut microbiota remodeling, lifestyle intervention, bariatric surgery

1. Introduction

Metabolic diseases, such as obesity, diabetes, hyperlipidaemia, and non-alcoholic fatty liver disease (NAFLD) have become widespread and significant public health problems (1). Specifically, the WHO released a report on the status of the obesity pandemic in Europe in May 2022, noting that 60% of citizens in the European region were overweight or obese, highlighting the impact of the obesity pandemic (2). The global prevalence of diabetes among individuals aged 20–79 was estimated to be 10.5% (536.6 million people) in 2021, and is expected to rise to 12.2% (783.2 million people) by 2045 (3). From 1991 to 2019, the estimated global prevalence of NAFLD increased sharply from 20 to 30% in the general population (4). Metabolic diseases are often caused by multiple factors, including excessive consumption of processed high-energy foods, lack of exercise, and environmental and genetic factors (5). However, current treatment strategies, including lifestyle changes, dietary and exercise interventions, and drug use, still have limited efficacy. Therefore, there is a need to identify reliable targets for the prevention and treatment of these diseases and their complications.

The intestinal barrier separates the human body from the intestinal microbes, viruses, food antigens, and environmental toxins. In healthy individuals, the intestinal barrier maintains normal gut microbiota and protects mucus layer physiological function (6) and balances epithelial cells and the gut immune system, which can maintain gut homeostasis and is crucial for the dynamic balance of the body (7). However, a significant increase in intestinal permeability has been observed in patients with obesity, NAFLD, and diabetes. The underlying mechanisms may be related to harmful changes in gut pathogenic bacteria and their products, which further increase barrier permeability. Host metabolic states, such as hyperglycaemia and hyperlipidaemia, have also been confirmed to decrease tight junction protein expression and disturb epithelial cell integrity. Both are considered crucial factors for intestinal barrier integrity (6, 8, 9). An impaired gut barrier leads to the translocation of microbiota-derived LPS into the circulatory system, and high circulating LPS levels, a condition referred to as metabolic endotoxemia is associated with obesity and related metabolic disorders (10). In addition, increases harmful gut bacteria, which can further aggravate gut infection and promote gut bacterial translocation to the blood and liver (11). Moreover, impaired gut barriers increase the transfer of intestinal-derived metabolites such as trimethylamine N-oxide, branched-chain amino acid, and indoxyl sulfate from the gut to the systemic circulation, which are also associated with the development of metabolic diseases (12, 13). Meanwhile, a compromised gut barrier can lead to the over-activation of the gut immune system, inducing chronic systemic inflammation or an impaired immune response, which promotes the progression of metabolic diseases (14).

In this review, we describe the critical molecular pathways and mechanisms underlying abnormal gut barrier function in metabolic diseases. Then, we summarize advances made in supporting the improvement of the intestinal barrier using natural medicines, gut microbiota remodeling, and lifestyle interventions. Lastly, we discuss the current challenges and prospects for treating metabolic diseases through the modulation of the intestinal barrier axis.

2. A short review of the intestinal barrier

The intestinal barrier is the primary defense against potentially harmful substances and pathogenic bacteria and consists of a physical barrier, a mucus barrier, and an immunological barrier (15–19). Intestinal physical barrier integrity is regulated by tight junctions and intestinal epithelial cell function. Tight junctions consist of transmembrane proteins such as claudin, occludin, zonula occludens-1 (ZO1), and cingulin between intestinal epithelial cells (20–22). The mucus layer is composed of many components: water, electrolytes, lipids, and about 30 proteins, most of which are produced by specialized secretory goblet cells (GCs), including mucin, human IgGFc-binding protein, calcium-activated chloride channel modulator 1, and zymogen granule protein 16. The mucus layer serves as a barrier covering the intestinal epithelium that prevents direct contact between antigens, toxins, gut flora, and epithelial cells while maintaining permeability to essential nutrients and macromolecules. In addition, at the same time, the outer mucus layer is used as the energy source of some bacteria to stabilize the balance of intestinal flora (23–25). The innate and adaptive immune systems in the intestinal tract are strictly regulated. The immune cells in the intestinal tract cooperate closely (macrophages, monocytes, neutrophils, dendritic cells, natural killer cells, eosinophils, and non-specifically recognized basophils) to achieve and maintain intestinal immune balance (26, 27). However, dietary disorders, diseases, and pressure affect the intestinal barrier function. Mechanically, intestinal barrier dysfunction is first caused by tight connection disorder, the loss of tight junctions causes intestinal mucus layer atrophy and secretory dysfunction (28), which in turn causes further immune cell activation by numerous antigenic molecules or microorganisms through the paracellular pathway, aggravating intestinal immune dysfunction (29).

3. The impaired intestinal barrier is a catalyst for the development of metabolic diseases

Intestinal barrier integrity is impaired in metabolic diseases, including diabetes, hyperlipidaemia, and cardiovascular diseases (30). Numerous studies have confirmed that metabolic disorders and diseases can cause harmful changes in the intestinal microenvironment, including abnormal lipid load, high glucose, high uric acid, and intestinal flora disturbances. This remodeling further damages the integrity of the intestinal barrier (31–33).

First, in the physical barrier section, metabolic diseases can impair the intestinal barrier by affecting the intestinal epithelial cell function and tight junction protein expression. High glucose levels can lead to abnormal intestinal epithelial cell function, abnormal lipids, and abnormal immune responses, and an intestinal microbiota imbalance can induce intestinal epithelial cell tight junction damage, further aggravating intestinal epithelial barrier dysfunction (34, 35). Additionally, metabolic diseases can contribute to abnormalities in the mucus barrier (36). Defects in the colonic mucus layer, characterized by increased permeability and reduced mucus growth rate, have been observed in obese mice. Moreover, the intestinal immune barrier is known to be impaired in metabolic diseases, wherein the main alteration is a decrease in the production of intestinal antimicrobial factors and the promotion of an increased release of pro-inflammatory cytokines, including IL-1b, IL-6, IL-12, and IL-18 (37).

As an accelerator of metabolic diseases, the impairment of the intestinal barrier exacerbates systemic inflammation, impaired energy metabolism, insulin resistance, and abnormalities in glucose and lipid metabolism, thereby accelerating the progression of metabolic diseases (38). For instance, damage to the intestinal barrier can increase intestinal endotoxins in the blood, leading to chronic low-grade inflammation, further promoting the development of metabolic syndrome (39). In a population study, an increased intestinal permeability in pregnant women and an impaired intestinal barrier was found to lead to increased insulin resistance and decreased insulin sensitivity (40). A study of mice fed a high-fat diet found that intestinal barrier dysfunction leads to higher glucose metabolism disorders and liver steatosis (41, 42). Although the pathway by which an impaired intestinal barrier exacerbates metabolic disease has been partially demonstrated, the underlying mechanism still requires

further exploration. With the development of high-throughput analysis, including serum and intestinal metabolomics and intestinal proteomics, the research depth and breadth of microbiota, intestinal barriers, and the host is likely to be further expanded in this future, and providing new directions for prevention and treatment metabolic diseases(Figure 1).

4. Improving the intestinal barrier as a target for the treatment of metabolic diseases

With advances in research, several strategies have been developed to improve the intestinal barrier in the treatment of metabolic diseases in rodent and clinical studies, including drug therapy, adjusting the composition of the intestinal flora, adding probiotics, exercise, diet, and bariatric surgery. In this section, we comprehensively summarize the experimental clinical studies on improving the intestinal barrier using these methods and explore the potential treatment mechanisms of metabolic disease (43–46).

4.1. Drugs improve gut barrier function in metabolic diseases

Currently, treatments for intestinal barrier damage are limited. Clinical drugs used to treat intestinal barriers include natural drugs, short-chain fatty acids, and those that improve intestinal inflammation (47). The main therapeutic targets of these drugs are increasing intestinal tight junction protein expression, improving intestinal cell function, and inhibiting intestinal inflammation. Studies have confirmed that in the clinical treatment of metabolic diseases, drugs such as metformin, berberine, and butyrate have been found to improve disease progression by modulating the intestinal barrier (48). Therefore, in this section, we mainly discuss the function and specific mechanisms of drugs, such as metformin, berberine, and butyrate, used to improve the intestinal barrier and provide new perspectives for treating metabolic diseases (Table 1).

4.1.1. Metformin

Metformin is an oral hypoglycaemic agent that is widely used as first-line treatment for type 2 diabetes. Metformin improves

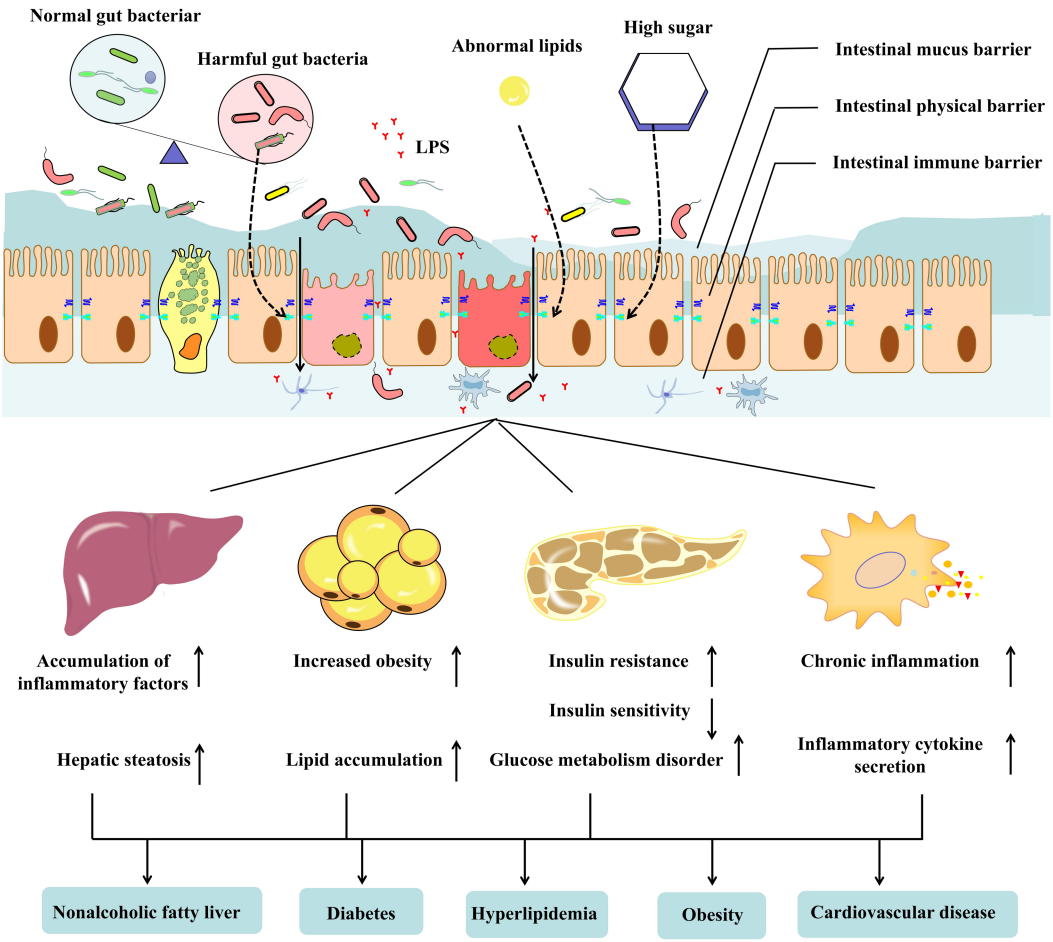

FIGURE 1
The gut barrier consists of gut commensal microbes, mucus, and immune cells in the intestinal epithelium and lamina. Metabolite disorders (hyperglycemia and abnormal lipids) and metabolic diseases, including obesity type 2 diabetes mellitus (T2DM) and non-alcoholic fatty liver disease (NAFLD), lead to gut dysbiosis and disrupt the integrity of the gut barrier. Harmful bacteria or lipopolysaccharides in the intestine enter the blood through the damaged intestinal barrier. It can affect the tissues and organs related to metabolic diseases, resulting in abnormal liver, adipose tissue, pancreas, and immune system functions, aggravating the occurrence and development of metabolic diseases further.

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TABLE 1 Drugs that improve the gut barrier.

Drug name	Source	Signal pathway	Effects	References
Metformin	Mice models	Activates the AMPK pathway	Increases ZO-1, occludin, and claudin-1 expression	(49)
	Mice models	AMPK1-dependent inhibition of JNK signaling activation	Increases ZO-1, occludin, and claudin-1 expression	(50)
	Mice models		Restores the tight junction protein occludin-1 levels	(51)
	Caco-2 cells	Inhibits the MLCK-MLC signaling pathway	Increases tight junction proteins	(52)
	Mice models	Inhibits Wnt signaling	Increases goblet cell mass and mucin production in the gut	(53)
	NAFLD mice		Mitigates the loss of tight junction proteins in the small intestine	(54)
	Caco-2 cells	Inhibits endoplasmic reticulum stress	Reduces intestinal epithelial cell apoptosis and increases tight junction protein expression	(55)
	IBS rats	Reduces PAR-2 expression inhibits ERK activation	Improves the interaction of clau-4 with ZO-1 and clau-4 expression	(56)
Butyrate	IEC cells	Promotes the interaction of transcription factor SP1 with the claudin-1 promoter	Increases claudin-1 transcription	(57)
	Diabetic mice		Increases intercellular adhesion molecules	(58, 59)
	Diabetic mice	Activated NLCR3 binds to GPR43 on colonic epithelial cells	Upregulates expression of the tight junction protein ZO-1/occludin	(60)
	H4 cell		Upregulates tight junction and mucus genes	(61)
	IPEC-J2 cells	Activates the Akt/mTOR-mediated protein synthesis machinery	Enhances the abundance of tight junction proteins	(18)
	IBD mice	Activates GPR109A and inhibits AKT and NF-κB p65 signaling	Improves intestinal epithelial barrier dysfunction	(62)
	CKD rats	Improves AMPK phosphorylation and increases GLP-1 secretion	Promotes colonic mucin and TJ proteins	(63)
Berberine	T2DM rat model		Improves the intestinal mucosa and immune barrier	(64)
	T2DM rat model		Increases ZO-1 expression and repairs damaged intestinal mucosa	(65)
	Caco-2 monolayers	Structural normalization and redistribution of the tight junction protein occludin	Improves intestinal epithelial tight junction injury	(66)
	HT-29/B6 human colon monolayers, rat	Mediated through the tyrosine kinase, pAkt, and NFκB pathways	Prevents the TNF-α-induced claudin-1 disassembly and upregulation of claudin-2	(67)
	T84 colonic epithelial cells	Ability to promote cell migration	Increased expression of intestinal tight junction and adhesion-linked proteins	(68)
	DSS-induced murine UC	Regulation of gut EGC-IEC-immune cell interactions	Improves mucosal inflammation	(69)
	DSS-induced murine UC	JAK–STAT pathway	Inhibits intestinal mucosal inflammation	(70)
	DSS-induced murine UC		Promotes anti-inflammatory and anti-oxidative stress responses	(71)
Infliximab	Crohn's disease patients		Anti-inflammatory effects of TNF-α blockade	(72)
	Crohn's disease patients	Normalization of apoptosis in colonic epithelial cells	Repairs the intestinal barrier	(73)
	Crohn's disease patients		Improves intestinal mucosal barrier	(74)
	Crohn's disease patients	Promotes Th22 cell differentiation and upregulates IL-22 production	Intestinal epithelial barrier repair	(75)
Oregonin	Caco-2 cells		Restores zonula occludens-1 and occludin expression	(76)

(Continued)

TABLE 1 (Continued)

Drug name	Source	Signal pathway	Effects	References
RIPK1 inhibitor	Intestinal epithelial cells	Inhibits necroptosis and the NF-κB signaling pathway	Reduces the disruption of tight junctions and accompanying oxidative stress	(77)
	Murine colitis model	FADD-RIPK1-caspase-3 signaling	Inhibits intestinal epithelial cell necrosis	(78)
Citrus nobiletin	Rat intestinal and Caco-2 cells	Inhibits the Akt-NF-κB-MLCK pathway	Restores damaged barrier function	(79)
Tofacitinib	Caco-2BBe intestinal epithelial cells		Improves intestinal epithelial-macrophage interactions	(80)
	Intestinal epithelial cells (IECs)		Restores claudin-2 expression levels	(6)

hyperglycaemia by inhibiting hepatic glucose production and increasing glucose uptake in muscles (81). Metformin has also been shown to reduce cardiovascular events and improve abnormal lipid metabolism and chronic inflammation (82). Furthermore, metformin has been found to improve the gut barrier, and its metabolic disease-treatment effect is partly based on modulating gut function (83).

Specifically, recent studies have confirmed that metformin can improve intestinal physical barrier function by increasing the expression of intestinal tight junction proteins (ZO-1, occludin, and claudin-1) by activating the AMPK pathway, reducing the entry of LPS into the blood and the inflammatory response to body stimuli (49–51). Cell experiments have also confirmed that metformin can stabilize and upregulate the expression of tight junction proteins by inhibiting the MLCK-MLC signaling pathway, thereby improving the tight junctions of intestinal epithelial cells (52). Moreover, metformin was found to improve the intestinal mucus barrier by beneficially regulating the quality of goblet cells and mucin production. It can be used to prevent and treat metabolic diseases in obese individuals and individuals on a western high-fat diet (53).

In addition, maintaining bile acid homeostasis can improve the intestinal barrier and prevent bacterial translocation in the intestinal tract, demonstrating efficacy in the treatment of metabolic liver disease (84). Interestingly, metformin can promote bile acid homeostasis in the liver and intestines. We speculated that metformin can target the homeostasis of the bile acid-intestinal barrier axis and be used to develop new methods for the treatment of metabolic diseases (85). Glucagon-like peptide-1 (GLP-1) is a peptide hormone in the gut that plays a central role in coordinating postprandial glucose homeostasis. The administration of GLP-1 and glucagon-like peptide-2 (GLP-2) receptor agonists promotes intestinal barrier function in mice (86). Interestingly, the insulin sensitiser metformin increased circulating GLP-1 concentrations and the relative number of intestinal L cells (87). Therefore, it is reasonable to believe that the improvement of the intestinal barrier by metformin is partly dependent on the regulation of GLP-1 (88). Notably, metformin concentrations are much higher in the gut than in the plasma. Although there is reason to believe that the maintenance of the gut barrier and gut axis plays a role in the efficacy of metformin, an understanding of the mechanisms by which metformin promotes a healthy gut barrier will require a systems-level approach.

4.1.2. Butyrate

Butyrate is a four-carbon short-chain fatty acid fermented by the intestinal flora through dietary fiber (89). It meets most of the energy needs of colonic epithelial cells and is required for cellular energy metabolism and the maintenance of intestinal homeostasis. Butyrate supplementation has been investigated for its potential protective and ameliorative effects on a wide range of human diseases, including type 2 diabetes, cardiovascular disease, dyslipidaemia, and non-alcoholic fatty liver disease (90). For example, a metagenomic analysis of type 2 diabetes found that butyrate-producing bacteria had a reduced proportion of the overall gut flora, whereas butyrate supplementation could treat diabetes by increasing the integrity of the gut barrier (91–93). Moreover, butyrate may reduce diet-induced barrier dysfunction to improve diet-induced obesity (58, 94). In summary, the improvement of metabolic diseases by butyrate mainly depends on the regulation of the intestinal barrier.

Next, we analyzed the specific mechanism by which butyrate regulates the intestinal barrier. Butyrate regulates the intestinal barrier by regulating the physical barrier. In diabetic mice, butyrate stimulates the expression of NLRC3 in colonic epithelial cells, increases the phosphorylation of AMPK, and upregulates tight junction proteins and TJs in colonic epithelial cells. In addition, butyrate can upregulate the transcription of tight junction and mucus genes in epithelial cells (H4 cells), increase claudin-1 expression, and stabilize intestinal epithelial cell functions (60). Additionally, butyrate regulates the repair of the intestinal mucus barrier by activating the macrophage/WNT/ERK signaling pathway (95). Furthermore, butyrate can effectively inhibit the activation, proliferation, and production of cytokines (IFNγ and IL-17) by CD4 T cells, thereby maintaining the intestinal immune barrier and regulating the integrity of the epithelial barrier (96). Numerous studies have also shown, butyrate affects epithelial O2 consumption through epithelial β-oxidation and maintains the stability of hypoxia-inducible factor (HIF). HIF is a transcription factor that coordinates the protection of the barrier, which is essential to maintain the integrity of the intestinal barrier (97–99). Therefore, the treatment and prevention of metabolic diseases from the perspective of butyrate and improving the intestinal barrier are considerable goals, highlighting the need to maintain normal levels of butyrate in the gut during metabolic disease.

Increased levels of intestinal butyrate have been reported in metabolic disease states, notably through the supplementation of

butyrate production and the number of bacteria producing butyrate, including *Clostridium butyricum* and *F. prausnitzii*. Other studies have demonstrated that adding modified high-amylose maize-resistant starch increases butyrate concentrations in feces and plasma, thereby decreasing blood sugar levels (31). In addition, the fructooligosaccharide supplement was sufficient to increase butyrate levels, contributing to the balance of host energy. These supplements can be fermented into short-chain fatty acids, such as butyrate, by the hydrolytic enzyme system of beneficial bacteria. Specific genetically modified *Escherichia coli* can promote butyrate levels in the intestine by increasing the production capacity of butyrate (100). A limited number of human studies have shown that butyrate has a good clinical effect in improving the intestinal barrier in metabolic diseases. However, more clinical studies are needed to further confirm its effectiveness and safety in treating metabolic disorders. In addition, the delivery of butyrate to peripheral tissues *via* oral administration is poor because it is absorbed and metabolized by the colon and liver. In treating metabolic diseases, the therapeutic action of butyrate is mainly exerted *via* improvements in the intestinal barrier to enhance the intestinal microenvironment and physiological function, rather than directly affecting peripheral tissues and organs (61, 63). The energy source of colon cells mainly depends on SCFA, especially butyrate, so it may improve the intestinal barrier by improving the function of intestinal epithelial cells. In a study of sodium butyrate for the treatment of intestinal inflammation, sodium butyrate was found to reduce harmful pathogenic bacteria in the gut (such as *Bacteroides, Clostridium, Helicobacter pylori*, and *Desulfovibrio*), suggesting that sodium butyrate may improve the intestinal barrier by beneficially modulating the intestinal microbiota (101, 102).

4.1.3. Berberine

A recent study on berberine demonstrated its metabolic and pathophysiological roles in metabolic disorders, suggesting that it plays a promising role in metabolic diseases, such as obesity, NAFLD, diabetes mellitus, and hyperlipidaemia. For example, it promotes insulin secretion, improves insulin resistance, inhibits adipogenesis, reduces adipose tissue fibrosis, reduces liver steatosis, and improves intestinal flora disturbances (103). Notably, similar studies further confirmed that the function of berberine in the treatment of metabolic diseases largely depends on regulation of the intestinal barrier (64, 104). In addtion, berberine supplementation can improve intestinal flora, regulate innate immunity and improve energy metabolism (105).

The improvement of intestinal barrier function by berberine is multifaceted. First, berberine modulates the intestinal barrier by regulating the physical barrier. The treatment of diabetic rats with berberine restored tight junction protein expression in the intestinal epithelial cells and improved the intestinal barrier (65). In addition, berberine can directly modulate the function of intestinal epithelial cells to repair damaged intestines by promoting differentiation of intestinal stem cells and enhancing cell migration. Second, berberine was found to significantly reduce chronic intestinal inflammation to maintain the intestinal immune barrier function (66). Further studies found that berberine ameliorated pro-inflammatory cytokine-induced tight junction damage in the intestinal epithelium by downregulating the aberrant activation of the TNF-α-NF-κB-MLCK pathway or inhibiting TNF-α, thereby upregulating the expression of tight junction proteins (67). In addition, berberine can ameliorate mucosal inflammation by modulating intestinal epithelial cell and immune cell interactions. Third, by modulating the intestinal barrier through the mucus barrier, oral berberine significantly increased the transcription of mucus-secreting genes and the production of host mucus proteins (68). In addition, berberine also stimulated the growth of the probiotic *Akkermansia*, suggesting that berberine's improvement of the intestinal barrier may be dependent on the restoration of beneficial intestinal bacterial populations (106).

Notably, although the natural alkaloid berberine has shown promising results in the treatment of the intestinal barrier and metabolic diseases, its clinical application is hampered by its poor gastrointestinal absorption, low bioavailability, and gastrointestinal side effects. In this context, metabolomics and proteomics can be used to explore the mechanism of its treatment of metabolic diseases and study its pharmacokinetics, metabolism, and general safety *in vivo* to improve the intestinal barrier more effectively, while ensuring that it has fewer side effects in humans, thus treating diabetes from multiple perspectives.

4.1.4. Drugs that regulate intestinal inflammation

The pathological state of intestinal inflammation leads to the recruitment of large numbers of immune cells and the release of inflammatory mediators, which affects the physical function of the intestinal barrier by disrupting the structure and intestinal epithelial intercellular junctions (74) and inhibiting intestinal mucosal repair (72). A large body of research has now confirmed that modulating intestinal inflammation is another strategy for restoring and improving impaired intestinal barriers and that intestinal immunomodulatory drugs focus on inhibiting immune factor release and immune cell activation in the first place. In the following, we present an overview of intestinal immunomodulatory drugs, highlighting that they improve intestinal barrier function as evidence and mechanisms for treating metabolic diseases (73).

According to the literature, immunomodulatory drugs can be divided into two categories based on the inhibition of inflammatory factors and the modulation of immune cells (75). First of all, immunomodulatory drugs can reduce the outbreak of intestinal inflammation by decreasing the inflammatory factors in the abnormal outbreak of metabolic diseases and the damage of inflammatory factors to the intestinal barrier and reducing the outbreak of intestinal inflammation (107). It has been noted that the eruption of intestinal pro-inflammatory cytokines (76), such as tumor necrosis factor-a (TNF-a), interleukin (IL)-1, IL-6, IL-9, IL-13, and IL-33, plays a role in the impairment of the intestinal barrier. Anti-IL-6 and anti-TNF-α therapy have been found to improve intestinal permeability by inhibiting the intestinal inflammation caused by the explosion of inflammatory factors (108), possibly mediated through the restoration of expression of intestinal tight junction protein zona occludens-1 (ZO-1) and occludin (109). They are also thought to regulate the reduction of intestinal pro-inflammatory factors, including the iron-binding glycoprotein lactoferrin (LF) and citrin, to restore impaired intestinal barrier function. The close interaction between intestinal epithelial cells and immune cells is essential for maintaining intestinal barrier function (79). Myocardial fibrosis improvement in type 2 diabetes has been reported in commonly used rat models of diabetes, and a recent study implicated it as a receptor-interacting protein kinase 1 (RIPK1) inhibitor (77). RIPK1 inhibitors have been shown to maintain the balance of the immune microenvironment, which normally improves the intestinal barrier by inhibiting the interaction

between intestinal epithelial cells (IECs) and immune cells *in vivo* and *in vitro* (110).

Although a large number of studies have shown that systemic inflammation is a potential driver for the development of metabolic diseases, it is believed that interventions to treat inflammation add to the burden of disease and complexity of healthcare. Therefore, we propose that personalized intestinal inflammatory interventions to improve the intestinal barrier for the treatment of metabolic diseases may be a future development in the management of diseases, such as obesity, T2DM, and NAFLD (111). However, the measures required to design effective personalized inflammatory interventions for treatment require considerable refinement. Therefore, in the future, more detailed information will be needed in clinical trials of drugs for the control of metabolic diseases than is currently documented in trials, and is essential for repurposing or developing immunomodulatory therapies to treat metabolic diseases.

4.2. Regulating the composition of the intestinal flora to improve the intestinal barrier

Over the past 20 years, research has shown that the gut flora under normal physiological conditions may help maintain the metabolic health of the human host (5). Further studies have found that metabolic disease states, such as obesity, dyslipidaemia, insulin resistance, and low inflammation, often lead to dysbiosis of the intestinal flora. Intestinal dysbiosis is manifested by an increased abundance of "pro-inflammatory" bacterial strains, such as *Ruminococcus gnavus* or *Bacteroides* species, in the gut, while "anti-inflammatory" strains, such as *Faecalibacterium prausnitzii*, show low abundance (112, 113). Microbial dysbiosis further aggravates the development of metabolic diseases by inducing the disruption of the intestinal barrier (114). A study of T2D patients found that diabetes can cause excessive growth of intestinal flora, increase intestinal permeability, and damage the intestinal barrier (115, 116). The conclusion drawn from extensive data is that dysbiosis of the intestinal flora leads to disruption of the intestinal barrier, mainly through the induction of increased intestinal oxidative stress in the intestinal epithelium, reduced expression of tight junction proteins (such as claudin, occludin, and zonula occludens), and increased mucus degradation (117, 118). Therefore, restoring intestinal barrier function by regulating intestinal flora dysbiosis represents a new approach for the prevention and treatment of metabolic diseases. Currently, there are three ways to modulate the composition of gut microbiota: (i) supplement probiotics, (ii) intervene with specific microbial species using drugs, and (iii) transplant normal intestinal flora to restore the normal intestinal flora ecosystem. The specific mechanism may involve correcting intestinal flora disturbance, increasing the expression of intestinal barrier function proteins, maintaining the normal function of intestinal epithelial cells, and improving intestinal barrier function (119, 120) (Figure 2).

4.2.1. Probiotic supplementation

In the last decade, probiotics [gram-negative anaerobic bacteria *Akkermansia muciniphila* (121), *Lactobacillus reuteri* (122), and gram-positive anaerobic bacteria *Bifidobacterium* (123), *Roseburia intestinalis* (124)] have been widely used to prevent and treat various diseases, especially metabolic diseases, such as obesity, diabetes, non-alcoholic fatty liver, and hyperlipidaemia, and to improve the microecological balance in the host gut to inhibit the proliferation of harmful bacteria and improve the barrier function of the gastrointestinal tract for the treatment of metabolic diseases (125). In this section, we explore the precise mechanisms by which improving the gut barrier can be used to treat metabolic diseases.

Although probiotics are the most commonly used substances that regulate intestinal flora homeostasis, which can strengthen the intestinal barrier by enhancing epithelial defense function and regulating intestinal microbiota, their application continues to face several challenges (126, 127). Studies have shown that *lactobacillus* maintains intestinal epithelial regeneration and repairs damaged intestinal mucosa (128). Numerous studies have shown that probiotics improve the mechanism of the intestinal barrier by influencing the renewal of intestinal epithelial cells, increasing the production of tight junction proteins, and increasing mucin secretion while promoting the immune system.

The use of probiotic *Bifidobacterium bifidum* strains in obese individuals has shown to improve gut barrier function (129, 130). *Akkermansia muciniphila* and its derived extracellular vesicles (AmEVs) can exert anti-diabetic effects by reducing intestinal barrier disruption and insulin resistance (131, 132). In addition, 14 probiotic species have been found to improve intestinal barrier function in db/db mice (114). Probiotics can improve intestinal epithelial cell function and tight intercellular junctions, and restore intestinal physical barrier function (133). *Escherichia coli* strain Nissle 1917 (EcN) is a Gram-negative probiotic found to modulate the expression and localization of intestinal tight junction proteins, and consequently, enhance intestinal barrier function (134, 135). The probiotic EcN repairs the intestinal epithelial barrier by decreasing the secretion of inflammatory cytokines while increasing the production of anti-inflammatory factors, eliminating reactive oxygen species at the site of inflammation, and effectively relieving symptoms of inflammation (136, 137). Furthermore, EcN *in situ* production of therapeutic protein matrices consisting of coiled nanofibers of trefoil factors (TFFs) can promote mucosal healing and restore mucus barrier function (138–140). In addition, probiotic therapy protects the intestinal epithelial barrier by mitigating a reduction in the expression of tight junction proteins that caused by an intestinal ecological disorder in metabolic diseases and increasing the rate of apoptosis (122, 141). Probiotics are widely used in the treatment of NAFLD as they improve the function of the intestinal immune barrier and inhibit the proliferation and translocation of harmful bacteria. Additional studies have shown that *Bifidobacterium* can enhance the function of the intestinal mucus layer and phagocytes through the activation of intestinal autophagy and calcium signaling pathways (142). Probiotic formulations have also been found to induce an increased expression and secretion of mucin (MUC1 and MUC3) in colonic epithelial cells, improving intestinal mucus barrier function (143). In general, these findings contribute to a better understanding of the complex and beneficial interactions between probiotics and colonic epithelial cells in the gut to restore impaired intestinal barrier function in metabolic diseases (144).

Further studies should screen out effective probiotics *via* the high-throughput analysis of the intestinal flora and, at the same time, explore how probiotics restore the imbalance of the intestinal flora. This is expected to improve the barrier function of the gut by targeting specific probiotics to modulate the dysbiosis of the intestinal flora.

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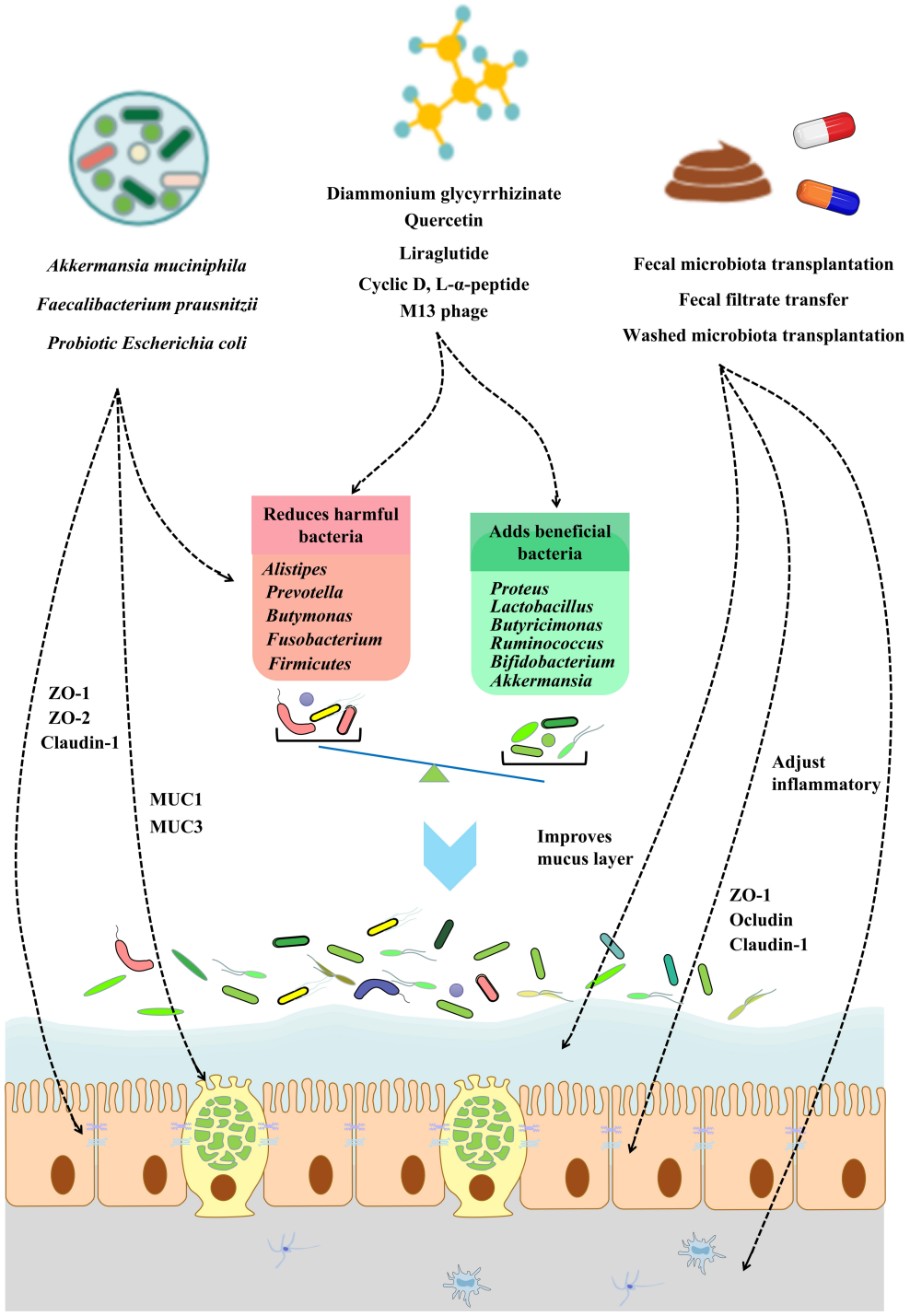

FIGURE 2
The role of modulating gut microbiota in improving gut barrier integrity. The gut barrier can be affected directly or indirectly by the gut microbiota. The modulation of the gut microbiota can occur *via* probiotics, small-molecule compounds, and fecal transplantation. Restoring the gut microbiota balance can restore gut barrier integrity *via* gut epithelial cell function and tight junction protein expression, improving the mucus barrier and adjusting inflammatory and inflammation. Further improvements of the intestinal barrier can be used to relieve or treat metabolic diseases.

With advances in technology, the targeting proteins of probiotics should be further identified through metabolomics and proteomics to provide a reliable theoretical basis for probiotic targeting and to improve the intestinal barrier. Oral probiotics are affected by metabolic disease-induced pathological inflammatory microenvironments, such as reactive oxygen species (ROS) and depleted mucus layers, which limit their survival and their colonization of the gut (137). Therefore, improving probiotic intestinal colonization by modulating the intestinal microenvironment or modifying probiotics is expected to provide an important perspective for the treatment of various metabolic diseases. In a previous study, a synthetic biology approach was used to develop an engineered probiotic with superior resistance

to the harsh environment of the gastrointestinal tract to enhance the colonization and growth of probiotics in the mucus layer (145). In addition, through a composite biomagnetic material composed of tiny magnetic particles and probiotics, an external magnetic field can capture and retain probiotics in the gastrointestinal tract of mice, thereby improving the accumulation and stable colonization of probiotics under specific conditions. In summary, in-depth research on probiotics has led to the development of a variety of effective and safe probiotics. In the later stages of research, the focus can be shifted to the transformation of probiotics to make them more suitable for the intestinal environment in the disease state to improve the intestinal barrier more effectively, maintain intestinal homeostasis, and exert their therapeutic effect on metabolic diseases.

4.2.2. Small molecule compounds engineer the gut microbiome

In some ways, the gut microbiota is the largest "organ" of the body, and its composition is species-diverse. Recently, it has been proposed that gut microbiota is involved in the development and progression of metabolic diseases. Host health and disease status can be maintained and improved by modulating the imbalanced gut microbiota, including beneficial and harmful bacteria, based on the symbiotic or antagonistic relationships between various microorganisms. The "gut microbiota barrier axis" can be used as an alternative target for the treatment of metabolic diseases. It restores the impaired intestinal barrier in metabolic disease states by altering the structure and composition of intestinal flora.

Specifically, diammonium glycyrrhizinate (DG) was found to reduce the ratio of *Firmicutes* to *Bacteroidetes* and endotoxin-producing bacteria, such as *Desulfovibrio*, and increase probiotics, such as *Proteus* and *Lactobacillus*, in animal models (146). It also increased the levels of short-chain fatty acid (SCFA)-producing bacteria such as *Ruminococcus* and *Lachnospira* (147), significantly alleviating low-grade intestinal inflammation, improving tight junction protein expression, goblet cell number, and mucin secretion, and enhancing intestinal barrier function to prevent non-alcoholic fatty liver disease in mice (146). In NAFLD treatment studies, obeticholic acid was found to lessen endotoxemia and inflammation levels by reversing gut flora imbalance, particularly increasing the abundance of *Blautia*, and restoring gut barrier function to improve NAFLD (148, 149). Furthermore, the therapeutic effects of liraglutide may be due to improved gut microbiota structure associated with hepatic steatosis (150). In a study on obesity and diabetes, it was found that the intestinal flora can be adjusted by drugs, such as Akebia saponin D (151) and Ganoderma lucidum (152) which significantly reduce the HFD-related *Alistipes* and *Prevotella*, increase the proportion of *Butyricimonas, Ruminococcus*, and *Bifidobacterium*, and increase the abundance of the anti-obesity bacterium *Akkermansia*. Reverse HFD-induced gut dysbiosis, such as reduced *Firmicutes* to *Bacteroidetes* ratios and endotoxin-carrying *Proteobacteria* levels, maintains gut barrier integrity, reduces metabolic endotoxemia, and improves obesity and diabetes (152, 153).

Overall, these results led us to speculate that these drugs could be used to treat metabolic diseases by improving the composition and structure of intestinal microbiota in mice to reduce intestinal permeability. However, the structure of the intestinal flora is complex and rich in diversity. The regulation of intestinal flora by common drugs is often accompanied by interference with the growth of normal intestinal flora.

Next, we discuss the therapeutic potential of targeting gut microbiota based on existing research. Cyclic D-and L-alpha-peptides, using an *in vitro* drug screening protocol, were selected to improve the integrity of the intestinal barrier and inhibit the development of atherosclerosis through the molecular reprogramming of the microbiome transcriptome *via* the selective alteration of bacterial growth. In a colorectal cancer (CRC) study, a specific M13 phage was screened using phage technology to achieve the specific clearance of Fusobacterium nucleatum and to remodel the tumor immune microenvironment (151). Recent studies have demonstrated a correlation between gut phage composition and host health, phage therapy as an antibacterial agent, and the application of genetically engineered phages in gut microbiome remodeling (154, 155). Directed chemical manipulation provides additional tools for deciphering the chemical biology of the gut microbiome and designing phage-containing supplements to target remodeling of the gut microbiota (156). The elimination of specific pathogens can correct gut dysbiosis and improve gut barrier function (157). Therefore, the targeted remodeling of the microbiome should be explored in the future for the treatment of metabolic diseases (158). In a colorectal cancer (CRC) study, a specific M13 phage was screened using phage technology to achieve specific clearance of *Fusobacterium nucleatum* and to remodel the tumor immune microenvironment. Recent studies have demonstrated a correlation between gut phage composition and host health, phage therapy as an antibacterial agent, and the application of genetically engineered phages in gut microbiome remodeling (159, 160). Directed chemical manipulation provides additional tools for deciphering the chemical biology of the gut microbiome and designing phage-containing supplements to target remodeling of the gut microbiota. The elimination of specific pathogens can correct gut dysbiosis and improve the gut barrier. Therefore, the targeted remodeling of the microbiome should be explored in the future for the treatment of metabolic diseases.

4.2.3. Transplanting the normal intestinal flora

Gut microbiota transplantation is where gut microbiota from a healthy donor is transplanted into a patient's gastrointestinal tract. Previously, this therapy was used to treat gastrointestinal diseases caused by pathogenic microorganisms or opportunistic microbial activities (161). However, a growing number of studies have recently reported on the use of fecal microbiota transplantation for metabolic syndrome, diabetes, and other diseases (162). Population studies have found that fecal microbiota transplantation leads to increased insulin sensitivity in patients with metabolic syndrome and improved glucose metabolism, with the effectiveness of treatment depending on improved gut microbiota and changes in plasma metabolites associated with increased beneficial intestinal metabolites (163, 164). The efficacy of fecal transplants in metabolic diseases is well documented and relies on improvement of the intestinal barrier. Washed microbiota transplantation has been shown to effectively improve compromised gut barrier function, significantly reducing the level of endotoxins and thus reducing the symptoms of gout patients (165). The transplantation of normal fecal microbiota into a mouse model of disease was found to normalize intestinal permeability, thereby significantly reducing metabolic endotoxemia, reversing weight gain, and achieving glucose tolerance (166).

With regard to the apparent restorative effect of intestinal flora transplantation on the intestinal barrier, the underlying mechanisms include restoring dysregulated intestinal flora or acting directly on the host intestine to improve the intestinal barrier. Firstly, flora transplantation improves intestinal tight junctions and increases the expression of intestinal barrier function proteins, including ZO-1, occluding, and claudin-1 (167, 168). Simultaneously, it improves the mucus layer components to protect the function of the mucus barrier. In addition, it significantly modulates the function of intestinal epithelial cells and reduces the loss of villi and epithelial cells by inhibiting epithelial cell apoptosis. Moreover, the beneficial effects of FMT on intestinal barrier function can reduce intestinal inflammation and inhibit inflammatory cell infiltration, thereby reducing the level of systemic inflammation and resulting in a significant reduction in systemic endotoxemia (169, 170). Studies have found that flora transplantation reduces intestinal epithelial cell damage caused by pathogens in the gut and restores the damage to the intestinal barrier caused by the dysbiosis of the intestinal flora. In addition, fecal microbiota transplantation reduced Bacteroidetes and Desulfovibrio, altering the imbalance in the gut microbiota and restoring the richness and diversity of intestinal flora (171). Thus, the intestinal inflammation and intestinal mucosal destruction induced by the dysbiosis of the intestinal flora in metabolic diseases are alleviated.

Although most existing studies show that fecal transplantation has beneficial effects, attention should also be paid to its safety, especially in patients with metabolic diseases that are often accompanied by systemic diseases that decrease immunity. Adverse events have been reported in seven patients who received FMT from fecal donors colonized with Shiga toxin-producing Escherichia coli (STEC) in a clinical study (172). In this context, improved screening and pre-transplant management may reduce adverse events. A preliminary study of five patients with CDI showed that the transfer of sterile filtrate from donor feces (FFT) containing bacterial fragments, proteins, antimicrobial compounds, metabolites, and oligonucleotides/DNA rather than intact microorganisms was sufficient to restore normal bowel habits and eliminate symptoms (173). This finding suggests that bacterial components, metabolites, or phages mediate many of the effects of FMT, and that FFT may be an alternative, especially in immunocompromised patients. In addition, another study proposed for the first time that washed microbiota transplantation (WMT) is safer, more precise, and quality-controllable than manual crude FMT (174). Overall, follow-up studies on fecal transplantation should focus on further strengthening its safety under the premise of ensuring efficacy. Modifying and optimizing the intestinal flora before transplantation is necessary, and it will be beneficial to improve the efficacy of intestinal flora transplantation and reduce the occurrence of adverse events.

4.3. Lifestyle interventions

An unreasonable lifestyle is one of the main factors leading to the high incidence of modern metabolic diseases (175, 176). Lifestyle interventions, including physical activity and healthy eating habits, have the aim of controlling weight and reducing the risk factors related to metabolic diseases (177). Studies have reported that a lack of physical activity and unhealthy diet are likely to lead to diabetes and significantly increase the risk of major cardiovascular events (178, 179). This emphasizes the importance of lifestyle changes. No matter the current metabolic state, maintaining a healthy lifestyle can reduce the risk of developing metabolic diseases.

4.3.1. Reasonable exercise

Exercise increases the body's metabolism and regulates the function of the body's organs (180), and has been regarded as a treatment prescription for metabolic diseases (181). In a clinical study, sustained moderate exercise increased insulin signaling, decreased lipogenesis and weight loss, and reversed the risk factors for metabolic syndrome (182). A randomized controlled trial found that exercise improved metabolic profile and insulin sensitivity, reduced abdominal fat, and maintained liver fat, blood sugar, and cardiorespiratory fitness in patients with type 2 diabetes (183, 184).

A growing body of research has focused on the use of exercise to treat metabolic diseases by improving the gut barrier. In a six-month exercise training study on 30 T2D patients, long-term exercise was found to reduce gut permeability, improve systemic hypoglycaemia and inflammation, and control diabetes (185). In addition, exercise has been shown to reduce HFD-induced obesity and intestinal barrier damage by modulating lipid metabolism (186) or activating the AMPK/CDX2 signaling pathway (187). This suggests a potential mechanism by which long-term exercise can improve gut barrier integrity (188). Furthermore, the function of exercise in the gut barrier is mainly dependent on the modulation of intestinal epithelial cell function and gut microbiota. For example, exercise can upregulate the expression of claudin-1 and occludin proteins, suggesting that exercise may regulate barrier integrity through tight junctions (189). Moreover, exercise can increase the number of beneficial microbial species, enrich the diversity of microbial communities, promote the development of commensal bacteria, and remodel the gut microbial ecosystem, thus protecting the gut barrier, preventing the dysregulation of the gut-liver axis, and reducing circulating LPS levels, thereby helping to relieve chronic inflammation (190, 191).

Although exercise is widely promoted as a healthy habit in contemporary society, excessive exercise represents a significant health concern. Studies have shown that excessive exercise often leads to impaired intestinal epithelial barrier integrity and gastrointestinal disease. Thus, determining the optimal exercise dose with which to manage metabolic diseases is vital. Recent studies suggest that metabolic disease can be improved significantly by 30 min of moderate-intensity cardio once a week (192). Therefore, an appropriate amount of exercise is suggested, especially in patients with metabolic diseases. Recent clinical trials have found that dietary exercise programs have shown positive effects. Notably, high-intensity interval training (HIIT) with time-restricted eating (TRE) improves cardiometabolic health in at-risk populations (193). In addition, with plant extracts of polyphenols, glycaemic control improves the oxidative capacity of skeletal muscle and intestinal mucosal function (194). Hence, there is an urgent need for more clinical trials on the delivery of rhythm-exercise diet interventions to investigate the long-term effects and feasibility of these interventions over longer durations.

4.3.2. Diet composition adjustment

Unhealthy dietary patterns can lead to hyperinsulinaemia, insulin resistance, dyslipidaemia, low-grade systemic inflammation, and endotoxemia. These pathological processes are closely related to metabolic diseases, such as obesity, type 2 diabetes, hyperlipidaemia,

cardiovascular disease, and non-alcoholic fatty liver disease (195). Furthermore, unhealthy dietary patterns promote altered gut function, leading to gut barrier dysfunction, increased permeability, and microbiota dysbiosis (196). Many population-based dietary intervention studies have found that metabolic diseases can be improved by optimizing the dietary structure and components, such as polyphenol-rich diets, increasing dietary fiber, specific vitamin supplements, and energy-restricted diets, which depend on improving intestinal barrier function (197). In the following, we will take a closer look at these diets and intestinal barrier function.

Polyphenols are a well-known class of bioactive compounds that are widely distributed in the plant kingdom and are abundant in plant-based and plant-derived foods. The biological activity of polyphenols has been studied using various *in vitro* and *in vivo* experimental models. These studies have shown their potential to help maintain health and prevent, delay, or reduce the number of chronic diseases (198). The biological functions of polyphenols include antioxidant, anti-inflammatory, and immunomodulatory activities at the intestinal and systemic levels (199). A study of life interventions in elderly subjects found that a diet rich in polyphenols can reduce serum zonulin levels and improve intestinal permeability. Although the precise molecular mechanism is not fully understood, polyphenols can, directly and indirectly, act on different levels of the intestinal barrier by regulating tight junction function, the production of numerous inflammatory cytokines, and the activation of antioxidant genes. This mechanism may improve the intestinal barrier by increasing the expression of tight junction proteins (ZO-1 and occludin) and mucin, and balancing the immune response interaction in the colon (200, 201). In addition, polyphenols undergo extensive alterations in the gut microbiota, thus affecting the gut microbial ecosystem (201, 202). In conclusion, these findings preliminarily reveal the complex relationship between dietary supplementation with polyphenols, intestinal barrier function, and metabolic diseases. The molecular pathways underlying this function using an integrated multi-omics approach (food components, microbiota, gut proteomics, and metabolomics) provide a theoretical basis for future population studies on polyphenol diets (203). In this context, further population studies will be needed to optimize the formulation of personalized polyphenol dietary interventions.

Dietary fiber contains various plant-based compounds that are not fully digested in the human gut, including insoluble fibers, such as cellulose, hemicellulose, and lignin, and soluble fibers, such as pectin, beta-glucan, and hydrocolloids (204). Dietary fiber is a crucial component of the diet. A meta-analysis of randomized controlled trials found that intake of soluble fiber supplements was effective in controlling blood glucose and improving insulin resistance and BMI levels in patients with type 2 diabetes (205). Results of the large-scale NutriNet-Santé prospective cohort study (2009–2019) showed that dietary fiber intake was inversely associated with the risk of mortality from several chronic diseases (cardiovascular disease, cancer, type 2 diabetes) (206). Studies have shown that chronic or intermittent dietary fiber deficiency can lead to the erosion of the colonic mucus barrier and intestinal barrier dysfunction (207, 208). Dietary fiber treatment can increase the thickness of mucus and the number and function of goblet cells (23), and prevent the increase of mucus permeabilityreduce mucus thickness, inflammation, and intestinal damage in mice (209), thereby strengthening the intestinal barrier (210). Specifically, fiber supplements have been found to improve the intestinal barrier of C57BL/6 and $Ldlr^{-/-}$ mice by increasing the colonic mucin layer, reducing systemic inflammation, and significantly reducing WD-induced metabolic disease. In addition, a high-fiber diet can improve intestinal barrier function by regulating immune regulatory cells and increasing intestinal tight junction proteins, thereby reducing the development of autoimmune hepatitis (211). In addition, dietary fiber intervention correlates with host gut microbiota. While identifying beneficial bacterial strains, dietary fiber reshapes the gut microbiome in metabolic diseases according to the preference of bacteria that use the specific and ingested dietary fiber (212). For example, the beneficial effect of dietary fiber on T2D is achieved by increasing butyrate levels and the abundance of beneficial bacteria (*Lachnobacterium, Parabacteroides, Faecalibacterium, Akkermansia*, some butyrate-producing bacteria and SCFA-producing strains) (213), while also reducing 12α-hydroxylation Production of bile acids, acylcarnitines, and metabolically harmful compounds such as indole and hydrogen sulfide (214). Thus, additional well-studied types and sources of dietary fiber are needed to determine the role of metabolic diseases and how dietary fiber diets become precision nutrition and metabolic disease treatment strategies by design.

Vitamin consumption through diet is crucial for controlling a variety of physiological functions, including metabolism. The intestinal tract is the primary absorption site for vitamins A, D, E, and K in the human diet (215).According to research, vitamins A and D may have an impact on the onset of obesity, type 2 diabetes, liver steatosis, and steatohepatitis (216, 217). Additionally, there is growing proof that intestinal barrier function will be harmed by vitamin deficit or excess (218). The topic of enhancing the intestinal barrier with vitamin supplements to treat metabolic illnesses will be covered next (219). First, the study discovered that by increasing tight junction protein expression, vitamins A and D can enhance intestinal barrier function (220–222). Specifically, vitamin A and vitamin D can strengthen the intestinal epithelial barrier function by stabilizing the mucosal immune system, thus affecting the process of intestinal inflammation (223). Additionally, the administration of vitamin A and vitamin D can influence intestinal microflora, including elevating the number of helpful bacteria (like *Clostridaceae*) and lowering the number of pathogenic bacteria (like *Streptococcaceae*) (224), which can also improve the relevant metabolites of the intestinal microflora, like elevating the production of SCFA (225), which can effectively improve metabolic diseases and restore the intestinal barrier function. Recent research has also demonstrated the ability of VB12 oral supplement to participate in the epigenetic modification of intestinal barrier genes, limit the colonization of harmful bacteria, and coordinate the functions of ileal epithelial cells (iEC) and intestinal microbiota (226). Although vitamins have a substantial causal role in metabolic disorders, further research is needed on how specific vitamin intake and type (whether in excess or deficiency) affect the gut barrier, gut bacteria, and the potential to treat metabolic disease.

Investigating the effects of dietary energy restriction (ER) is an active area of research. Dietary ER protocols involve dietary regimens associated with limiting the total daily energy intake or allocating energy intake to specific periods of the day. Dietary ER has been shown to be feasible and effective for weight loss, as well as for the treatment of other metabolic diseases by improving insulin sensitivity and inflammatory markers (227). In addition, one study found that the expression of the tight junction markers claudin-2 and zonula occludens-1 was elevated in the colon of mice in the ER-treated group,

suggesting that ER may treat metabolic diseases by regulating the intestinal barrier (228, 229). It should not be overlooked that both energy and protein deficiencies may contribute to age-related bone loss, and patients with osteopenia or osteoporosis must exercise caution when implementing severe energy restrictions (230, 231). In addition, although energy-restricted diets are effective in weight loss, they are difficult to maintain after the resumption of feeding. Studies have found crosstalk between microbiota and bile acids in weight regain and the addition of *Parabacteroides distasonis*, a potential probiotic that could prevent rapid weight gain after calorie restriction diets (232). Further research into dieting and weight regain in humans is therefore necessary to develop nutritional supplements to replace the beneficial bacteria that individuals lose and reduce the incidence of malnutrition.

4.3.3. The spatiotemporal regulation of the diet

High-frequency excessive food intake and an irregular diet often lead to metabolic diseases, intestinal barrier damage and intestinal dysfunction (233). Studies have found that dietary content and rhythm regulate transcription in the intestinal epithelial cells. Changes in the timing or content of feeding can lead to intestinal epithelial cell homeostasis and disrupt intestinal barrier function (234). Multiple trials in adult populations worldwide have examined the efficacy of various dietary timing regimens, including time-restricted eating, short-term fasting, and intermittent fasting. Time-restricted eating involves shortening the eating window to a pre-specified number of hours per day (6 to 10 h) and fasting for several hours the remainder of the time without changing diet quality and quantity (235). Time-restricted eating improves insulin sensitivity, beta-cell responsiveness, blood pressure, oxidative stress, and appetite (236). Clinical studies have found that time-restricted eating reduces the risk of metabolic diseases in healthy individuals when treating metabolic syndrome (237). For instance, time-restricted eating can lower HbA1c levels in individuals with diabetes, which enables achieving blood sugar control and weight loss (238). A previous study found that a 12-week time-restricted eating intervention in 19 subjects with metabolic syndrome improved cardiometabolic health in treating metabolic syndrome (237). Abnormal feeding timing and increased gut permeability are associated with obesity, which in turn modulates feeding rhythms and improves gut barrier function, providing new opportunities to combat metabolic dysfunction (239).

Recent studies have found that dietary rhythms can regulate the bi-directional interaction between the intestinal circadian clock, gut microbiota, and host metabolic system, and enhance the circadian rhythm of adipocytes to improve metabolism (240). In addition, studies on short-term fasting have found that it can protect the viability of small intestinal stem cells in the small intestine of mice and act as a barrier (241, 242). A recent study found that food stimulation activation-induced expression of the neuropeptide vasoactive intestinal peptide significantly enhanced IL-22 production and epithelial barrier function (243). Intermittent eating with moderate rhythm balances the body's energy intake, regulates intestinal homeostasis, and improves the intestinal barrier through food stimulation and rhythm. In conclusion, regulating feeding timing may improve metabolic diseases through gut barrier function. Although the underlying mechanism has not been fully elucidated, animal experiments have yielded impressive data on the prevention or reversal of obesity-related metabolic diseases. Therefore, more rigorous human studies are needed to assess the efficacy, mechanisms, and sustainability of the meal-timing modulation of the gut barrier in a wide range of populations and diseases. It has been suggested that time-restricted eating may improve the efficacy of pharmacological treatment. Therefore, further population studies should be conduced in the future to explore how time-restricted eating can improve the pathways associated with metabolic diseases.

4.4. Metabolic surgery

Bariatric surgery is an effective treatment for patients with metabolic disorders and includes gastric bypass (RYGB) and sleeve gastrectomy (SG), the two most commonly performed procedures (244). These account for 76% of the procedures currently performed in bariatric surgery. They have shown surprising efficacy in improving hyperglycaemia, insulin sensitivity, hyperlipidaemia, and steatosis in patients with metabolic diseases (245). As bariatric surgery remodels the digestive tract, the specific mechanisms for the treatment of metabolic disorders can be further explored in terms of gut function (246–248). A study on SG in morbidly obese patients found that the procedure can significantly improve intestinal barrier damage (249).

Specifically, metabolic surgery improved the intestinal barrier by relying primarily on the increased expression of the intestinal epithelial tight junction proteins ZO-1, occludin, and claudin-1 to maintain intestinal epithelial cell proliferation and restore the physical barrier of the intestine (250). This optimizes mucosal function, increases submucosal thickness, and improves the intestinal mucosal barrier. In addition, it increases the number of Paneth cells and the depth of the crypt, alleviates the intestinal inflammatory response, and enhances the intestinal immune barrier (251). In addition, bariatric surgery can increase the intestinal secretion of molecules, such as glucagon-like peptide 1 (GLP-1) (252) and glucagon-like peptide 2 (GLP-2) (253). Previous studies have shown that the intestinal secretion of GLP-2 inhibits epithelial cell apoptosis and promotes cell proliferation. In addition, GLP-1 can increase intestinal gland secretion and mucin expression, protecting the intestinal barrier (254). Therefore, we speculate that bariatric surgery may treat metabolic diseases by enhancing intestinal barrier function by increasing the secretion of GLP-1 and GLP-2 in the intestine (255). Interestingly, the serum levels of bile acids, such as tauroursodeoxycholic acid (TUDCA) and lithocholic acid (LCA), increase after bariatric surgery (256, 257). LCA and TUDCA can improve intestinal barrier function by reducing intestinal inflammation, suggesting that bariatric surgery improves bile acid metabolism and may further strengthen intestinal barrier function for the treatment of metabolic diseases (258–260).

Existing research has focused on the management of metabolic diseases through weight loss and energy restriction in bariatric surgery. However, complications, such as protein malnutrition, micronutrient deficiencies, and small intestinal bacterial overgrowth, which can occur after bariatric surgery, should not be overlooked. Therefore, metabolic disorders may be better managed through supplemental pharmacological interventions and the development of new surgical modalities. Further improvements in the intestinal mucosa, enteric nervous system, hormonal responses, and intestinal barrier function after gastric bypass surgery were achieved through

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supplementation with α-ketoglutarate (261). Compensatory antibody responses may help reduce systemic inflammation by neutralizing the immunogenic components of the intestine, thereby enhancing intestinal barrier function after bariatric surgery. Therefore, the modulation of the postoperative intestinal immune response is a potential strategy. Further studies have shown that endoscopic sleeve gastroplasty is a safer intervention that can result in significant weight loss and reduced postoperative complications. Future research could focus on improving the intestinal barrier to develop a more rational approach to bariatric surgery by restoring intestinal barrier function in patients with metabolic diseases, reducing chronic inflammation both in the gut and systemically, and improving the secretion of hormones, such as GLP-1, in the gut. Serum metabolomics and proteomics should also be used to search for key effectors to reveal the role of gut barrier-targeted bariatric surgery in the treatment of metabolic diseases, such as improved host metabolic disorders, insulin sensitivity, and adipokine secretion, as well as to develop effective postoperative interventional agents to further reduce the incidence of postoperative complications (Figure 3).

5. Conclusion

At present, the burden of metabolic diseases, particularly diabetes mellitus, obesity, and NAFLD, is increasing globally. However, there are limited means and efficacy for the treatment of these metabolic diseases. Recent studies have shown that multiple factors in metabolic diseases affect gut barrier function, wherein

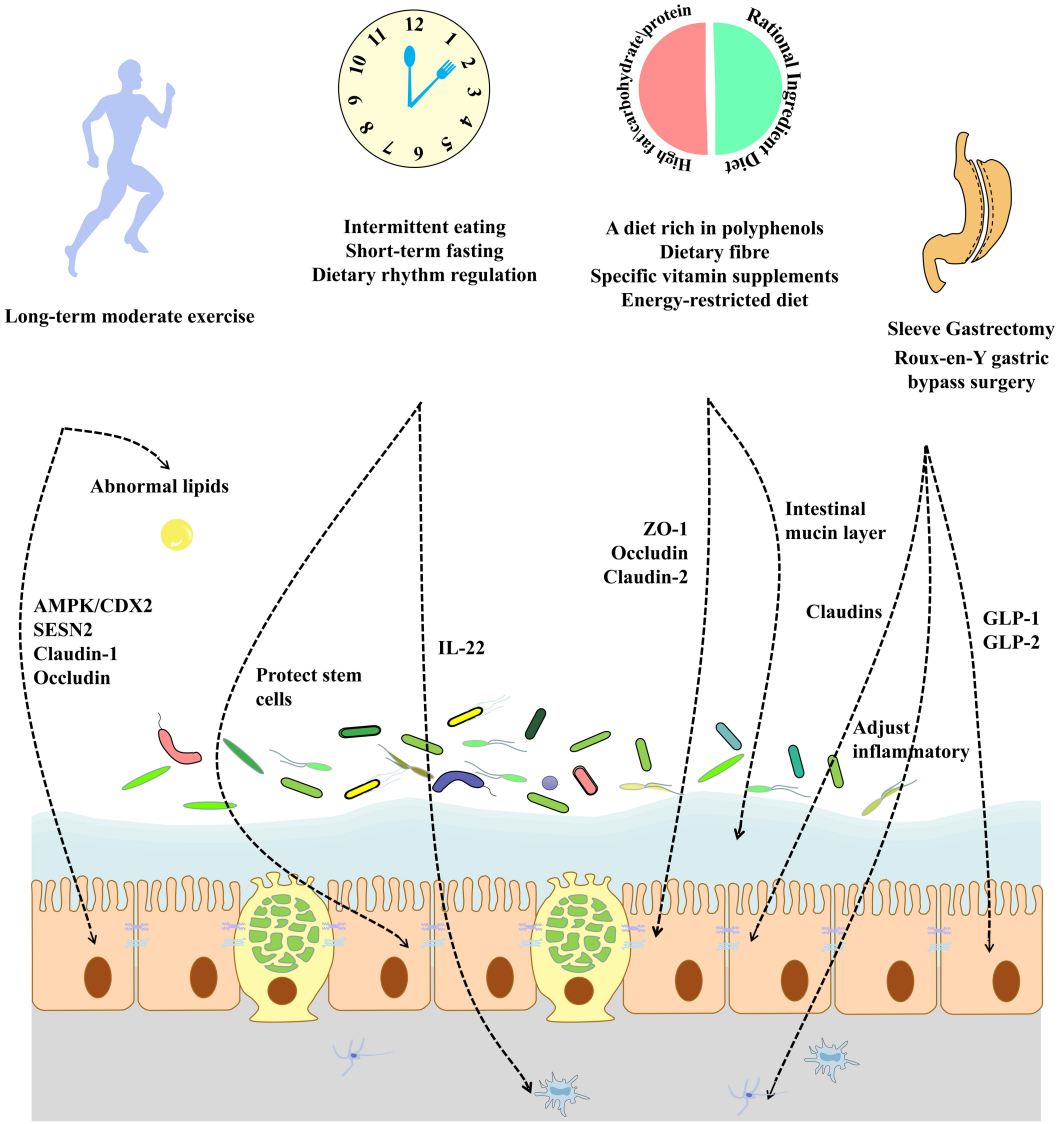

FIGURE 3
An unhealthy lifestyle and diet can play an important role in the development of metabolic diseases by affecting the intestinal barrier. Exercise over the long-term improves intestinal tight junction protein expression and ameliorates abnormal lipids in the gut. The modulation of dietary rhythms can improve intestinal stem cell function, alleviate chronic inflammation, and improve the intestinal barrier. The modification of the dietary composition increases tight junction protein expression while improving the intestinal mucus barrier. Bariatric surgery in obese patients can adjust intestinal inflammatory and inflammation and increase the secretion function of intestinal epithelial cells by increasing the expression of intestinal tight junction proteins.

the impairment of the gut barrier can further exacerbate metabolic disease progression and severity. New therapeutic strategies for manipulating the gut barrier, including drugs, probiotics, diet, or natural products, have been tested clinically and in various diseases, repairing gut barrier dysfunction in many cases. In particular, rational medication coupled with lifestyle interventions represents a safe and effective means to intervene early in chronic metabolic diseases, and may have significant health benefits by modulating the gut barrier. In summary, improving our understanding of the relationship between the gut barrier and metabolic disease can provide detailed mechanistic insights into the pathogenesis and reveal possible pathways for the modulation of disease prevention.

Thus, defining a "healthy" gut barrier and developing new multi-omic technologies in the form of biomarkers and therapeutic tools will significantly advance the research on metabolic diseases. This will allow researchers to investigate the dynamic relationship between metabolic diseases and intestinal barrier function and to provide translational opportunities for therapeutic strategies for metabolic diseases that use the intestinal barrier as a target organ, including drug design, microbial transplantation, and science-based lifestyle. Multi-omics studies can help us to understand the gut barrier-metabolic disease axis and could lead to the development of personalized medicine. Therefore, there is a need to demonstrate causality in metabolic diseases, with a detailed understanding of the gut barrier function, using transcriptomic, proteomic, and metabolomic technologies. The gut barrier-metabolic disease axis is an exciting area of exploration for unraveling the mechanisms that support the therapeutic regulation of metabolic diseases, and an in-depth understanding of these complex systems can be used to develop new preventive and therapeutic strategies.

Author contributions

YZ, XZ, XY, and PN wrote the manuscript. QG and KY supervised the writing and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Strategies involving low-molecular-weight heparin for the treatment and prevention of venous thromboembolism in patients with obesity: A systematic review and meta-analysis

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Introduction: Recent studies have indicated that the dosage of LMWH in patients with specific weights may be controversial. Therefore, we conducted a meta-analysis to explore an appropriate dosage of LMWH for the prevention and treatment of venous thromboembolism (VTE) in patients with obesity.

Materials and methods: We searched the PubMed, EMBASE, and Cochrane Library databases up to July 23, 2022. Study selection, bias analysis, and information extraction were performed by three independent reviewers. The occurrence or recurrence of VTE and bleeding events were the primary outcomes we assessed.

Results: Eleven studies (a total of 6266 patients) were included in the prevention group, and 6 studies (a total of 3225 patients) were included in the treatment group. For VTE prophylaxis, compared with the standard-dosage group, the high-dosage group had a lower incidence of VTE (OR: 0.47, 95% CI: 0.27-0.82, *P*=0.007) and a similar incidence of bleeding events (OR: 0.86, 95% CI: 0.69-1.08, *P*=0.020). For VTE therapy, compared to the standard-dosage group, the reduced-dosage group had a similar incidence of VTE recurrence (OR: 0.86, 95% CI: 0.11-6.84, *P*=0.89) but a lower incidence of bleeding events (OR: 0.30, 95% CI: 0.10-0.89, *P*=0.03).

Conclusion: In patients with obesity, increasing the dosage of LMWH is a more appropriate option for the prevention of VTE. Due to the limited evidence,

reducing the therapeutic dosage of LMWH requires careful consideration. Larger-scale, well-designed randomized controlled trials are necessary.

Systematic Review Registration: https://www.crd.york.ac.uk/prospero/display_record.php?, identifier ID=CRD42022298128.

KEYWORDS

venous thromboembolism, obesity, anticoagulant, low-molecularweight heparin, treatment, prevention

1 Introduction

Venous thromboembolism (VTE), including deep vein thrombosis (DVT) and pulmonary embolism (PE), is one of the major causes of in-hospital morbidity and mortality (1, 2). Obesity (BMI> 30 kg/m^2) is an established strong and independent risk factor for the development and recurrence of VTE. The risk of VTE is estimated to be approximately 2.3 times higher in patients with obesity than in normal-weight patients (BMI 18.5-29.9 kg/m^2) (3, 4).

Anticoagulation is the most crucial element in preventing and treating VTE. However, the strategies for anticoagulation in specific weight groups are still controversial. While several guidelines recommend the same agent and dosage as those given to normal-weight patients (5, 6), others do not address patients with specific weights (7, 8). Furthermore, according to the International Society on Thrombosis and Hemostasis (ISTH) guidelines, direct oral anticoagulants (DOACs) are not recommended for patients with a BMI>40 kg/m^2 or a weight >120 kg (9).

Low-molecular-weight heparin (LMWH) is commonly used for the prophylaxis and treatment of VTE. Currently, patients with BMI>30 kg/m^2 are often administered the same dosage as normal-weight patients. However, significant differences have been found in pharmacology between patients with obesity and normal-weight patients. After subcutaneous injection of a weight-dose (1.5 mg/kg) of enoxaparin, obese healthy volunteers had a higher level of anti-Xa exposure and a lower total body clearance than normal-weight volunteers (10). Furthermore, the lower volume of distribution in obese volunteers suggested that LMWH did not distribute into adipose tissue. Whether patients with BMI >30 kg/m^2 need a dosage adjustment of LMWH is a topic of increasing concern in clinical decision-making.

Recent research shows that the use of a standard dosage of LMWH for prevention in this specific weight group may be inadequate (11–13). In a large-scale retrospective cohort study, despite receiving chemoprophylaxis, critically ill obese patients had a significantly higher incidence of VTE than nonobese patients (3). Moreover, for the treatment of obese VTE patients, some researchers have proposed a reduced dosage of LMWH, instead of 1 mg/kg twice daily (14, 15). Higher anti-Xa exposure at the same dosage may lead to a higher incidence of supratherapeutic anti-Xa levels. Research has shown a positive correlation between anti-Xa levels and BMI (15).

Thus, we performed a systematic review and meta-analysis to evaluate a higher dosage of LMWH for prophylaxis of VTE and a reduced dosage of LMWH for treatment of VTE in patients with obesity.

2 Methods and materials

In accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement, this systematic review and meta-analysis was registered with PROSPERO as CRD42022298128.

2.1 Search strategy

PubMed, EMBASE, and the Cochrane Library databases were searched systematically and comprehensively for all available comparative studies from inception until July 23, 2022. The combination of search terms included “venous thromboembolism”, “low molecular weight heparin” and “obesity”. Additionally, we searched the references of the included studies for other relevant articles that were not found in the literature search. We present the complete search strategies for each database in Supplementary Material (1).

2.2 Study selection

All the retrieved studies were imported into a citation manager (Endnote 20, Clarivate, Philadelphia USA). After removing duplicate studies, two authors (JJ Liu and X Qiao) systematically screened the remaining studies according to the titles, abstracts, and full text. Any disagreement was discussed in the case of a conflict, and a consensus

Abbreviations: BMI, body mass index; LMWH, low-molecular-weight heparin; VTE, venous thromboembolism; NOS, Newcastle–Ottawa Scale; DVT, deep vein thrombosis; PE, pulmonary; ISTH, International Society on Thrombosis and Hemostasis; DOACs, direct oral anticoagulants; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; RCT, randomized controlled trial; PCS, prospective cohort study; RCS, retrospective cohort study; USA, United States of America; UFH, unfractionated heparin; AF, atrial fibrillation; CAHD, coronary atherosclerotic heart disease.

was reached. Eligible studies met the following criteria for: (1) type of studies, namely, randomized controlled trials and high-quality cohort or case–controlled studies; (2) participants, namely, patients with BMI >30 kg/m^2 needing prophylactic or therapeutic anticoagulation; (3) comparators, namely, use of a different dosage of LMWH; and (4) outcomes, namely, occurrence or recurrence of VTE, bleeding events, and anti-Xa levels.

Studies were excluded if they met the following criteria: (1) conference abstracts, letters, and comments; (2) studies not in English; (3) studies that involved patients who were pregnant or had renal impairment [creatinine clearance (CrCl)<30 ml/min]; and (4) studies with fewer than 10 participants in each group.

2.3 Definition of outcomes

The primary outcomes were the occurrence or recurrence of VTE, including PE or DVT, and the incidence of major and minor bleeding events during hospitalization or follow-up. Major bleeding was defined as a drop in hemoglobin of more than 2 g/dL, a transfusion of 2 or more units of blood products, or a retroperitoneal, intraocular, or intracranial hemorrhage. After major bleeding was ruled out, the remaining bleeding events were considered minor bleeding events (16).

The secondary outcomes were the incidence of supraprophylactic (supratherapeutic) anti-Xa levels and subprophylactic (subtherapeutic) anti-Xa levels. Although currently controversial (12, 17, 18), the prophylactic and therapeutic levels of anti-Xa are 0.2-0.4 IU/ml and 0.5-1.0 IU/ml, respectively.

2.4 Data extraction and risk of bias

We developed a predesigned data extraction sheet, and the data, including the study design, geographic location, sample size, intervention, and baseline data (i.e., age, BMI, sex, duration, renal function), were extracted independently by two coauthors (JJ Liu and MD Wu). Disagreements were resolved by discussion between the two review authors. If no agreement could be reached, a third author (B Tang) made the decision. Not all included studies monitored patients' anti-Xa levels, and the characteristics of the enrolled studies that did not include patients' anti-Xa levels were analyzed as a subgroup. Emails were sent for further information that was not provided in the full text.

The quality and risk of bias assessment of the studies included were independently evaluated by two coauthors (JJ Liu and MD Wu). The Cochrane Collaboration tool was used to assess the potential bias of randomized controlled trials. We used the Newcastle–Ottawa Scale (NOS) tool for cohort and case–controlled studies.

In addition, we used funnel plots and the Egger test to assess publication bias. A P value<0.05 indicated significant publication bias.

2.5 Statistical analysis

The occurrence or recurrence of VTE was used to evaluate the effectiveness of different dosages of LMWH for prophylaxis or treatment. The incidence of bleeding events was used for safety evaluation. As an adjunct, anti-Xa levels were included as another measure of efficacy if a small number of studies were included. The proportions and adjusted odds ratios (ORs) with 95% confidence intervals of the respective outcomes were calculated through meta-analysis. The inconsistency index (I^2) statistics and the Q test were calculated for heterogeneity assessment. Low heterogeneity was defined as a P of > 0.1 and an I^2 of <25%. When heterogeneity was low, a fixed-effects model was used; otherwise, a random-effects model was used. A P value < 0.05 was considered statistically significant. Subgroup analyses for the outcomes were performed according to the types of LMWH, bariatric surgery patients for prophylaxis, and supratherapeutic anti-Xa levels and subtherapeutic anti-Xa levels for treatment.

Review Manager v5.4 (Cochrane Collaboration, Copenhagen, Denmark), Stata v16.0 (StataCorp, Texas, USA), and R version 4.1.0 (R Foundation, 2021) were used for all of the statistical analyses.

3 Results

The searches of the three databases provided 296 records. After adjustment for duplicates and other reasons, 168 studies remained. Of these, 113 studies were discarded because the papers did not appear to meet the criteria after review of the title and abstracts. Another 6 studies were excluded because they were conference abstracts. The full text of the remaining 49 citations was examined in detail. Thirty-two studies did not meet the inclusion criteria. Finally, a total of 17 studies involving 11 trials for prophylaxis (19–29) and 6 trials for treatment (30–35) were identified for inclusion in this meta-analysis. See the flow diagram in Figure 1.

The data on the number of patients who were administered LMWH could not be extracted from the full text of Wang TF (28) because a higher dosage of chemoprophylaxis consisting of unfractionated heparin (UFH) and LMWH was studied as a whole. An email was sent to obtain more detailed research data.

3.1 Characteristics of the included studies

3.1.1 Prophylaxis

Table 1 shows the baseline characteristics of the 11 included studies for prophylaxis. A total of 3153 participants with a standard dosage and 3113 with a higher dosage were included. These studies included 4 randomized controlled trials (19, 20, 23, 25), 5 prospective cohort studies (21, 22, 24, 26, 27), and 2 retrospective cohort studies (28, 29). Patients following bariatric surgery were the main target population for clinical VTE prevention (19–21, 24, 25, 27, 29). Of all the LMWH drugs, enoxaparin was most frequently provided for chemoprophylaxis in patients with BMI >30 (21, 23–29).

As in normal-weight patients, most studies used subcutaneous 40 mg daily of enoxaparin as the standard dosage and 60 mg daily or 30 mg twice daily as the higher dosage (23–25, 27–29). Few studies used a weight-based dosage (0.5 mg/kg^2) as a prophylactic option (26). It is worth noting that the standard dosage of the

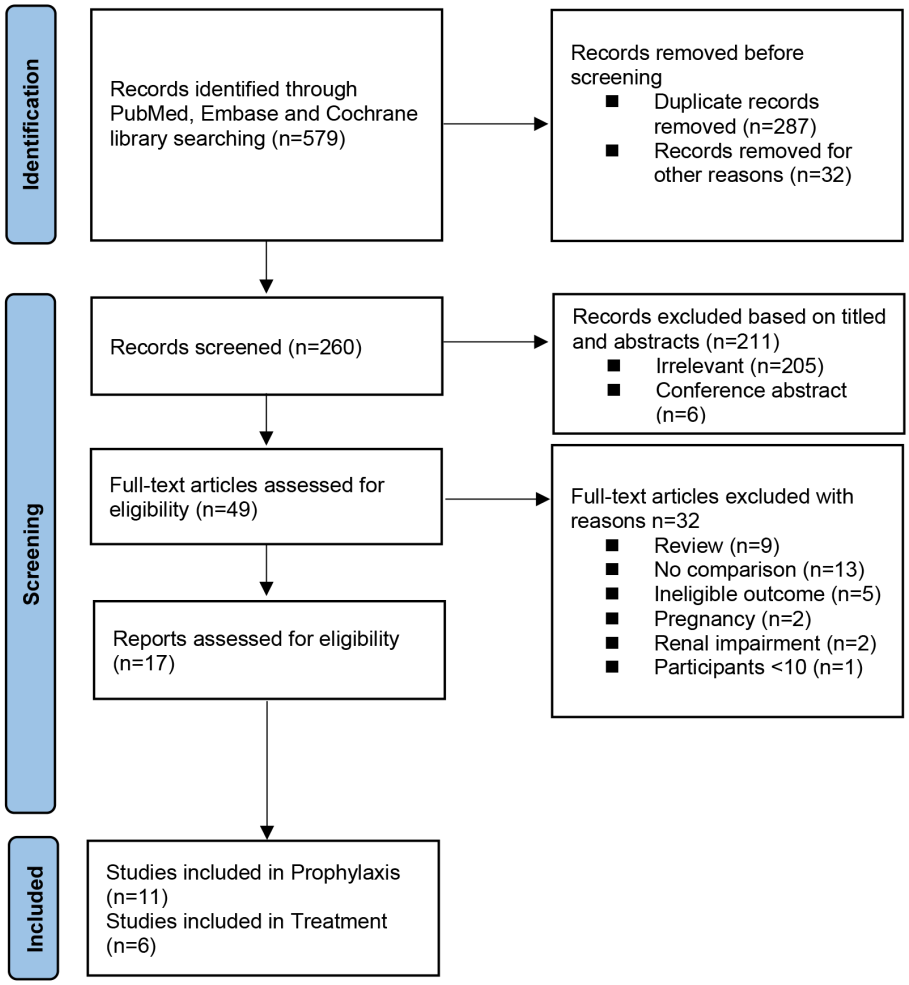

FIGURE 1
Flow diagram showing the progress of the included studies. The flow diagram template is derived from the open source template published in the 2020 version of the PRISMA guidelines, and is available for free download and use for all system reviews. https://guelphhumber.libguides.com/c.php?g=213266&p=1406923#:~:text=What%20is%20PRISMA%3F,a%204%2Dphase%20flow%20diagram.

prophylaxis group at a few institutions was higher (40 mg twice daily) (27). To explore whether the inclusion would affect the research conclusion, we conducted a sensitivity analysis.

Dosage selection for the remainder of the studies was performed using site-specific criteria according to different types of LMWH drugs and different medical centers. Five studies recommended a higher dosage as a prophylactic strategy in patients with obesity (21, 23, 25, 27, 28), 3 studies maintained the traditional strategy (19, 20, 22), and an additional 3 studies considered the current findings controversial because the studies were small scale and the evidence was insufficient (24, 26, 29).

3.1.2 Treatment

Table 2 shows the baseline characteristics of the 6 treatment studies, including 1 randomized controlled trial (32), 1 prospective cohort study (31), and 4 retrospective cohort studies (30, 33–35). A total of 3225 participants were included in this group (2616 with a standard dosage vs. 609 with a reduced dosage). Enoxaparin remained the preferred type of LMWH for patients with BMI >30 (31–33, 35). A weight-based dosage of 1.0 mg/kg q12h was commonly used as a standard dosage, and a reduced dosage of LMWH referred to less than 1.0 mg/kg, which was approximately 0.8 mg/kg (30–32, 35). Almost all studies recommended dosage reduction for treating VTE in patients with BMI>30 (30–33, 35), except for one retrospective cohort study that used dalteparin (34).

3.2 Bias assessment

For the quality and potential bias assessment of RCTs, selection bias, performance bias, detection bias, attrition bias, and reporting bias were evaluated with the Cochrane Collaboration tool, and no disagreements occurred between the two coauthors. The NOS tool was used for cohort studies. We assessed a total of 8 items in terms of selection, comparability, and outcome and scored each study with a total score of 9. A score of less than 6 was considered a high risk of bias study. All bias assessment results are presented in Supplementary Material (2).

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TABLE 1 Characteristics of the included prophylaxis studies.

Study	Design	Population	Agent	Dosage	Number of patients-n	Occurrence of VTE-n	Bleeding events-n	Suggest
Imberti (19) 2014	RCT	Bariatric	Parnaparin	S: 4250 IU/day H: 6400 IU/day	131 119	2 1	8 6	Standard
Kalfarentzos (20) 2001	RCT	Bariatric	Nadroparin	S: 0.6 ml/day H: 1.0 ml/day	30 30	0 0	0 2	Standard
Scholten (21) 2002	PCS	Bariatric	Enoxaparin	S: 30 mg/12 h H: 40 mg/12 h	92 389	5 2	1 1	Higher
Vavken (22) 2009	PCS	Orthopedic	Bemiparin	S: 3500 IU/day H: 5000 IU/day	83 667	1 2	0 0	Standard
Miranda (23) 2017	RCT	Medical inpatients	Enoxaparin	S: 40 mg/day H: 60 mg/day	45 46	0 0	3 2	Higher
Gelikas (24) 2017	PCS	Bariatric	Enoxaparin	S: 40 mg/day H: 60 mg/day	31 23	0 0	0 1	Controversial
Steib (25) 2015	RCT	Bariatric	Enoxaparin	S: 4000 IU/day H: 6000 IU/day	44 44	0 0	1 2	Higher
Gibson (26) 2021	PCS	Medical inpatients	Enoxaparin	S: 0.5 mg/kg/day H: 40 mg/12 h	40 40	0 0	0 0	Controversial
Simone (27) 2008	PCS	Bariatric	Enoxaparin	S: 40 mg/12 h H: 60 mg/12 h	24 16	0 0	1 0	Higher
Wang (28) 2013	RCS	Medical inpatients	Enoxaparin/ UFH	S: 40 mg/day enoxaparin 5000 IU/8or12h UFH H: 40 mg/12h enoxaparin 7500 IU/8 h UFH	2369 1559	35 12	200 112	Higher
Hamad (29) 2005	RCS	Bariatric	Enoxaparin	S: 40 mg/day H: 60 mg/12 h	264 180	0 2	3 3	Controversial

S, Standard dose. H, Higher dose

VTE, Venous thromboembolism. RCT, Randomized controlled trial. PCS, Prospective cohort study. RCS, Retrospective cohort study. USA, United States of America. UFH, Unfractionated heparin.

3.3 Meta-analysis of the included studies

In the meta-analysis, we excluded studies with no outcome events occurring in either the experimental group or the control group. Ultimately, 5 studies for efficacy assessment and 9 studies for safety assessment in the VTE prevention group were enrolled. Only 2 studies for efficacy assessment and 4 articles for safety assessment in the treatment group were included. Considering that few studies were included to evaluate the effectiveness of the reduced dosage of LMWH, we included anti-Xa levels in the effectiveness evaluation and compared the incidence of subtherapeutic anti-Xa levels. The higher the incidence was of not reaching anti-Xa therapeutic levels, the lower the efficacy. A therapeutic level referred to 0.5-1.0 IU/ml. The anti-Xa levels of the included studies are presented in Supplementary Material (3). A meta-analysis was performed for each level group, and 4 studies were included in each subgroup.

3.3.1 Prophylaxis

The results of the meta-analysis in the prophylaxis group are presented in Figure 2. Overall, increasing the dosage of LMWH tended to decrease the incidence of VTE events. Combining all studies, the incidence of VTE was approximately 0.65% (n=2914) in the higher-dosage group, which increased to 1.50% (n=2939) in the standard-dosage group (OR: 0.47, 95% CI: 0.27-0.82, P=0.007). In addition, the heterogeneity test suggested that the heterogeneity between studies was low (P=0.17; I^2 = 38%).

Bleeding events occurred in 129 of 2406 patients in the higher-dosage group and 217 of 3030 in the standard-dosage group. A higher dosage of LMWH did not significantly increase the risk of bleeding in patients with obesity, and the incidence of bleeding events was similar between the two dosages (OR: 0.86, 95% CI: 0.69-1.08, P=0.020). No significant heterogeneity was observed in the meta-analysis (P=0.83; I^2 = 0%).

TABLE 2 Characteristics of the included treatment studies.

Study	Design	Population	Agent	Dosage	Number of patients-n	Recurrence of VTE-n	Bleeding events-n	Suggest
Mirza (30) 2020	RCS	VTE	LMWH	S: 20865 IU/day R: 18000 IU/ day	2392 454	6 2	24 0	Reduced dose
Thompson-Moore (31) 2015	PCS	VTE/AF/ CAHD	Enoxaparin	S: 1.0 mg/kg/12 h R: 0.83 mg/kg/ 12 h	18 23	0 0	4 4	Reduced dose
Curry32 2018	RCT	VTE/AF	Enoxaparin	S: 1.0 mg/kg/12 h R: 0.8 mg/kg/12 h	26 28	0 0	0 0	Reduced dose
Van Oosterom (33) 2019	RCS	VTE	Enoxaparin	S: >0.85 mg/kg/ 12 h R: <0.85 mg/ kg/12 h	67 66	2 0	2 0	Reduced dose
Smith (34) 2003	RCS	VTE/AF/ CAHD	Dalteparin	S: 126.2 units/ kg/12 h R: 196.5 units/ kg/day	11 10	0 0	0 0	Controversial
Maclachlan (35) 2019	RCS	VTE	Enoxaparin	S: 1.0 mg/kg/12 h R: <1.0 mg/kg/ 12 h	102 28	0 0	4 0	Reduced dose

S, Standard dosage. R, Reduced dosage
VTE, Venous thromboembolism. AF, Atrial fibrillation. CAHD, Coronary atherosclerotic heart disease
RCT, Randomized controlled trial. PCS, Prospective cohort study. RCS, Retrospective cohort study. USA, United States of America.

3.3.2 Treatment

The recurrence of VTE after anticoagulant treatment was low. Only 10 obese patients in two included studies experienced recurrent VTE (2 of 520 with a reduced dosage vs. 8 of 2459 with a standard dosage). After a meta-analysis of the two studies, there was no significant difference in the incidence of recurrent VTE between the reduced-dosage group and the standard-dosage group (OR: 0.86, 95% CI: 0.11-6.84, *P*=0.89) (Figure 3). Considering the bias caused by the small number of included studies, we performed a meta-analysis of rates of subtherapeutic anti-Xa levels in the

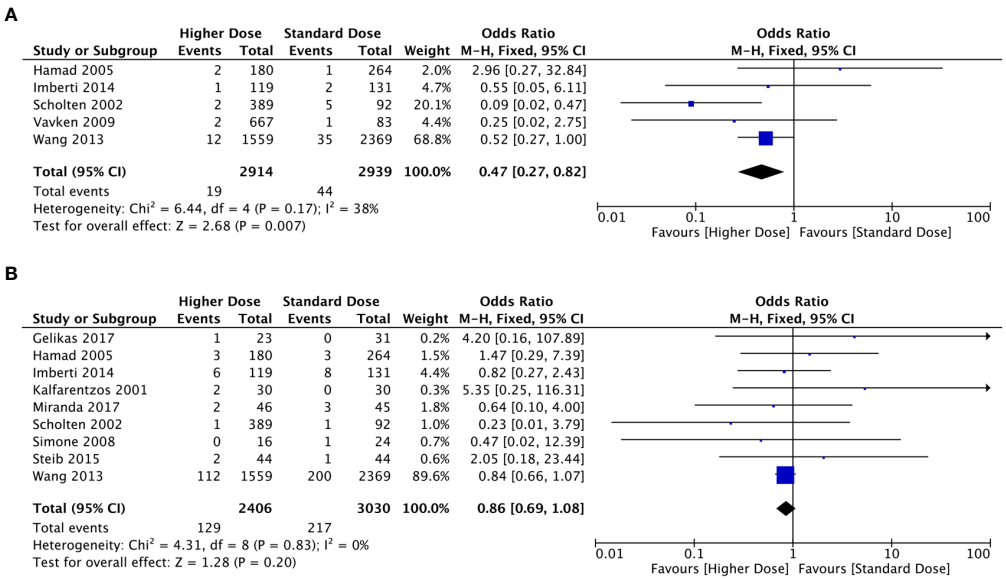

FIGURE 2
Forest plots involving higher dosage versus standard dosage of LMWH for the prophylaxis of VTE in obese patients. **(A)** occurrence of VTE; **(B)** bleeding events.

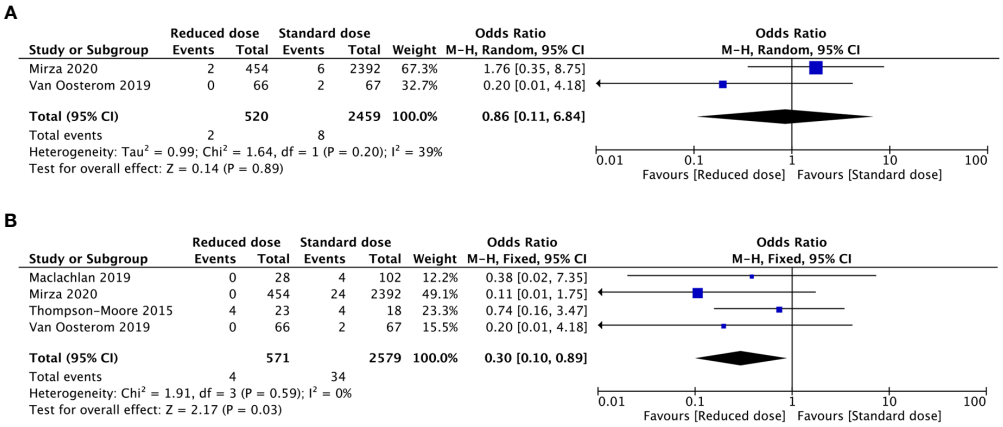

FIGURE 3
Forest plots involving reduced dosage versus standard dosage of LMWH for the treatment of VTE in obese patients. **(A)** recurrence of VTE; **(B)** bleeding events.

included studies (Figure 4). Compared with the standard-dosage group, the proportion of patients in the reduced-dosage group who did not reach the therapeutic level was significantly higher (OR: 4.23, 95% CI: 1.97-9.07, *P*=0.0002). No significant heterogeneity was observed.

After an overall meta-analysis of the incidence of bleeding events between the reduced-dosage group and the standard-dosage group, the use of a reduced dosage in obese patients was significantly associated with a lower incidence of bleeding events (OR: 0.30, 95% CI: 0.10-0.89, *P*=0.03). No significant heterogeneity was observed among the 4 included studies (*P*=0.59; I^2 = 0%).

3.4 Subgroup, sensitivity analysis, and publication bias

We performed a subgroup analysis of the meta-analysis of prophylaxis in obese patients (Figure 5). Groups were divided according to the use of enoxaparin only and bariatric surgery patients, and the efficacy and safety were both analyzed. The results for each subgroup were basically consistent with the overall statistical results.

Sensitivity analysis excluded the study by Wang et al. because UFH was incorporated in this study. The results of the meta-analysis after this exclusion were consistent with the main results, and no heterogeneity was found in the efficacy and safety analysis. In addition, we performed another sensitivity analysis of the remaining low-risk studies after excluding higher-risk studies based on the quality bias analysis of each study. The quality of the included studies did not significantly affect the conclusions of the studies.

We performed the Egger test for several major studies, including the efficacy and safety of the prophylactic group and the safety of the therapeutic group, with respective Egger test values of P=0.894, 0.485, and 0.097. No publication bias was found. Funnel plots are presented in Supplementary Material (4).

4 Discussion

The use of LMWH for anticoagulation in patients with BMI > 30 kg/m^2 is controversial. There is the possibility of insufficient anticoagulation with a standard dosage. Moreover, an aggressive dosing strategy will cause excessive anticoagulation and increase the bleeding risk. Pharmacological findings also suggest that our research is necessary. To the best of our knowledge, this is the first meta-analysis to evaluate LMWH strategies in patients in this specific weight group.

In this meta-analysis, 11 studies were included in the prevention group, and 6 studies were included in the treatment group. The final

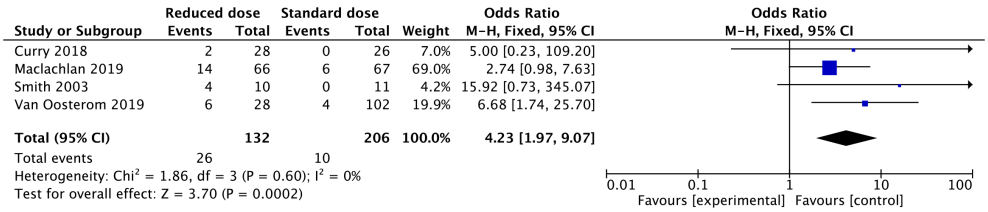

FIGURE 4
Forest plots involving reduced dosage versus standard dosage of LMWH for the treatment of VTE in obese patients: patients below the therapeutic anti-Xa level;.

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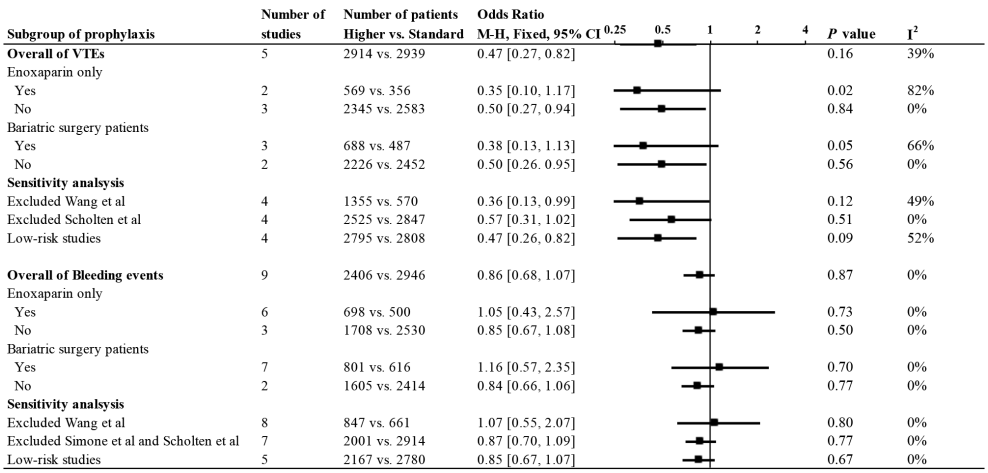

FIGURE 5
Subgroup analysis and sensitivity analysis.

statistical analysis of the prophylaxis group found that a higher dosage of LMWH reduced the incidence of VTE without increasing the risk of bleeding. In the treatment group, a reduced dosage was associated with a reduced incidence of bleeding events. After combining the two studies on the efficacy of LMWH treatment, we found that the outcome was favorable for reducing the dosage in obese patients. When we attempted to address this issue by including anti-Xa levels in the efficacy evaluation, the results were contradictory. A reduced dosage of LMWH failed to provide adequate anti-Xa levels in patients with obesity. Due to the limited number of studies, whether to reduce the therapeutic dosage of LMWH in obese patients needs further verification.

In a large retrospective cohort study, Wang et al. combined LMWH and UFH to observe the effect of high-dosage chemoprophylaxis on anticoagulation in obese patients (28). However, the full text of the article does not mention the statistical data for enoxaparin. We learned by email from the authors that 69% of obese patients were administered enoxaparin, while another 31% were administered UFH. Considering the large number of patients included in this study and the similarities in the pharmacology of LMWH and UFH, this article was still included in our study by a unanimous decision of the three investigators. To explore the impact of this study on the final results, we conducted a sensitivity analysis. After the study was removed, the statistical results were still consistent with the main statistical results.

A standard prophylactic dosage of LMWH in most institutions is 40 mg QD, and higher dosages of LMWH are total dosages of 60-80 mg daily (19, 20, 22–26, 28, 29). We included two additional studies using 30 mg BID and 40 mg BID as standard dosages and 40 mg BID and 60 mg BID as higher dosages. The two studies ultimately supported the higher dosage recommendation as well. This suggests that an increased dosage of prophylaxis is warranted; however, a total dosage of 60 mg daily may not be optimal. Further research is needed to explore optimal prophylactic dosages for patients in this specific weight group. A sensitivity analysis of the two included studies was conducted, and after excluding them, the results remained consistent.

Gibson et al. compared the difference between a weight-based dosage (0.5 mg/kg) and a fixed dosage (40 mg twice daily) (26). There was no significant difference in anti-Xa levels between the two regimens. However, due to the small size, no outcome events occurred. Larger-scale studies are therefore needed to investigate the applicability of weight-based versus fixed dosages in patients with BMI>30.

There was no clear reduced dosage as a treatment option for obese patients, and 0.8 mg/kg q12h appeared to be the choice in some institutions (31, 32). However, due to the low incidence of recurrent VTE and the limited number of included studies, only two studies had recurrent VTE events. After a meta-analysis of the two studies, the results were in favor of reducing the dosage in obese patients. To address this, we introduced anti-Xa factor levels. Anti-Xa levels below the lower limit of the therapeutic standard (0.5 IU/ml) were considered inadequate. Notably, the results indicated that the dosage of the reduced group was significantly insufficient.

Therefore, the current evidence does not directly demonstrate that reducing the therapeutic dosage in patients with obesity can achieve the same effect. Even the incidence of bleeding events was significantly reduced. We still have doubts about whether reduced dosages would result in insufficient anticoagulation. Therefore, conclusions should be considered with caution until further research takes place.

We performed subgroup analysis and sensitivity analysis only on the meta-analysis of prophylaxis. Owing to the fact that the number of studies included in the meta-analysis of the treatment group was small. This showed that the conclusions of the treatment group needed to be treated with more caution. In subgroup analyses, we discussed the effect of enoxaparin and bariatric surgery on the study findings. Effectiveness analysis showed that although the grouping created heterogeneity, the forest plot showed a trend that did not be changed. We attribute this to the small

number of included studies. In addition, in the safety analysis, subgroup and sensitivity analysis were consistent with the final conclusion, and there was no statistical heterogeneity. Due to the limitation of the number of research studies, we performed publication bias testing on the efficacy and safety of studies in the prevention group and the safety of studies in the treatment group, and the results were negative.

The need for anti-Xa monitoring in obese patients is another controversial topic. Routinely, some studies did not recommend anti-Xa monitoring unless the patient was at significant risk of major bleeding, especially for prophylaxis (17, 35–37). Several other studies suggested that anti-Xa monitoring in obese patients is necessary to make dosage adjustments (32, 38). In this meta-analysis, after combining the included studies, the incidence of VTE was 0.65% with a higher dosage for prophylaxis and 0.38% with a reduced dosage for treatment. Therefore, given the low incidence of VTE, anti-Xa monitoring is not recommended for obese patients unless further studies demonstrate a benefit.

Our study excluded obese patients with atrial fibrillation. The anticoagulant strategy for patients with atrial fibrillation is mainly based on out-of-hospital oral anticoagulants (warfarin and DOACs), and LMWH is mainly used as bridging anticoagulation in the perioperative period of patients with atrial fibrillation (39). In addition, related research was limited. It showed that this may be an overlooked area and further research is needed to explore perioperative anticoagulation strategies in this particular population.

Furthermore, since our study focused on hospitalized obese patients administrated LMWH, the use of DOACs in obese patients was not included in our research. DOACs in obese patients also face challenges. Relevant systematic studies and meta-analyses had shown that the use of DOACs in obese patients was safe, and the efficacy of DOACs in various weight groups might not be affected by body fat (40, 41). In addition, DOACs could reduce the risk of bleeding in obese patients compared with warfarin. DOACs may be a safe and effective option for out-of-hospital obese patients.

This systematic review and meta-analysis has several limitations. First, although randomized controlled trials were included in both the prevention and treatment sections, they included small sample sizes. Large-scale studies are still being conducted retrospectively. This may have an unpredictable effect on our statistical analysis. Because of the instability of dosage maintenance in retrospective studies, grouping by dosage is not strictly controlled. Second, due to individual differences in drug types and clinical centers, not all studies had the same standard dosages and altered dosages. Such dosage differences across studies, especially in the prevention component of meta-analyses, may affect the incidence of outcomes. Third, the number of studies included in the reduced dosage efficacy analysis for treatment was smaller due to the low recurrence rate of VTE. Even though we assessed anti-Xa levels as a supplementary analysis, the reference treatment level of anti-Xa was not clearly defined. Due to the different therapeutic levels of anti-Xa, we analyzed the subtherapeutic group and the supratherapeutic group to ensure the combination ability of each study group, but it did not reflect the real effectiveness of the reduced dosage in obese patients. Further large-scale studies are needed to verify the efficacy. Fourth, although we included studies in obese patients with BMI >30, in fact, the BMI values of obese patients in each study varied from 30 to 60, so the analysis of drug dosages for patients with a higher BMI is not accurate. Unfortunately, due to the lack of baseline data, a subgroup analysis of BMI values could not be performed in this study.

5 Conclusion

Our systematic review and meta-analysis show that compared with the standard dosage, a higher dosage of LMWH to prevent VTE in patients with obesity can reduce the incidence of VTE without increasing the risk of bleeding. Due to limited evidence, the option of reducing the therapeutic dosage should remain cautious until further studies are available. Larger-scale, well-designed randomized controlled trials are necessary.

Data availability statement

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

Author contributions

JL: study design, literature retrieval, data collection, data analysis and manuscript writing. XQ: study design, data collection, data analysis and manuscript writing. MW: literature review, data collection and manuscript writing. HW: literature retrieval, data analysis, and manuscript writing. HL: study design and manuscript revision. HZ: conception and manuscript revision. JS: data analysis, manuscript revision. YC: data analysis, manuscript revision. BT: conception, manuscript revision All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1084511/full#supplementary-material

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Progress and obstacles in transplantation of brown adipose tissue or engineered cells with thermogenic potential for metabolic benefits

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Transplantation of brown adipose tissue (BAT), engineered thermogenic progenitor cells, and adipocytes have received much attention for the improvement of obesity and metabolic disorders. However, even though the thermogenic and metabolic potential exists early after transplantation, the whitening of the brown fat graft occurs with metabolic function significantly impaired. In this review, specific experiment designs, graft outcomes, and metabolic benefits for the transplantation of BAT or engineered cells will be discussed. The current advancements will offer guidance to further investigation, and the obstacles appearing in previous studies will require innovation of BAT transplantation methods.

KEYWORDS

brown adipose tissue, uncoupling thermogenesis, transplantation, engineering, energy expenditure, metabolism

Background

The obesity pandemic along with the high morbidity rate of concomitant diseases constitutes a serious danger to worldwide population health (1). Quite a number of cardiovascular, neoplastic, infectious, and autoimmune diseases have been reported to be involved in the pathological course of obesity, which significantly causes functional damage to multiple systems and a decline in the patient's quality of life and longevity (2–4). Limited to the individual's poor subjective initiative, keeping a balanced diet and regularly exercising to lose weight seem difficult for most people. Invasive methods such as liposuction and sleeve gastrectomy have their intrinsic risks, which definitely set restrictions on their development on a larger scale (5, 6). In addition, weight-loss drugs currently approved by the FDA, most of which are based on appetite suppression or reduction of energy absorption, such as Orlistat, usually elicit adverse reactions (7, 8). On the other hand, especially for bedridden patients with severe obesity, increasing energy expenditure in the resting state can theoretically improve metabolic status. Therefore, how

to safely and effectively increase the basal metabolic rate has become the focus in the field of obesity research in recent decades (9).

There are three major types of adipose tissue in humans: white adipose tissue (WAT), brown adipose tissue (BAT), and beige adipose tissue (beige AT), as verified by anatomy, imageological examination, and histological and functional features (10, 11). WAT was firstly recognized by researchers with adipocytes in both subcutaneous and visceral depots possessing a large unilocular lipid droplet and few mitochondria and subserving the function of energy storage in the form of triglycerides (12). By contrast, BAT, primarily identified in newborns and the supraclavicular and paravertebral regions of adults, usually overexpresses uncoupling protein 1 (UCP1) in the mitochondrial membrane and is characterized by shorter cell diameter, multilocular lipid droplets, and abundant mitochondria in adipocytes (13, 14). Histologically, BAT possesses more substantial vascularization and innervation in morphology and substantially more active substrate uptake for lipid and glucose metabolism, and more lipolysis and uncoupling thermogenesis in metabolic aspects than WAT (15). Beige AT, which resembles classic BAT, can be induced within WAT depots under stimuli such as cold exposure, *in-vivo* application of β3 adrenoreceptor agonists, peroxisome proliferator-activated receptor (Pparγ) agonists, etc. (16–18). Non-shivering heat production is the most remarkable feature of BAT, which relies on UCP1 to uncouple the respiratory chain from oxidative phosphorylation and acts as an important way to increase the energy consumption without relying on muscle unit exercise in the resting state (19). In addition, BAT could also be regarded as a secretory organ to exert beneficial effects on metabolic disorders (20). It has been demonstrated that several BAT-derived molecules, among which fibroblast growth factor 21 (FGF21), interleukin 6(IL6), and neuregulin 4(NRG4) are identified first, act in a paracrine or endocrine manner to regulate the metabolism of other tissues and organs (21).

Thermogenic adipose tissues, including BAT and beige AT, directly dissipate chemical energy as heat with significant weight loss in mammals and markedly improve glucose and lipid homeostasis especially after endogenous or exogenous stimulation (22, 23). As observed in many animal models with metabolic diseases, the abnormally elevated serum glucose, lipid, and other featured metabolic substrates showed nonnegligible improvement in the presence of sufficient thermogenic adipose tissues. The regained metabolic homeostasis appeared to be both long-lasting and comprehensive after further *in vivo* examination showed enhanced insulin sensitivity, glucose and lipid tolerance, and substrate uptake and consumption rate, as long as the thermogenic adipose tissues continued to function (24). In humans, positron emission tomography-computed tomography (PET-CT) scans provided researchers with explicit descriptions of BAT distribution, and statistical analysis of study cohorts indicated that individuals with detectable thermogenic BAT had lower body mass index (BMI) and lower prevalence of type 2 diabetes, dyslipidemia, coronary artery disease, cerebrovascular disease, congestive heart failure, hypertension, and other diseases associated with metabolic disorders (25, 26).

Regretfully, there is a significant decrease in the volume and activity of BAT especially in elderly or obese mammals, which is called the whitening of BAT (27). It has been validated that the reduced function of BAT can lead to obesity and other related complications (28). Multiple approaches targeted at restoring BAT function have been validated in relevant research, among which BAT transplantation seems to be an appropriate method (29–31). However, according to our previous studies, there still exist many technical difficulties in BAT transplantation, and unsatisfactory results, such as low retention rate or rapid whitening of transferred BAT, allograft induced systemic immune reaction, or disruption to the sympathetic system, might occur (32, 33). In this review, we expound recent advancements in BAT transfer, highlighting the promising application fields, alternative transplantation methods, regional or systemic risks, and possible approaches to enhance the transplantation results.

Diseases correlated with the functional decline of BAT

BAT can function as a "heat production factory", with the UCP1 protein in mitochondria inner membrane mediating uncoupling thermogenesis when fueling lipids and glucose (34). However, the thermogenic potential of BAT can be significantly impaired with aging or the development of obesity, which is called the whitening of BAT (35). In elderly obese individuals, BAT in specific anatomical regions showed reduced content with less vascularization and lightened brown color. In histology, the main feature of whitening BAT is the conversion of brown adipocytes to white-like unilocular cells, and the factors triggering the whitening process always lead to massive macrophage infiltration, programmed apoptosis of brown adipocytes, and a scattered crown-like structure, which can usually be seen in traditionally transferred WAT and indicate tissue necrosis (36). Regarding cytoarchitecture, the whitened brown adipocytes, surrounded by an increased number of collagen fibrils, are characterized by enlarged endoplasmic reticulum, cholesterol crystals, and some degenerating mitochondria (37). In molecular biology, the gene expression pattern of whitening BAT might show upregulated inflammasome activation, ER stress markers, and a deficiency in markers of vascularization,β-adrenergic signaling, electron transport chain, rate-limiting enzymes regulating substrate breakdown, specific membrane receptors, etc. (38–40).

The disease spectrum associated with BAT whitening is dominated by obesity and obesity-related metabolic diseases, and the therapies aiming to reverse the whitening process have been confirmed to have varying effects on these diseases by many studies (41–43). First of all, obesity is the phenotype consequence of energy intake exceeding energy expenditure in a certain time, therefore the functional decline of BAT is destined to increase the risk of obesity. Previous studies revealed that brown adipocytes can efficiently ingest substrates such as lipids, carbohydrates, and even succinic acids in blood circulation (44, 45). The classic intracellular lipid metabolism starts from the gradual lipolysis by lipolytic enzymes, such as adipose triglyceride lipase (ATGL), hormone-sensitive

lipase (HSL) and monoglyceride lipase (MGL), and then the fatty acids produced can be transported into the mitochondria by special transport proteins on the mitochondrial membrane (46–48). Ultimately, the UCP1 protein on the mitochondrial inner membrane eliminates the potential energy gap inside and outside the mitochondria, resulting in the inability of high-energy protons to be transferred to energy carriers such as adenosine triphosphate (ATP) and directly converted into thermal energy instead. The thermogenic potential of BAT, through dissipating stored chemical energy as the more disordered form, is dramatic when activated (49). For an adult, the amount of BAT is generally less than 200 grams, which is an order of magnitude less than WAT. To our surprise, BAT can increase the daily energy consumption of 25 to 211 kcal after being activated by cold exposure, and the heat production of BAT is significantly enhanced by approximately 200 kcal per day after the administration ofβ-adrenergic receptor agonists. As proved by the detected volume in PET-CT scans, the importance of BAT in adults was validated by the amount of BAT being inversely correlated with body-mass index, especially in older people (50).

Even though the ability of BAT to protect against chronic metabolic diseases has traditionally been attributed to its capacity to utilize glucose and lipids for thermogenesis, it also plays a secretory role, which enables the establishment of extra protection against metabolic diseases, such as type 2 diabetes mellitus and dyslipidemia (51, 52). Most of the BAT-derived molecules, usually referred to as batokines, promote hypertrophy and hyperplasia of BAT, vascularization, innervation, and other processes that are all associated with BAT recruitment when thermogenic activity is enhanced (53, 54). The paracrine or autocrine batokines construct positive feedback to stimulate the potential of BAT. On the other hand, the batokines secreted into systemic circulation have substantial impact on targeted organs or tissues, which directly influences the progression of many metabolic diseases (55). Bone morphogenetic protein 7(BMP7), as a classical batokine, could lead to an acute decrease in food intake partly through a central rapamycin-sensitive mTOR-p70S6 kinase pathway after Intracerebroventricular administration. In addition, among a variety of batokines, FGF21, IL6 and ANGPTL8 might directly target the pancreas and improve insulin secretion and β-cell function, while NRG4 and IGF1 had been demonstrated to attenuate lipogenesis in the liver (56–58). In addition to contributing to the obese phenotype and dysfunction of glucose and lipid metabolism, whitened BAT could cause the onset of polycystic ovary syndrome, gut flora disorder, and cardiovascular diseases as well (59, 60). Therefore, BAT transfer, as an effective way to combat the whitening process, has been adopted by many researchers in the treatment of obesity and other diseases correlated with metabolic disorder.

Transplantation of brown adipose tissue

Autologous transplantation of traditional subcutaneous WAT or its mechanical processed products was usually utilized for the reconstruction of soft-tissue defects in clinical practice (61, 62). Interestingly, the spontaneous browning of transferred WAT was observed in some studies, which did not last long and might be part of the survival mechanism where the browned subcutaneous WAT participates in adaptive tissue remodeling following grafting and contributes to adipose tissue repair under the extreme ischemic and hypoxic environment after transplantation (63–65). Of course, the temporarily transformed brown-like adipocytes did not exert much influence on overall energy expenditure and metabolism, which also highlighted the importance of browning characteristic maintenance in fat transplant with the aim of long-term metabolic improvement.

Contrary to the large reserves and easy access of subcutaneous WAT, BAT depots are of low reserves and confined to specific anatomical regions in mammals (66). Under such circumstances, the brown fat transplants mainly included *in vivo* surgically harvested BAT and engineered brown adipocytes, beige adipocytes, or preadipocytes (67, 68). Generally, the transplantation methods included autologous transplantation, syngeneic transplantation, and cross-species transplantation, with the immune response greatly eliminated by selecting the immunocompromised mammals, such as nude mice, as recipient subjects (69).

BAT obtained in mammals yielded different outcomes after transplantation because of the variation of the strain, age, donor sites, receiving sites, total transferred volume, perioperative treatment, etc. (Table 1) (70). According to the survival and regeneration theory proposed by Yoshimura et al., large-volume fat grafting would lead to necrosis of the core area due to the ischemic and hypoxic microenvironment in the transplanted areas, and only the transplanted fat spheres with a diameter of no more than 3.2 mm could be completely vascularized under ideal conditions (71, 72). In addition, although BAT itself has brown adipocytes with a larger surface-to-volume ratio and presents higher microvessel density than both WAT and beige AT, it exhibits the lowest retention rate after transplantation, probably due to the necrosis of the high oxygen consumption brown adipocytes and the local persistent inflammatory state (32). Therefore, the transplantation of the whole brown fat pads or large volume of mechanically processed brown fat fragments would result in massive necrosis in the core zone of the fat graft, which suggests the BAT acquired in mammals should be broken into pieces in advance, and the total transferred volume in one recipient site should be limited for better survival. In addition, the implantation of BAT fragments with extremely small volume would lead to the complete disappearance of the fat graft, where it has been previously reported that subcutaneously transferred brown fat weighing 1-3 mg did not form fat pads (73). The explanation for this outcome could lie in the insufficiency of regeneration signals, leading to failure of the recruitment and directed differentiation of stem cells, and causing clearance mediated by rapidly recruited M1 type macrophages.

BAT acquired from WT mice presented beneficial effects to the metabolism of recipient WT or obese mice, including the reduction of body weight, decrease of total body fat mass, and increase of energy expenditure, and improvement of insulin resistance, thyroid hormone sensitivity, and liver steatosis (74). Regretfully, the transferred BAT did not keep its thermogenic and metabolic

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TABLE 1 Previous studies of transplanted BAT or engineered cells with thermogenic potential are summarized.

Graft sources	Recipient sites	Treatments	Graft outcomes and metabolic effects
Brown fat from WT mice	Perirenal in WT mice	None	No innervation after 2 weeks. Increased cell size and lipid content. Metabolic effect not detected.
Brown fat from WT or ob/ob mice	Perirenal In WT mice or ob/ob mice	(1)Exposure to 4°C for consecutive 5 weeks (2)Exposure to 23°C or 33°C for consecutive 5 weeks (3)Exposure to 4°C for consecutive 5 weeks followed by another 3 week exposure to 23°C	(1) Long time exposure to cold temperature completely transformed lipid size and mitochondrial structure to that of host. Satisfactory innervation and vascularization. This phenomenon was not observed in host ob/ob mice. (2) Warm or hot temperature partially transformed lipid size and mitochondrial structure to that of host. Very few innervations or vascularization. (3) Cold adaptation followed by warm temperature exposure still maintained complete transformation to that of host. Satisfactory innervation and vascularization . This phenomenon was not observed in host ob/ob mice.
Brown fat from WT mice weighing 1–3 mg	Subcutaneous in WT mice	None	Subcutaneous fat pad not formed.
Brown fat fragments from rats	Intramuscular in rats	None	Intramuscular fat pad formed. Metabolic effect not detected.
Brown fat fragments from WT mice	Dorsal subcutaneous region in WT mice	None	Subcutaneous fat pad formed. A significant reduction of body weight. Increased oxygen consumption and decreased total body fat mass. Improvement of insulin resistance and liver steatosis. Up-regulation of thyroid hormone sensitivity . Increased β3-adrenergic signaling and fatty acid oxidation in WAT.
Brown fat fragments from WT mice	(1) Dorsal subcutaneous region in ob/ob mice (2) Deep into the quadriceps femoris muscle in ob/ob mice	None	Subcutaneous fat pad formed with the retention rate being 24.2% ± 3.5% and histological and gene expression pattern features showing fat transplant whitening after 16 weeks. Short-term but no long-term effect on the reduction of body mass gain, increase of energy expenditure or reduction of subcutaneous adipose tissue inflammation. Intramuscular fat pad formed with the retention rate being 22.0% ± 3.6% and histological and gene expression pattern features showing maintained BAT features after 16 weeks. Both short-term and long-term effect on the reduction of body mass gain, increase of energy expenditure and reduction of subcutaneous adipose tissue inflammation.
Brown fat fragments from WT mice	Flank subcutaneous region in WT mice	None	Compared to the transferred beige fat or white fat, the grafted brown fat showing the lowest retention rate and more severe inflammation and adipocyte apoptosis early after transplantation. Whitening of transferred BAT. Metabolic effect not detected.
Beige adipose tissue from ex vivo browning of subcutaneous WAT in WT mice	Re-implantation subcutaneously into the host WT mice.	None in this research, but could potentially act with pharmacological approaches.	Endogenous BAT increased. Metabolic effect not detected.
VEGF-A-overexpressing adipose tissue	diet-induced obese mice	None	Systemic metabolic benefits, associated with improved survival of adipocytes and a concomitant reduced inflammatory response.
Brown adipose tissue from rats	(1)PCOS rats (2)PCOS mice	None	(1)Recovered the ovarian function of PCOS rats. (2)Significantly prolonged the fertility of aging mice and did not cause severe rejection reaction, and significantly recovered ovarian functions. improved insulin resistance.
Isolated brown adipocytes or preadipocytes	Perirenal in rats	None	Fat pad not formed.
Brown adipose progenitor cells (BAPCs) from WT mice	Limb skeletal muscles in WT mice	VEGF administration	The ectopic formation of UCP1+ adipose tissue with long-term engraftment (>4 months). Combining VEGF with the BAPC transplant further improved BAT formation in muscle.
C3H10T1/2 cells	Subcutaneous in athymic mice	BMP7 administration	UCP1+ brown fat pad formed. Increased energy expenditure. Dreased body weight gain decreased.

(Continued)

TABLE 1 Continued

Graft sources	Recipient sites	Treatments	Graft outcomes and metabolic effects
MEFs	Subcutaneous in athymic mice	Transduced with PRDM16 and C/EBP-β	Formed brown fat pad with UCP1-positive multilocular and unilocular fat cells. Glucose uptake into fat pad. Increased basal respiration.
CRISPR-engineered human brown-like adipocytes	In thoracic-sternum region of diet-induced obese nude mice	Fed with 45% HFD for a further 4 weeks	Reconstitute functional adipocytes and activate endogenous murine BAT in mice. Facilitate glucose metabolism and thermogenesis and display long-term metabolic benefits in mice.
White adipose tissue-derived multipotent stem cells (ADMSCs) within optimized hyaluronic acid-based hydrogels	Subcutaneous implantation in WT mice	None	Distinct UCP1-expressing implants that successfully attracted host vasculature and persisted for several weeks. Elevated core body temperature during cold challenges, enhanced respiration rates, improved glucose homeostasis, and reduced weight gain.

The graft sources, recipient sites, perioperative treatments and transplantation outcomes are listed in detail.

characteristics for long, with the whitening process aggravated over time in brown fat graft especially when transferred to obese mice (75). Syngeneic intramuscular transplantation of brown fat fragments in rats showed that fat pads formed well in the recipient site based on the magnetic resonance imaging and ultrastructural studies (76). Our previous study showed that the transplanted BAT from WT mice deep into the quadriceps femoris muscle in ob/ob mice could maintain the BAT features up to 16 weeks, exerting a lasting influence on the reduction of body mass gain, increase of energy expenditure, and reduction of subcutaneous adipose tissue inflammation (33). The better microvascular foundation and more abundant secretion of paracrine factors acting as browning agents, such as irisin and other myokines in muscle microenvironment, were the possible mechanism for the long-term browning feature maintenance of intramuscular BAT transplantation (77–79). In addition, exposure to cold after transplantation enabled the brown fat graft to maintain satisfactory innervation and vascularization among adipocytes similar to that of BAT in WT mice for a longer time (80).

However, BAT resected from both diet-induced and genetically abnormal obese mice showed decreased UCP1 expression, sparse mitochondria distribution, and increased cell size and lipid content of adipocytes (81). While transplanting the whitening BAT from obese mice to WT mice, the transplant itself regained some browning features similar to that of the host but had hardly any beneficial effect on the metabolism of the host (80). Transplantation of BAT between obese or WT mice also indicated that the host environment, rather than the donor of the fat graft, determined the morphological and functional change of the transplanted BAT in the early phase after transplantation.

Autologous or syngeneic transplantation of beige adipose tissue, either induced *in vivo* by cold exposure and browning agents or from ex vivo browning of subcutaneous WAT from WT mice, showed increased endogenous BAT content and improved metabolic status early after transplantation (82, 83). Even though the retention rate of transferred beige adipose tissue was the highest compared to WAT and BAT transplantation, its thermogenic and metabolic potential was much weaker than BAT, which made it unsuitable as a fat transplant for improving metabolic abnormalities (32). Transplantation of VEGF-A-overexpressing adipose tissue from a doxycycline-inducible adipocyte-specific mouse model to diet-induced obese mice showed systemic metabolic benefits associated with improved survival of adipocytes and a concomitant reduced inflammatory response (84). This study showed the importance of rapid vascularization in transplants in the maintenance of BAT function after transplantation.

Rat-to-mouse BAT xenotransplantation, as reported recently, did not cause obvious immunorejection and significantly recovered the fertility of mice with polycystic ovarian syndrome (PCOS), accompanied by the recovery of oocyte quality, up-regulation of multiple essential genes and kinases connected to ovarian function, and the improvement of insulin resistance (85). This study provided feasibility of BAT xenotransplantation by the quantitative description of immune response, which has always been assumed to be strong by default on most occasions. In the future, research will continue to search for a BAT xenograft that elicits little immune response or drugs with the potential to dramatically reduce immune rejection of a BAT xenograft when transferred to humans.

Transplantation of engineered thermogenic progenitor cells or adipocytes

The methods of obtaining BAT *in vivo* are limited by the inadequate storage and the inevitable large trauma to the donor sites; thus, utilizing ex vivo culturing, expansion, and engineering of thermogenic progenitor cells or adipocytes for the brown fat transplants seems like a feasible alternative choice (Table 1). First of all, implantation of isolated brown adipocytes or preadipocytes under the kidney in rats yielded no newly formed fat pads, which indicated that the perirenal area, lacking sufficient vascularization and cytokine secretion, might be unsuitable as the recipient site for preadipocyte or brown adipocyte transplantation (76).

Transplantation of isolated and expanded brown adipose progenitor cells (BAPCs) into limb skeletal muscles showed the

ectopic formation of UCP1 positive adipose tissue with long-term engraftment and augmented energy expenditure, and combining VEGF with the BAPC transplant further improved BAT formation in muscle (86). Furthermore, a previous study had CRISPR-engineered human brown-like adipocytes transferred to the thoracic-sternum region of diet-induced obese nude mice, with the result showing facilitated glucose metabolism and thermogenesis and presenting long-term metabolic benefits in the group fed with a 45% high-fat diet(HFD) for a further 4 weeks (69). Taken together, transplantation of the engineered thermogenic progenitor cells or adipocytes favored well-vascularized recipient sites since the transplant survived the extreme microenvironment partly by the infusion of nutrients and oxygen through newly formed microvessels.

However, some engineered cell lines still showed satisfactory browning features when transferred subcutaneously into recipients. *In-vitro* cultured C3H10T1/2 mesenchymal progenitor cells were reported to be capable of changing into brown adipocytes and increasing energy expenditure and mitochondrial biogenesis, and decreasing weight gain after BMP7 administration when transplanted subcutaneously into athymic mice (87). Likewise, MEFs transduced with retroviral PRDM16 and C/EBP-β showed brown adipocyte differentiation within the formed fat pads with high expression of UCP1, active glucose uptake, and increased basal respiration after subcutaneous transplantation into athymic mice (88).

Transplantation of white adipose tissue-derived multipotent stem cells (ADMSCs) conjugated with optimized hyaluronic acid-based hydrogels into the subcutaneous region of WT mice successfully attracted host vasculature and persisted for several weeks, and functionally elevated core body temperature during cold challenges, enhanced respiration rates, improved glucose homeostasis, and reduced weight gain (89). In this study, the hyaluronic acid-based hydrogels worked as cell scaffolds for supporting the directed differentiation of ADMSCs and the eventual establishment of functional brown fat-like depots. This attempt proved the importance of the extracellular matrix (ECM) in the regeneration process after engineered adipocyte or preadipocyte transfer. Biomaterials providing extracellular scaffolds and inducing directed differentiation towards UCP1-expressing adipocytes, such as the acellular adipose matrix (AAM), might be useful for assisting the transplantation of engineered adipocytes or preadipocytes (90, 91).

Possible methods for preventing the whitening of transferred BAT or engineered cells

First of all, the intrinsic histological and functional characteristics of the graft itself and the selection of the transplant recipient sites all affect the biological performance of the graft, including the alteration of UCP1-mediated thermogenesis and systemic metabolic activity (92). BAT obtained from aged or obese individuals exhibited impaired thermogenic and metabolic potential and produced much fewer metabolic benefits to the host after transplantation; thus, selecting young and healthy donors could improve transplantation results (93). In addition, the engineering method of BAT or even isolated adipocytes and preadipocytes aiming to enhance the thermogenic potential, including the application of CRISPR and iPSC technology, showed varying graft destiny and manifestations of its browning characteristics after transplantation. Diverse engineering methods have endowed BAT or brown fat-like cells with greater thermogenic potential, which has drawn much attention from researchers recently (94).

In addition, the well-vascularized and active cytokine secreting recipient areas facilitated the survival and maintenance of browning features of brown fat graft (95). Therefore, compared to perirenal or subcutaneous transplantation, intramuscular transplantation of either BAT resected *in vivo* or adipocytes and preadipocytes engineered *in vitro* showed better retention and prolonged BAT feature maintenance (Figure 1).

Long-term cold exposure was adopted by many researchers for the induction of beige adipose tissue or the enhancement of browning features of BAT (96). In several studies conducted on mammals, recipients exposed to cold after BAT transplantation manifested the prolonged browning feature maintenance of fat graft and systemic metabolic benefits (80, 97). In addition, local injection or oral administration of browning agents, such asβ3 adrenergic receptor agonists, PPARγ agonists, estrogen analogs, etc., could promote the re-browning of transferred brown fat undergoing a whitening process (98, 99). In addition, local administration of growth factors and small molecule nutrients to recipient sites might promote the vascularization and regeneration of newly developed brown adipose tissue (100, 101). Sufficient uptake of food supplemented with nutrients with browning potential, such as capsaicin, synephrine alkaloids, etc., might help the maintenance of browning features of transplanted brown fat (102, 103). However, these approaches affect not only the transferred brown fat but also the function of BAT and WAT in the recipient and cause a multi-systemic response. Consequently, the overall functional change of target organs should be monitored in the practice of brown fat grafts for better risk control.

Combined transplantation of BAT and adipose-derived stem cells (ADSCs) might help the survival and regeneration of fat grafts since the transferred ADSCs have exhibited the potential to differentiate into vascular cell components or directly into mature adipocytes and to secrete paracrine cytokines or chemokines for the further recruitment and differentiation of stem cells from the host in previous research regarding the technology of cell-assisted lipotransfer (CAL) (104–106). Another recent discovery in the field of BAT research was that the gut microbiota modulated the metabolism and activity of BAT, and that depletion or imbalance of gut microbiota impaired thermogenesis of BAT (107–109). Under such a remarkable regulatory relationship, it would be reasonable to conduct gut microbiota transfer simultaneously with BAT transplantation for pronged thermogenic and metabolic benefit maintenance.

Conclusion

Unlike the utilization of subcutaneous WAT in volume augmentation for esthetic and reconstructive surgery, the preserved

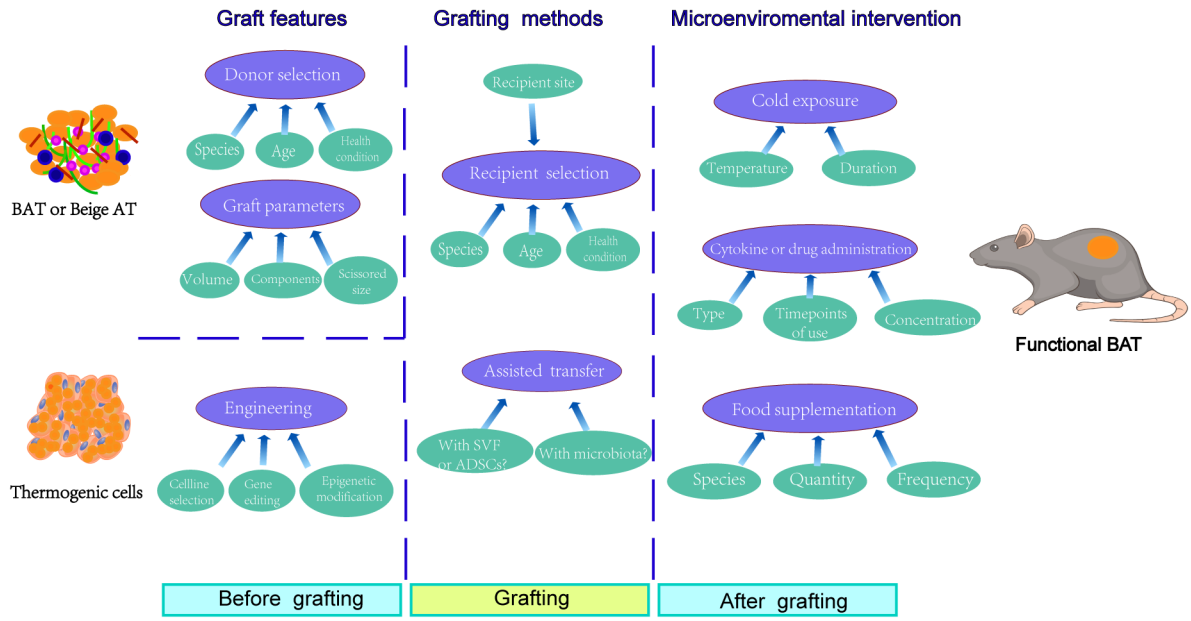

FIGURE 1
Factors involved in graft outcomes and function maintenance of transferred BAT or thermogenic cells. Before grafting, the graft itself dramatically determined the transplantation outcomes, which mainly involved the donor selection and the mechanical pretreatment of adipose tissue. As for thermogenic cells, the engineering methods, including cell line selection, gene editing, and epigenetic modification, affected the transfer results. During grafting, the recipient selection was also relevant to the graft outcomes, especially for the choice of recipient site. In addition, the simultaneous transplantation of stromal vascular fraction (SVF), adipose derived stem cells (ADSCs), or gut microbiota from healthy donors seemed like adoptable approaches for improving grafting results. After grafting, however, the microenvironmental intervention using cold exposure, cytokine or drug administration, or even specific food supplementation provided a promising way for the function maintenance of transferred BAT or thermogenic cells.

thermogenic and metabolic potential of transferred BAT or engineered adipocytes and preadipocytes inspired researchers to tentatively apply transplantation of thermogenic adipose tissue or engineered cells for metabolic improvement. Brown adipose tissue *in situ* functions as a heat production factory with the UCP1 protein mediating uncoupling thermogenesis based on the adequate vascularization for sufficient substrate and oxygen supply and appropriate stimulation of certain cytokines, such as BMP7 and PRDM16, for directed differentiation and functional protein expression of progenitor cells. Unfortunately, the brown fat graft was in an extreme microenvironment where the oxygen and nutrient supply was obviously insufficient and the signaling molecules were differently expressed.

Whitening of the transferred BAT or engineered adipocytes and preadipocytes remain a barrier to the long-term improvement of energy expenditure and metabolism. The specific mechanisms and possible solutions for whitening of transferred BAT are still poorly understood. Therefore, it is necessary to analyze recent progress in the improvement of brown fat graft methods and to further elucidate the possible influencing factors in order to prevent the whitening of transferred BAT or engineered brown fat-like cells and create a mobile metabolic factory for sustainable thermogenesis.

Author contributions

TZ and SJ conceived and wrote the manuscript, while XC searched the database and provided some relevant literature. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Signaling metabolite β-aminoisobutyric acid as a metabolic regulator, biomarker, and potential exercise pill

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Signaling metabolites can effectively regulate the biological functions of many tissues and organs. β-Aminoisobutyric acid (BAIBA), a product of valine and thymine catabolism in skeletal muscle, has been reported to participate in the regulation of lipid, glucose, and bone metabolism, as well as in inflammation and oxidative stress. BAIBA is produced during exercise and is involved in the exercise response. No side effect has been observed in human and rat studies, suggesting that BAIBA can be developed as a pill that confers the benefits of exercise to subjects who, for some reason, are unable to do so. Further, BAIBA has been confirmed to participate in the diagnosis and prevention of diseases as an important biological marker of disease. The current review aimed to discuss the roles of BAIBA in multiple physiological processes and the possible pathways of its action, and assess the progress toward the development of BAIBA as an exercise mimic and biomarker with relevance to multiple disease states, in order to provide new ideas and strategies for basic research and disease prevention in related fields.

KEYWORDS

BAIBA, exercise mimic, biomarker, metabolic regulation, inflammation, ROS

1 β-aminoisobutyric acid: a signaling metabolite produced in response to exercise

Organisms maintain their life activities through the transformation of substances into energy; small-molecule products in this process are called metabolites (1). Some metabolites that participate in the transmission of information between cells are referred to as signaling metabolites (2). They represent important control points in homeostasis and may serve as biomarkers of diseases. Previous studies had suggested β-aminoisobutyric acid (BAIBA) as a muscle factor (myokine); however, BAIBA, being an amino acid metabolite, is neither a protein nor an active peptide, and may be more appropriately referred to as a muscle signaling metabolite.

BAIBA is a catabolic metabolite of thymine and valine in skeletal muscle (3). It is present as D- and L-enantiomers in mammals, D-BAIBA being an intermediate product of thymine degradation in the cytoplasm, and L-BAIBA being produced by L-valine decomposition (3). In 2014, Roberts et al. (4) had observed a significant increase in BAIBA synthesis in myoblasts, after PGC-1α treatment, by liquid chromatography-liquid mass spectrometry (LC-MS). Subsequent *in-vivo* and *ex-vivo* experiments confirmed that BAIBA might promote the differentiation of pre-adipocytes into brown fat, promoting free fatty acid (FFA) oxidation (4, 5) Further, BAIBA was considered to indirectly up-regulate browning and fatty acid oxidation in the liver, maintain lipid homeostasis, and prevent and improve lipid metabolism disorders (4, 6–15). In addition, BAIBA was reported to improve glucose homeostasis in mice (8, 13, 16); it could regulate the differentiation of osteoprogenitor cells, affect the balance between the number of osteoblasts and osteoclasts, maintain bone mass homeostasis (17, 18), reduce inflammatory response and oxidative stress (17, 19), and improve related diseases (4, 8, 14, 15, 20). Overall, BAIBA has drawn abundant attention of researchers recently.

The synthesis and metabolism of BAIBA are closely related to exercise, and no adverse effect has been observed in subjects, animals, or cells receiving different doses of BAIBA, suggesting its potential to be developed as an exercise pill. In addition, BAIBA has been associated with the development of osteoporosis and neo-coronary pneumonia; however, differences in conditions used by different investigators may be fundamental to BAIBA's role as a disease marker. The only review on BAIBA till date has reported its relationship with lipid metabolism (21), and the mechanisms of action of BAIBA are still mostly speculative. Therefore, in this review, we discussed the biological functions of BAIBA, its relationship with exercise, drug applicability, and considerations of it being a disease marker, in order to provide a theoretical basis for the study of BAIBA and related diseases.

2 BAIBA metabolism process

Thymine and dihydro pyrimidine dehydrogenase ((DPYD)) and hydrogen reacted to form two Thymine (Dihydrothymine) (22). Dihydrothymine combined with dihydropyrimidinase (DPYS) to form N-carbamoyl-β-amino-isobutyric acid (N-carbamoyl-BAIBA). After further reaction of D-BAIBA by N-carbamoyl-BAIBA and beta-ureidopropionase (UPB1), D-methylmalonic semialdehyde (D-MMS) was finally produced through AGXT2 in mitochondria (23). Studies have detected the expression level of AGXT in various organs such as the brain, liver, pancreas, spleen, heart, lung, esophagus, stomach, small intestine, colon, skeletal muscle, kidney, thymus, testis, ovary, uterus, prostate, tongue and skin. It was found that the expression level was higher in the liver and kidney.

L-BAIBA was produced by catabolism of the branched amino acid L-VALINE (24). L-VALINE was formed under ammonia, and the oxidation reaction of methyl malonyl half aldehyde (L-methylmalonylsemialdehyde, L-MMS). L-MMS produced L-BAIBA in reaction with mitochondrial enzyme 4-aminobutyrate aminotransferase (ABAT) (25, 26). It has been reported that the production of L-BAIBA by ABAT is a bidirectional reaction, so the same enzyme can catalyze the conversion of L-BAIBA to L-MMS. At the same time, the Malonate – semialdehyde dehydrogenase (MMSDH), can L-oxide MMS and D-MMS propionyl coa (24). L-BAIBA is converted to D-BAIBA and vice versa through the stereoisomerization pathway between L-MMS and D-MMS (24, 27). MMSDH has been less reported in organs and seems to be concentrated in the liver.

3 Biological functions of BAIBA

3.1 BAIBA and lipid metabolism

3.1.1 BAIBA promotes browning of white fat and enhances fat metabolism

Fat is an important energy source that provides energy to the body in the presence of a variety of enzymes and is important for maintaining life activities. However, disturbances in fat metabolism can induce the development of diseases, such as obesity and non-alcoholic fatty liver (28). The 2022 a study reported no significant change in body weight and fat mass in mice fed a high-fat diet along with BAIBA intervention, compared to that in the normal diet group (20), suggesting that BAIBA might play an important role in the prevention of high-fat diet-induced fat deposition. Fat tissue can be classified morphologically and functionally as white, brown, or beige. White fat cells have large lipid droplets and few mitochondria, which facilitate energy storage. Brown fat cells have small lipid droplets and many mitochondria, which facilitate the oxidative release of energy. Beige fat cells have properties intermediate between those of white and brown fat cells. When stimulated by cold or exercise, white fat cells can convert to beige or brown fat cells (29). Both beige and brown fat present highly specific molecular markers, such as the beige marker T box 1 transcription factor (TBX1), the browning marker uncoupling protein 1 (UCP1), cell death-inducing DFFA-like Effector A (CIDEA), PR domain-containing 16 (PRDM16), and elongase of very long chain fatty acids (ELOVL3) (30).

BAIBA treatment induced morphological changes and increased the levels of browning markers in pre-adipocytes (5). Comparing the effects of different doses of BAIBA and differentiation time on morphological changes and beige/browning markers of lipid droplets in 3T3-L1 (preadipocytes) cells, the number of lipid droplets were found to be increased and the surface area decreased in 3T3-L1 cells, after high BAIBA intervention, on day 4 of differentiation. Beige markers TBX1, browning markers UCP1, CIDEA, PRDM16, and ELOVL3, and the mitochondrial biogenesis marker mRNA were significantly increased, although the effects disappeared after 10 days, suggesting that BAIBA acts early in differentiation. Previous studies had shown that PGC-1α, a functional factor in white fat browning, alleviates mitochondrial dysfunction and increases FFA oxidation in obesity (4, 31). PGC-1α treatment of myocytes led to

an increase in BAIBA synthesis, along with increased FFA oxidation, and up-regulation of PPARα, a transcriptional activator. BAIBA enhanced the expression of several other markers in pluripotent stem cells via PPARα (4). This effect was dose-dependent, suggesting that BAIBA could improve lipid deposition by increasing white fat browning, up-regulating coupling, inhibiting phosphorylation, down-regulating adenosine triphosphate (ATP) production, converting bioenergy to heat, and promoting FFA metabolism (4). Overall, it improved lipid deposition.

In summary, BAIBA may regulate lipid metabolism by promoting FFA oxidation through PPARα-mediated white fat browning, and this regulation may occur early in adipocyte differentiation (4, 5, 20). Studies on BAIBA-mediated changes in lipid metabolism are still scarce, and the possible involvement of BAIBA in white fat browning under cold stimulation or fasting is yet be explored.

3.1.2 BAIBA regulates hepatic lipid metabolism

BAIBA may affect fat metabolism in the liver, the main site for FFA synthesis and oxidation. *In-vivo* studies have shown that BAIBA promotes FFA oxidation in the livers of normal mice (4, 6), whereas *in-vitro* experiments suggested no significant change in FFA oxidation following BAIBA treatment of hepatocytes (10). Liver lipid browning is mainly regulated by sympathetic activity, and L-BAIBA is known to improve oxidative stress in H_2O_2-treated mouse pheochromocytoma cells (32). BAIBA may not directly regulate hepatic FFA oxidation but might play an indirect role (4, 6, 10, 32, 33); confirmation will require further exploration of nerve-liver crosstalk to clarify the specific regulatory role of BAIBA.

In FFA synthesis, phosphorylated adenosine 5'-monophosphate-activated protein kinase (AMPK) inhibits hepatic fatty acid and triglyceride (TG) production and downregulates FFA synthase expression by inactivating sterol regulatory element-binding proteins (SREBPs)-1c through autophosphorylation. Shi et al. (13) reported that BAIBA attenuated glucosamine-induced ER stress in human hepatocellular carcinoma HepG2 cells and ameliorated the decrease in AMPK phosphorylation under pathological conditions, suggesting that activation of AMPK by BAIBA during ER stress might facilitate lipid metabolism and reduce intracellular lipid ectopic accumulation in hepatocytes (13). Interestingly, the study reported no significant change in ER stress in the medium without the addition of aminoglucose. The latest study in this regard has shown that aminoglucose ions and glucose play a central role in lipid metabolism by synergistically regulating SREBPs expressed in HepG2 and ER membranes, which are activated by ER-Golgi-nuclear translocation processes, suggesting that aminoglucoseions may mediate BAIBA regulation of lipid metabolism. The results suggested that ammonia ions may be critical in mediating the regulation of hepatic lipid synthesis by BAIBA. However, further studies would be required to prove this inference (34).

Although the role and mechanism of BAIBA in hepatic FFA oxidation and white fat browning are not fully clear yet, ammonium ions may play a role in regulating hepatic lipid synthesis in pathological states, suggesting possible future research directions (4, 8–10, 13, 33, 35).

Prevention of elevated lipid levels in blood could be an important approach for treating abnormal lipid metabolism. The role of BAIBA in lipid homeostasis is currently controversial, with some studies showing over expression of PGC-1α, a factor upstream of BAIBA in skeletal muscle, to not affect plasma lipid levels (35). Similarly, oral administration of BAIBA to lean mice had no significant effect on serum lipid parameters, including FFA, TG, total cholesterol, and phospholipid concentrations (9). Shimba et al. (35) reported no significant change in serum TC, HDL-c, LDL-c, or TG levels in ApoE-knockout mice (ApoE-KO) following oral administration of BAIBA. The findings suggested that BAIBA could be an important factor in the maintenance of lipid homeostasis. Shi et al. (13) observed that oral administration of BAIBA upregulated serum fasting FFA, TG, and LDL levels in mice with high-fat diet/low-dose streptozotocin-induced type 2 diabetes mellitus (T2DM). Considering that blood plays an important role in transporting glycerol and FFA, which are carried to the liver or other organs for synthesis of TG or breakdown by oxidation, BAIBA may upregulate lipolysis in adipocytes to compensate for the decrease in serum FFA levels or even upregulate serum FFA levels and maintain lipid homeostasis.

Leptin promotes FFA oxidation in humans and mice (11, 12, 36). Leptin-deficient obese ob/ob mice showed no change in lipid parameters after BAIBA treatment (9, 10), although heterozygous ob/+ mice showed low postprandial TG and fasting cholesterol levels (10). D-BAIBA is degraded by alanine-glyoxalate aminotransferase 2 (AGXT2) to D-methylmalonate semialdehyde (MMS) in mitochondria (23). Rhee et al. (37) showed that serum BAIBA is downregulated after AGXT2 mRNA knock out, suggesting that AGXT2 may regulate lipid metabolism via a response to L-BAIBA or other substrates; future studies would need to monitor the changes in L-BAIBA or other substrates to verify the role of BAIBA in lipid regulation (Figure 1).

3.2 BAIBA affects carbohydrate metabolism

BAIBA significantly lowers blood glucose levels in T2DM mice and in mice with insulin resistance (IR). At the same time, one large human cohort study showed serum BAIBA levels to be negatively correlated with serum insulin concentrations (4). BAIBA has been hypothesized to possibly regulate blood glucose by regulating insulin levels; however, since the mentioned study was cross-sectional, the causal relationship between BAIBA and insulin remains unclear. Mitochondria in pancreatic b-cells influence the oxidative phosphorylation of glucose to regulate ATP synthesis and eventually insulin secretion (38). Barlow et al. (39) reported that BAIBA downregulates mitochondrial energy metabolism and insulin release in INS-1832/3 cells *in vitro*. However, this was seen only upon stimulation with the next highest concentration of glucose, and BAIBA had no significant effect on insulin release in INS-1832/3 cells upon glucose and palmitic acid addition. Glucose uptake by tissues can occur through both insulin-dependent and insulin-independent pathways (40), and BAIBA has been speculated to regulate blood glucose levels in IR or T2DM in an insulin-independent manner. However, it has also been shown that

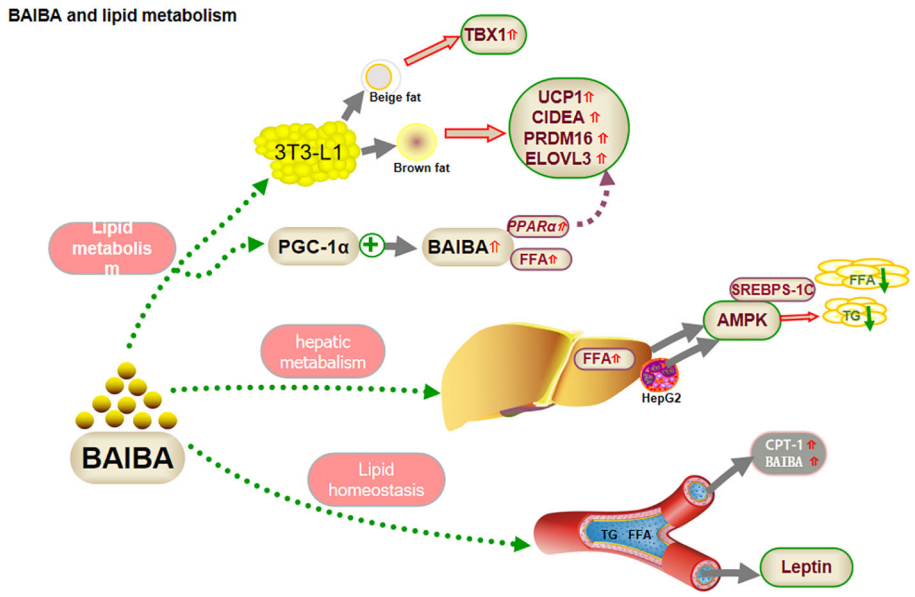

FIGURE 1
BAIBA and lipid metabolism.

glucose uptake by tissues may occur through both insulin-dependent and non-insulin-dependent pathways (40). Insulin is the only hormone in the body that lowers blood glucose levels; however, it also promotes lipid synthesis (41). PI3K-AKT-mTOR is the classical pathway that responds to insulin signaling, with insulin binding to cell surface receptors and activating the PI3K-AKT pathway via IRS1 to directly promote glucose uptake while activating mTORC via AKT to further utilize glucose (42). Increased AMPK activity is positively correlated with GLUT4 translocation to the plasma membrane and rapid glucose uptake (43). Although several experiments with BAIBA intervention (8, 13, 16) had shown serum insulin levels to remain unaltered, significant reductions in blood glucose were observed, and significant improvements in AMPK, AKT, and IRS-1 expression were observed, suggesting that BAIBA may increase insulin sensitivity and lower blood glucose without affecting insulin secretion (13, 16). Future studies on the role of BAIBA in the regulation of glucose metabolism and its mechanisms may represent a new direction for the treatment of IR and metabolic diseases (Figure 2).

3.3 BAIBA affects bone metabolism

Previous studies had suggested that the action of skeletal muscle on bone is mediated exclusively through mechanical stimuli; recent studies have shown that BAIBA can exert effects on bone proliferation and differentiation (44). Wang et al. (45) reported a positive correlation between hip T-score and serum D-BAIBA levels in healthy elderly, and bioinformatic analysis showed the genes related to bone metabolism to be closely associated with the synthesis and action of BAIBA, suggesting that BAIBA is a potential signaling molecule for bone metabolism. TGF-b promotes early differentiation and matrix production while inhibiting late differentiation and matrix mineralization, and serum BAIBA levels were correlated with TGF-β expression in patients with Duchenne muscular dystrophy when BAIBA was used in an intervention (46); this suggested that BAIBA might regulate bone metabolism via osteoprogenitor cells.

Osteoblasts are multifunctional cells that regulate bone reconstruction (18, 47). Kitase et al. (18) observed that oral administration of L-BAIBA reversed the reduction in bone trabecular volume in mice after two weeks of hindlimb suspension, and that mRNA expression of osteoclast metabolic factors was not affected by suspension. The regulation of bone metabolism by L-BAIBA has been suggested to not occur through sclerostin or RANKL/OPG regulation of osteoblasts. As mentioned above, the improved reduction in trabecular volume and the induction of BMSCs in the bone matrix by TGF-b to migrate to sites of bone resorption to promote osteoblast formation (46, 48–50). This suggested that the regulation of bone metabolism by BAIBA may revolve around osteoblasts rather than osteoclasts.

Reactive oxygen species (ROS) are important messengers in osteoblast proliferation and differentiation, and play an important role in the proliferation of pre-osteoblastic cells. Zhu et al. (17) observed that BAIBA activates the reduced nicotinamide adenine dinucleotide phosphate (NADPH)/ROS signaling pathway in a dose- and time-dependent manner, thus promoting osteoblast differentiation. BAIBA intervention in MC3T3-E1 (mouse embryonic osteoblast precursor cells) cells resulted in a significant increase in ROS production, a significant acceleration of osteoblast proliferation and differentiation, and a significant increase in osteoblast transcriptional regulators, with mRNA expression increasing in a dose-dependent manner (17). The mRNA expression of osteoprotegerin (OPG) increased in a dose-dependent manner. In addition, NADPH oxidase (NOX) 4 expression was significantly upregulated in BAIBA-treated

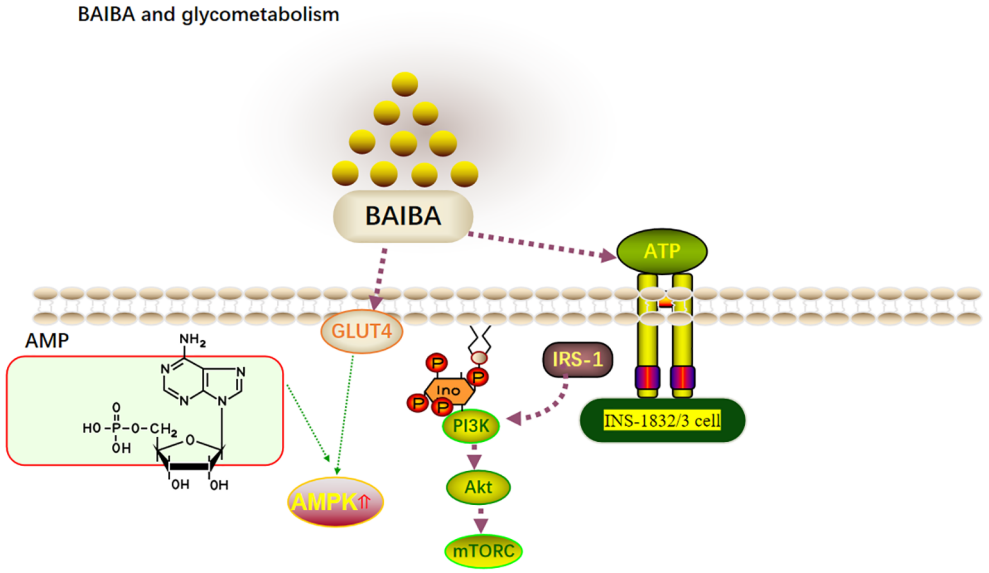

FIGURE 2
BAIBA and glycometabolism.

MC3T3-E1 cells, although it had no effect on the expression of NOX1 and NOX2 proteins in their family, suggesting that BIBA may induce ROS production in MC3T3-E1 cells through NOX4 to promote osteogenic differentiation. The Mas-related G protein-coupled receptor type D (MRGPRD) antagonist MU6840 blocked the effect of L-BAIBA on ROS induction (19), suggesting that L-BAIBA may have a direct effect on osteoblasts (18). The MRGPRD receptor was found to be significantly expressed in osteoblasts from juvenile mice, but was reduced in osteocytes from aged mice, which possibly indicated that the protective effect of BAIBA diminishes with age, not due to a diminished capacity of the muscle to produce BAIBA. The regulation of BAIBA function should be noted for changes in MRGPRD content in future studies. This could have important implications in the understanding of delay in skeletal aging due to exercise (18).

In summary, given the current new concept of muscle-bone crosstalk, the regulatory function of skeletal muscle signaling metabolites in bone may be more important than mechanical loading, with BAIBA promoting osteoblast proliferation and differentiation through NOX4-induced ROS production by osteoblast precursor cells, and TGF-b family members participating in cellular activity and metabolism during osteogenesis through the stimulation of BMSCs (51–53). Therefore, BAIBA may play an important regulatory role from bone progenitors to osteoblasts; however, further validation would be required to confirm this conclusion, since evidence is limited (Figure 3).

3.4 BAIBA improves inflammation

Inflammation is a defensive response of the body to stimuli; short-term inflammation helps to clear inflammatory substances, but chronic inflammation leads to structural and functional abnormalities in tissues and organs. Chronic inflammation of the hypothalamus, induced by saturated fatty acids, leads to proliferation and morphological changes in microglia, which are important regulators of energy metabolism (54–56). Park et al. (20) had reported that BAIBA treatment reverses palmitic acid-induced hypothalamic inflammation and microglia activation, and significantly reduces the expression of the inflammatory response factor cyclooxygenase 2 protein, hence suggesting that BAIBA may play a role in regulating chronic inflammation and protecting the body's health. The energy regulators AMPK, protein kinase B (Akt), and insulin receptor substrate 1 (IRS-1) have been shown to regulate long-term inflammation through multiple pathways (57–59). Jung's team explored the potential relationship between BAIBA and various other factors (8, 15, 16), and found that BAIBA significantly upregulates AMPK, AKT, and IRS-1 expression in 3T3T-L1 cells (15). Further studies showed that BAIBA significantly reverses the phosphorylation of pro-inflammatory factor IκBα, nuclear translocation of nuclear factor κB (NF-κB), serum tumor necrosis factor α (TNFα), and monocyte chemotactic protein-1 (MCP-1) in high-fat diet-fed mice and palmitate C2C12 skeletal muscle cells via the AMPK/PPARδ pathway. A follow-up study showed that BAIBA increased MCP-1 levels (8). The team subsequently showed that BAIBA could downregulate TNFα and MCP-1 expression in lipopolysaccharide-stimulated adipocytes and monocytes via the Akt/IRS-1 pathway and reduce lipopolysaccharide-induced monocyte adhesion to the endothelium (16).

Inflammation and impaired plasma lipid metabolism underlie the pathology of atherosclerosis (60). BAIBA inhibit TNFα-induced VCAM-1 and MCP-1 expression, and reduce atherosclerotic plaque area in ApoE-KO mice while having no effect on plasma lipids. This suggests that BAIBA could improve the inflammatory response and

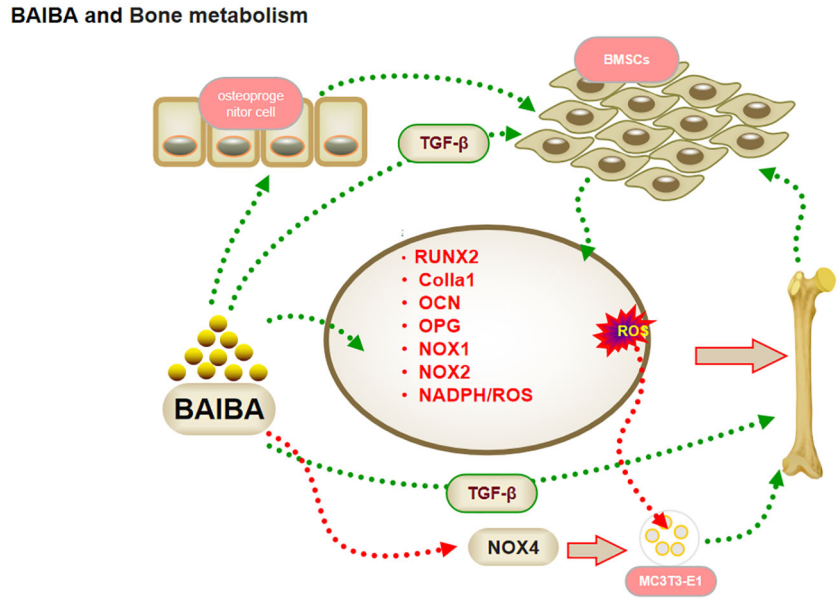

FIGURE 3
BAIBA and bone metabolism.

thus reduce VCAM-1 and MCP-1 expression, as well as the macrophage infiltration of plaque, ultimately inhibiting the development of atherosclerosis (35, 61).

In summary, BAIBA might regulate the inflammatory response through AMPK and AKT pathways (Figure 4). Significant upregulation of the anti-inflammatory proteins, tyrosine protein kinase receptor and interleukin 2 receptor alpha chain protein, after high-dose BAIBA intervention was detected by serum proteomics, suggesting that BAIBA may directly affect the two proteins to mediate inflammation; notably, both were reported to be closely related to AKT expression (62, 63), although their relationship with AMPK expression has not been reported yet. Exploring the relationship between AMPK and the two proteins may be important to further reveal the anti-inflammatory effects of BAIBA in future.

3.5 BAIBA ameliorates oxidative stress

ROS and antioxidants are dynamically balanced in healthy organisms. When this balance is disturbed, increased ROS leads to impaired mitochondrial function and decreased nitric oxide synthase (NOS) expression, which in turn induces increased production of ROS in the electron transport chain, ultimately leading to oxidative stress (64, 65). One study reported that plasma BAIBA is downregulated in patients with novel coronavirus pneumonia and in health care workers severely exposed to SARS-CoV-2, and that citric acid, a marker of mitochondrial metabolic efficiency, is significantly downregulated (66). The significant correlation between BAIBA and mitochondria suggested that BAIBA may be closely related to oxidative stress (67). Several studies have shown that myocardial tissue lipid peroxidation, creatine kinase, malondialdehyde (MDA), and TNFα are downregulated, and hepatic MDA protein expression levels are decreased in diabetic mice after BAIBA injection (68), whereas the expression level of the antioxidant protein glutathione is upregulated (69). Wang et al. (70) observed that BAIBA pretreatment significantly improved the oxidative stress factor angiotensin II (Ang II) in NRK-49F (rat renal fibroblasts), possibly due to the decrease in ROS levels and increase in antioxidant factors induced by BAIBA pretreatment. Interestingly, AGXT2 showed the highest mRNA and protein expression levels in the kidneys (71). Therefore, L-BAIBA may play an important role in the amelioration of oxidative stress.

AMPK is an important factor for ameliorating oxidative stress, promoting NO synthesis, and improving mitochondrial function. 2022 research showed that BAIBA upregulated AMPK phosphorylation levels, NO, L-arginine, malate (substrates for NO synthesis), NOS activity, and ADP/ATP ratio levels in the renal medulla of hypertensive rats (72, 73). BAIBA may improve oxidative stress by phosphorylating AMPK and increasing NO levels (72, 73). Further studies showed that BAIBA improved mitochondrial morphology, downregulated the expression of optic atrophy protein 1 and mitochondrial dynamics-related protein, and downregulated ATP production in the left ventricle of rats with heart failure through AMPK/miR-208b, thereby improving mitochondrial quality (74); this suggested that AMPK may be central to BAIBA-mediated mitochondrial function and amelioration of oxidative stress.

Sawada et al. (65) had reported that BAIBA can significantly reduce oxidative stress in vascular endothelial cells by regulating the mitochondrial function-related factor PGC-1β-estrogen-related receptor (ERRα)/PPAR-δ/PPAR-γ pathway (65). BAIBA intervention significantly upregulated the oxidative stress

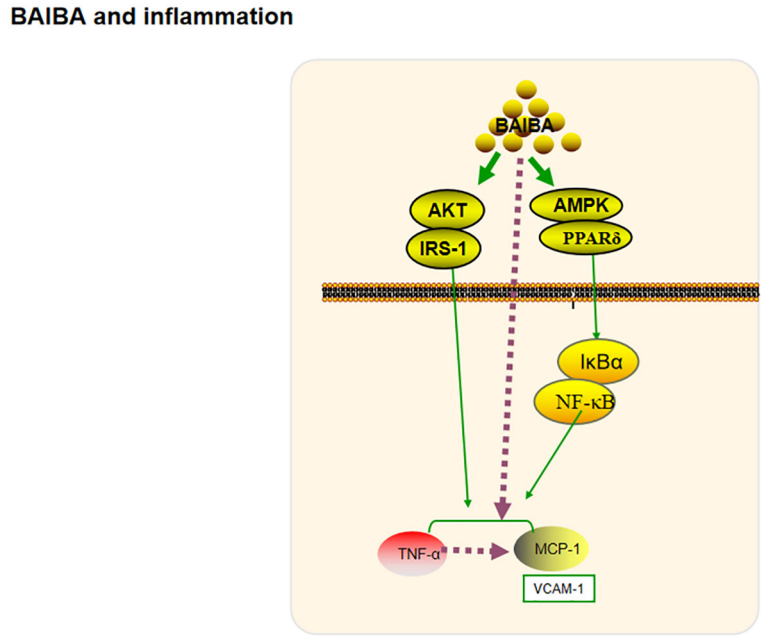

FIGURE 4
BAIBA and inflammation.

response of TNF cells. Moreover, BAIBA intervention significantly upregulated the TNF-α-mediated antioxidant factors CAT, SOD, thioredoxin, and γ-glutamylcysteine ligase in aortic endothelial cells and human umbilical vein endothelial cells, mitochondrial biogenesis-related molecular nuclear respiration Factor 1 and mitochondrial transcription factor mRNA levels. Notably, BAIBA treatment was observed to increase the expression of PGC-1β-downstream factors PPAR-δ, PPAR-γ, and ERRα before activating antioxidants and mitochondrial effects, hence suggesting that BAIBA takes longer to exert its biological effects by ameliorating oxidative stress and could involve multiple transcriptional steps (65). This study differed from previous reports, since significant changes in AMPK, Akt, and NOS phosphorylation were not observed after long- and short-term BAIBA treatment, which could be due to the different cell types used, suggesting that future studies on the mechanisms of BAIBA-mediated oxidative stress in different cells.

In summary, BAIBA affects mitochondrial genesis and ROS synthesis capacity via AMPK, thereby regulating oxidative stress (Figure 5).

4 Exercise regulates BAIBA synthesis and metabolism

BAIBA acts as a signaling metabolite and an antioxidant that is regulated by exercise, as validated at multiple levels (animal, cellular, protein, and genetic) (73–75).

Serum BAIBA levels are not significantly altered in adult male participants either immediately after acute aerobic exercise (70% of maximal oxygen uptake), or after 1 h, or even 4 h (75). Interestingly, serum D-BAIBA and L-BAIBA levels increased by 13% and 20%, respectively, after 1 h of acute aerobic exercise (40% maximal power) in 15 subjects (12 female and 3 male) with a polymorphism (rs37369) of a different (AGXT) genotype, which could be due to the fact that AGXT upregulated and catabolized D-BAIBA similarly during acute exercise (76), and L-BAIBA production increased, possibly due to the oxidation of the upstream species L-valine (18). This suggested that future assessment of exercise-mediated changes related to BAIBA would require special attention to isomeric differences and changes in AGXT (75, 77).

With regard to long-term exercise, in the follow-up of patients undergoing renal dialysis, plasma BAIBA levels were found to be significantly lower in sedentary patients than in those who remained physically active over long periods of time; however, there was no significant difference in the data across subjects with different levels of exercise over a long term. Unfortunately, owing to its cross-sectional nature, a causal relationship between plasma BAIBA levels and physical activity could not be established in the study (76). Morales et al. (75) reported a significant increase in serum BAIBA levels after 20 weeks of aerobic training. A study including both normal and obese adolescents showed that serum BAIBA levels increased after prolonged exercise (aerobic or resistance) regardless of obesity (78). This was confirmed by the results of an animal model, in which four weeks of hypoxic training increased BAIBA levels in the gastrocnemius and blood of obese rats (79). Roberts et al. (4) found serum BAIBA levels to be significantly higher in mice subjected to 3 weeks of free wheel exercise than in quiet control mice, and the BAIBA levels in

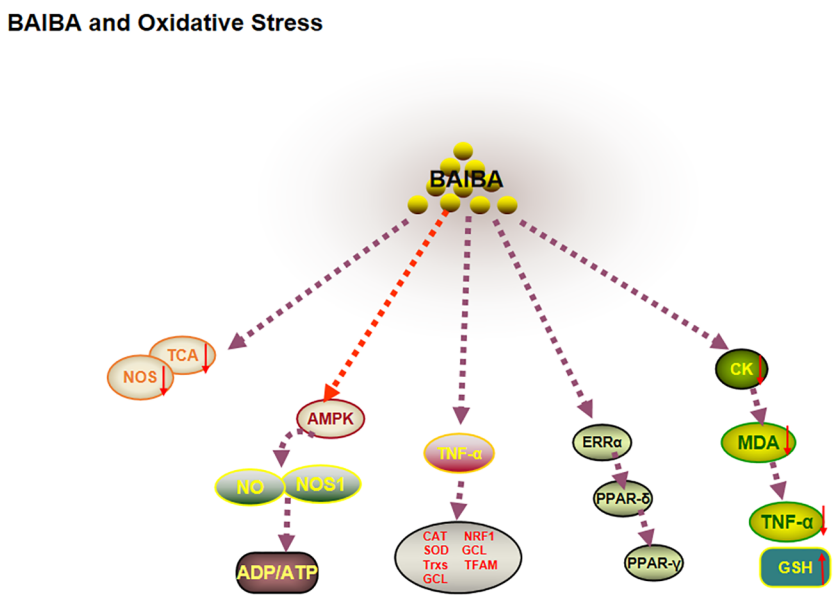

FIGURE 5
BAIBA and oxidative stress.

gastrocnemius and quadriceps to be increased 5.2-fold and 2.2-fold, respectively. This suggested that exercise significantly up-regulates BAIBA levels in muscle and serum. Differences in the modulation of BAIBA between different forms of exercise would be worth noting (80). Research observed that high-intensity interval training (HIIT) and moderate-intensity continuous training (MICT) significantly upregulated the expression of HADH, ACADS, and HADHA, key genes involved in BAIBA biosynthesis, in APOE-KO mice fed a high-fat diet. The mRNA expression of HADH, ACADS, and HADHA, key genes involved in BAIBA biosynthesis, was significantly upregulated in the HIIT group. Interestingly, after two weeks of single-leg immobilization, the femoral artery and femoral plasma concentrations in each leg were significantly higher in healthy male subjects at rest and during knee extension exercise in both legs. Venous plasma BAIBA concentrations were increased, but static-exercise BAIBA concentrations were decreased after leg immobilization (39). This pulsatile pattern of release and uptake may be a result of BAIBA uptake by skeletal muscle and may also be related to the metabolism of thymidine as a result of exercise.

In summary, although the modulatory effects of acute exercise on BAIBA levels have been studied relatively less, different forms of long-term exercise significantly increased BAIBA levels in serum and skeletal muscle, and HIIT has a more significant upregulation effect on serum and skeletal muscle BAIBA However, comparisons between different forms of exercise are currently scarce; therefore, the effect of factors, such as intensity, duration, and rest, on BAIBA levels would need to be explored further, and the question of whether exercise regulates synthesis and breakdown of BAIBA or uptake of BAIBA by skeletal muscle will be central to uncovering the relationship between exercise and BAIBA.

Little is known about the mechanisms by which BAIBA mediates exercise to regulate metabolism. 2022 research showed that BAIBA levels are reduced in hypoxic environments, whereas BAIBA expression is increased in blood and gastrocnemius muscle of mice after hypoxic exercise and was positively correlated with inguinal fat PPARα expression levels (81). Notably, both the hypoxic environment and hypoxic exercise upregulated PPARα mRNA expression in adipose tissue (81). Whether hypoxic exercise, as an effective form of fat loss, can promote fat metabolism by regulating adipose PPARα levels through BAIBA, and the mechanism of hypoxic environment being an external stimulus in BAIBA production during exercise would be important directions for future research towards revealing the mechanism of hypoxic exercise for weight loss.

To date, studies have shown that moderate exercise produces ROS, which promotes AMPK activation. Intense exercise, on the other hand, impairs mitochondrial function and inhibits AMPK activity and expression. Antioxidant supplementation may ameliorate the damage caused by excessive ROS production from intense exercise, but it counteracts the beneficial effects of moderate exercise at the same time, resulting in two incompatible interventions (82). A recent study comparing exercise and antioxidant interventions in T2DM rats found that moderate exercise can promote redox reactions in the liver by increasing antioxidant capacity. In contrast, antioxidant interventions impair redox homeostasis by inhibiting oxidative stress (82). It is interesting to note that BAIBA acts as a signaling metabolite and antioxidant, regulated by exercise; this has been validated at multiple levels (animal, cellular, protein, and genetic) (72–74). The validation of AMPK as a downstream target of BAIBA suggests that BAIBA supplementation may generate moderate

ROS and serve as a suitable intervention to promote redox homeostasis while avoiding the damage caused by intense exercise (82).

5 Prospects of BAIBA being used as an exercise pill?

Studies have shown that BAIBA plays an important role in health, not only by effectively improving glucose and lipid metabolism, but also by improving bone homeostasis and downregulating inflammatory responses and oxidative stress. A number of these effects appear to parallel benefits obtained via exercise-generated BAIBA, suggesting that BAIBA could potentially be applied as a drug or exercise mimetic.

In clinical applications, it is important to achieve drug efficacy without toxic side effects. A toxicological study showed that L-BAIBA can be delivered without adverse effects at 900 mg/kg/day for 90 days, which is the highest level tested (83). In addition, in human studies, approximately 2–20% Caucasians and 50% Asians were found to be deficient in AGXT2 (84, 85), with high urinary D-BAIBA levels. Kittel et al. (86) detected three-fold higher plasma BAIBA concentrations in a small number of subjects with AGXT2 gene deficit; however, none of the studies reported any adverse effect of increased endogenous BAIBA, suggesting that endogenous and exogenous factors resulting in high plasma BAIBA levels do not have any pathological effect (87). Notably, in pathological situations with substantial DNA degradation, such as malignancy (84, 88), radiation (89), and short-term starvation (90), the levels of BAIBA in urine are elevated. However, the current study was unable to define a causal relationship between BAIBA and these pathological conditions, and given that BAIBA inhibits oxidative stress in cancer cells (13), the feedback elevation of BAIBA was speculated to be a protective strategy of the body itself, thereby providing a theoretical basis for the clinical application of BAIBA as a new drug.

6 Applicability of BAIBA as a useful biomarker?

Although BAIBA can be detected in both blood and urine, the time course in the two matrices may not be the same. After an intervention with the DNA-based drug BC007, plasma BAIBA concentrations were found to increase after 3 min, reached a maximum after 60 min, and declined to baseline levels (87). The same intervention led to a rise in urinary BAIBA levels within a maximum of 2–4 h and a decline to pre-intervention levels over 8–12 h. BAIBA was considered to be metabolized rapidly in plasma, with urine representing a better matrix for testing, since sampling is non-invasive and the time profile is more favorable for testing.

Wang et al. reported that D-BAIBA is predominantly synthesized in humans while L-BAIBA is predominantly synthesized in mice (45); this was consistent with the recent finding that human D-BAIBA plasma concentrations are 67-fold higher than those of L-BAIBA (77). Most studies have shown that D-BAIBA is the major enantiomer of BAIBA in human urine (4, 91). However, in the mammalian kidney, AGXT2 levels are high (71), and D-BAIBA is considered the preferred substrate (23); therefore, D-BAIBA would be difficult to detect in urine (92). Since the lowest levels of urinary BAIBA were not observed in the 24-h observation time frame, D-MMS could have been further metabolized. Therefore, detection of the catabolite D-MMS may be the next step in determining whether D-BAIBA can be used as a urinary disease biomarker.

Biomarkers are characterized by their specificity, sensitivity, stability, non-invasiveness, and reproducibility. BAIBA is considered a biomarker for osteoporosis (45), and recent studies have shown it to possibly be a biological marker for the coronavirus infection as well (67). While two histological screens showed that BAIBA specificity and reproducibility met requirements (45, 68), and non-invasive testing in urine is attractive, there are several issues regarding the detection of enantiomers and metabolic degradation of BAIBA, owing to which stability and sensitivity of assays for BAIBA as a biological marker would need to be explored further.

7 Concluding remarks and future perspectives

BAIBA is a signaling metabolite that maintains lipid homeostasis by promoting white fat browning and increasing FFA oxidation, lowers blood glucose (possibly through an insulin-independent pathway), promotes osteogenic differentiation, and reduces inflammation and oxidative stress. Exercise promotes BAIBA production in skeletal muscle and inhibits its uptake. BAIBA has effects in a variety of diseases and has become a biomarker for osteoporosis and the coronavirus infection. Exogenous BAIBA can effectively perform biological functions without causing any side effects. Thus, it may have a promising future as a drug and biomarker.

However, there are many questions still that need to be addressed in future. 1. Since most existing epidemiological and experimental studies do not distinguish between D-BAIBA and L-BAIBA, there is a need to further define the biological functions of each subtype. 2. Since osteoclasts form an important factor in bone metabolism, it would be important to explore whether BAIBA can directly mediate the number or function of osteoclasts, and what would be the mechanism involved. 3. There are only few studies that have compared the regulation of BAIBA levels by different exercise modalities, and investigated whether exercise regulates health via BAIBA; hence, the relationship between exercise and BAIBA synthesis and secretion should be explored in detail in future using different times, intensities, and exercise frequencies.

Author contributions

XY, YY is responsible for topic selection, frame design and writing. TL, ML, TY, GH and GW were responsible for document

retrieval and chart design. BC conducted literature research and final revisions. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Comparison of computed tomography and dual-energy X-ray absorptiometry in the evaluation of body composition in patients with obesity

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Objective: a) To evaluate the accuracy of the pre-existing equations (based on cm2 provided by CT images), to estimate in kilograms (Kg) the body composition (BC) in patients with obesity (PwO), by comparison with Dual-energy X-ray absorptiometry (DXA). b) To evaluate the accuracy of a new approach (based on both cm2 and Hounsfield Unit parameters provided by CT images), using an automatic software and artificial intelligence to estimate the BC in PwO, by comparison with DXA.

Methods: Single-centre cross-sectional study including consecutive PwO, matched by gender with subjects with normal BMI. All the subjects underwent BC assessment by Dual-energy X-ray absorptiometry (DXA) and skeletal-CT at L3 vertebrae. CT images were processed using FocusedON-BC software. Three different models were tested. Model 1 and 2, based on the already existing equations, estimate the BC in Kg based on the tissue area (cm2) in the CT images. Model 3, developed in this study, includes as additional variables, the tissue percentage and its average Hounsfield unit.

Results: 70 subjects (46 PwO and 24 with normal BMI) were recruited. Significant correlations for BC were obtained between the three models and DXA. Model 3 showed the strongest correlation with DXA (r= 0.926, CI95% [0.835-0.968], p<0.001) as well as the best agreement based on Bland – Altman plots.

Conclusion: This is the first study showing that the BC assessment based on skeletal CT images analyzed by automatic software coupled with artificial

intelligence, is accurate in PwO, by comparison with DXA. Furthermore, we propose a new equation that estimates both the tissue quantity and quality, that showed higher accuracy compared with those currently used, both in PwO and subjects with normal BMI.

KEYWORDS

obesity, morbid obesity, body composition, computed tomography, dual-energy X-ray absorptiometry

Introduction

Obesity is a chronic and relapsing disease which prevalence is significantly increasing worldwide and its related comorbidities suppose a high cost for healthcare system (1–3).

At present body mass index (BMI) remains a categorical diagnostic criterion for obesity. Nevertheless, BMI has serious limitations and do not provide information on the body composition (BC) and the metabolic condition of the subjects (4). Recently, efforts have focused to identify more specific prognostic factors and biomarkers of obesity and its metabolic complications (5–7). The American Association of Clinical Endocrinologists (AACE) proposed a new definition for obesity: "adiposity-based chronic disease (ABCD)", which has also adopted by the European Society of Obesity (EASO) (8, 9). The concept "adiposity-based" refers not only to the total quantity of body fat, but also to its distribution and/or functionality (4, 10).

Furthermore, besides body fat, the muscle mass has a very relevant and complex role in the BC and body homeostasis and metabolic condition (11–13). Sarcopenia, which is the loss of muscle mass and strength of physical function synergistically worsen the adverse effects of obesity (14). However, the study of BC is crucial for identifying sarcopenia, especially in PwO, where body volume can mask low muscle mass if only anthropometric data is used for the clinical assessment (15). Recently, the European Society for Clinical Nutrition and Metabolism (ESPEN) and EASO have agreed a definition and diagnostic criteria for sarcopenic obesity, a condition in which sarcopenia and obesity coexists and leads to a cumulative risk derived from the two clinical situations (15, 16). In addition to measure the amount of adipose tissue or muscle mass, it is important to know its distribution and proportion, given its significant role in pathologies such as metabolic syndrome and associated complications (17–20). However, methods to assess BC are not taken into consideration in the daily clinical practice in the obesity management due to the lack of simple and reliable tests.

Dual-energy X-ray absorptiometry (DXA) has long been considered a reference technique to assess BC and it is still a reference method (14). It provides the mass of the different tissues, measured in kilograms. However, DXA does not provide information on the distribution of adipose tissue at the abdominal level (SAT or VAT) (21). Furthermore, in many clinics, accessibility to DXA is limited and except for the assessment of bone density, it is not a test that is performed in the usual clinical routine (22, 23).

In this scenario, CT emerges as a technique widely used in clinical practice that contains very precise information for assessing BC (24–28). Regional analysis of fat and fat -free mass at the third lumbar vertebra was shown to have a high correlation with total BC, and provides significant additional information of tissue quality and myosteatosis, based on the Hounsfield units (HU) (29–32). To obtain the CT image, the emission of radiation is necessary (approximately 10 mSv). Nonetheless, at present the assessment of BC by means of a CT image is been largely used in clinical research, especially in those pathologies in which the CT evaluation is part of the protocol, such as some types of cancer or abdominal pathologies (33).

Currently, BC assessment by CT is obtained by manual or semi-automatic marking software that provide an area parameter (cm^2) and average of Hounsfield Units of the of target tissue (34). The use of this type of software requires trained staff able to manually correct the images, therefore, the evaluation of these results is time consuming and unfeasible in the current clinical practice at large scale. However, emerging technologies, such as artificial intelligence (AI), have promoted the development of new software tools able to rapidly and precisely analyze the images obtained by CT resulting in qualitative and quantitative information (24, 25, 27, 35). As far as we know at present no such tool coupled with AI is used for the BC assessment based on CT scan images in PwO.

The accuracy of the BC assessment by CT image was evaluated by comparison with DXA, as the reference method (36, 37). For this purpose, equations were developed (i.e., those described by Mourtzakis et al) (24) to convert the cm^2 information provided by the CT-scan into Kg provided by DXA. It should be noted that these equations have been validated in subjects with mean BMI<27 kg/m^2, most of them with cancer and malnutrition. At present there is no data regarding the accuracy of these equations in PwO. Furthermore, these equations were based only on the area parameter (cm^2) provided by the CT-scan and did not take into the account the HU.

On this basis, we designed the present study aimed to a) evaluate the accuracy of the pre-existing equations (based on cm^2 provided by CT images), to estimate in Kg the BC in PwO, by comparison with Dual-energy X-ray absorptiometry (DXA). b) evaluate the accuracy of a new approach (based on both cm^2 and HU parameters provided by CT images), using an automatic software and AI to estimate the BC in kg in PwO, by comparison with DXA.

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Materials and methods

Patient selection

We performed a single-centre cross-sectional study including consecutive patients with morbid obesity, matched by gender with normal BMI subjects at Vall d´Hebron University Hospital, between April and September 2021. Control group was randomly drawn from patients with DXA performed in our centre with normal BMI (20-25 kg/m^2).

The study was approved by the local Ethics Committee (PR (AG)510/2021) and carried out in accordance with the Declaration of Helsinki. All the patients signed the informed consent form before the participation in the study.

Inclusion criteria: a) age between 18 and 60 years; b) morbid obesity (BMI >40 kg/m^2 or BMI > 35 kg/m^2 with at least one comorbidity related to obesity).

Exclusion criteria: a) any condition except obesity that can affect the body composition, (ex. myopathy, neurodegenerative disease, renal, liver and heart failure etc); b) any treatment that can affect the body composition as per investigator criteria (ex. corticosteroids, growth hormone, etc); c) unable to perform both DEXAand CT scan; d) antropometric data above the usual CT-scan machines (such as body weight >205kg or the presence of an abdominal circumference greater than the ability to obtain an image in a cut (>200cm corresponding to CT-scan gauntry diameter, usually of 70cm); e) metal plates or artifacts that can affect the radiodensity measurements (Hounsfield Units).

Clinical data collection

All the subjects underwent within a maximum of 30 days from the inclusion in the study: complete medical history, anthropometric data (weight-kg, height-m), biochemical analysis, body composition DXA and skeletal CT centered at L3 vertebrae.

BMI was calculated using the following formula: weight (kg)/ height^2 (m^2).

DXA analysis

Body composition DXA was performed using GE Lunar Prodigy dual-energy X-ray absorptiometry (DXA) scanner (GE Healthcare, Madison, WI, USA) by a certified technician. The software used for the total and regional body composition estimation was Encore (GE Healthcare) version 15. Each region was automatically analysed and then supervised by a Nuclear Medicine Physician. The following variables were registered: fat mass (Kg), fat free mass (Kg), appendicular skeletal muscle mass (Kg), appendicular skeletal muscle mass index (Kg/m²).

CT data extraction

Skeletal CT images focused at L3 vertebrae were obtained using a multidetector computed tomography scanner (Aquilion Prime SP, Canon Medical Systems, Japan), using the following technical parameters: 135 kV (tube voltage), 1mm 80 row (detector configuration), tube current modulation, and 0.8 sec/rotation (gantry rotation). The following variables were registered: skeletal muscle mass area or SMA (cm² and %), skeletal muscle mass index or SMI (cm²/m²), intramuscular adipose tissue area or IMAT (cm² and %), intramuscular adipose tissue index or IIMAT (cm²/m²), area of visceral fat mass (VFA) (cm² and %), subcutaneous fat (SFA) (cm² and %), visceral fat mass index (VFI) (cm²/m²), and subcutaneous fat (SFI)(cm²/m²), and average Hounsfield Units (HU) value per each segmented tissue.

The CT images centered at the third lumbar vertebra (L3) were analysed using FocusedON-BC software. This software presents a user-friendly interface and includes a semiautomatic labeling tool that allows the user to modify the body mass segmentation automatically carried out by the software. To determine skeletal muscle and abdominal adipose tissue area we analysed the cross-sectional CT images at the third lumbar vertebra (L3) using FocusedON-BC software. A total of 16 slices for each patient were assessed. The muscles involved in the analysis were: psoas, erector spinae, quadratus lumborum, transversus abdominis, external and internal obliques, and rectus abdominis muscles. Adipose tissue was classified as subcutaneous, visceral, and infiltrating the muscles. All areas were measured in cm^2 and normalized for height. Tissue quality was assessed based on its average Hounsfield Units (HU) value. Standard thresholds were employed as follows: -29 to 150 HU for skeletal muscle, -190 to -30 for subcutaneous adipose tissue and -150 to -50 for visceral adipose tissue (32, 38).

Models used for the body composition evaluation

The research works carried out so far have demonstrated the good correlation between the muscle and fat tissues area, measured in cm^2 at the L3 vertebral level, with the total amount of muscle and body fat mass, measured in kg by DXA. Mourtzakis et al. (24) has proposed a linear regression model, referred in this work as Model 1, which has also been used by Tewari et al. (39). However, these research works have been carried out using a relatively short number of patients with similar BMI values, and none of them have included people living with morbid obesity. For these reasons, the first goal of this work is to evaluate the suitability of Model 1 to assess muscle mass for patients with a wider range of BMI values, include people living with obesity.

Model 1: This is the most widely used in clinical practice (24) and estimates the fat mass (FM) and fat-free mass (FFM) using the area labelled as fat (visceral and subcutaneous) and muscle in a CT slice at L3 vertebral level (measured in cm²).

This model is described by Equation 1 (Mourtzakis et al. model) (24):

$$FM_{(kg)} = 0.042 \cdot FM\_CT\_L3_{(cm^2)} + 11.2 \quad \text{(eq 1.a)}$$

$$FFM_{(kg)} = 0.3 \cdot Muscle\_CT\_L3_{(cm^2)} + 6.06 \quad \text{(eq 1.b)}$$

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Values of $FM\_CT\_L3_{(cm^2)}$ and $Muscle\_CT\_L3_{(cm^2)}$ for each patient are directly obtained from the FocusedON-BC software. These values were used to estimate the FM and FFM in kilograms using this model.

Model 2: This model has been generated to evaluate if Model 1 methodology is suitable to estimate muscle and fat mass in kg for patients with different BMI values, including people living with obesity. Concretely, least squared method has been used to adjust the linear regression model described in Model 1 (24) to better fit our patient's data, and hence, better results are expected. This model is described by Equation 2:

$$FM_{(kg)} = 0.058 \cdot FM\_CT\_L3_{(cm^2)} + 7.35 \quad \text{(eq 2.a)}$$

$$FFM_{(kg)} = 0.27 \cdot Muscle\_CT\_L3_{(cm^2)} + 13.31 \quad \text{(eq 2.b)}$$

FocusedON Model: is a new model proposed by our group based on the present study, using FocusedON software. The goal of this approach is to provide a solution that can be used to precisely estimate the body fat mass and fat free mass (in kg) based on the data extracted from a CT scan with independence of the patient BMI. This approach takes in consideration both the tissue area, measured in percentage, and its average density, measured in HU. These data are directly obtained from FocusedON-BC software. This model is described by Equation 3:

$$\rho \approx \frac{HU}{1000} + 1 \quad \rightarrow \quad \left(\frac{kg}{cm^3}\right) \quad \text{(eq 3.a)}$$

$$FM_{(kg)} = 0.0069 \cdot \frac{Weight}{\rho_{ROI}} \cdot FAT_{(\%)} \cdot \rho_{FAT} + 4.53 \quad \text{(eq 3.b)}$$

$$FFM\ (kg) = Weight\ (kg) - FM\ (kg) \quad \text{(eq 3.c)}$$

First, this model considers the tissue density according to its HU average value, as shown in Equation 3.a. This equation estimates the tissue density in kilograms per cubic centimeter based on its radiodensity measured in HU. As FocusedON-BC provides the average patient radiodensity (for the analyzed images), the patient volume can be extrapolated based on this value and the patient weight as $V_{total} = Weight/\rho_{ROI}$. Then, since FocusedON-BC provides each tissue quantity measured in area percentage, we can extrapolate to estimate the total tissue volume as $V_{tissue} = (V_{total} \cdot Tissue\ percentage)/100$. Finally, tissue mass measured in kilograms can be estimated according to its density as $M_{tissue} = V_{tissue} \cdot \rho_{tissue}$. Following this procedure for fat tissue we obtained $FM_{(kg)} = m \cdot (Weight/\rho_{ROI}) \cdot FAT_{(\%)} \cdot \rho_{FAT} + n$. If we replace the weight, density and tissue percentage with our patient data and apply least squared method to calculate m and n we obtain Equation 3.b. Since we already know patients' weight and we are estimating the amount of fat mas in kilograms, we can directly know the amount of fat free mass as the difference (Equation 3.c).

Statistical analyses

Statistical analysis was performed using Python 3.8. Continuous variables are presented as mean ± standard deviation (SD) for normal distributed variables and median ± interquartile range (IQR) for non-normal distributed variables. Categorical variables are presented using percentages. Statistical significance was accepted at $p<0.05$. Quanti Quanti Plot (QQ) has been used to assess the normal distribution of the dataset. The correlation between analysed methods and the DXA was assessed using linear correlation coefficient (Pearson). Bland-Altman plots were used to estimate agreement between analysed methods and DXA. Additionally, simplified error grid plots have been also used to assess the error obtained in the estimations. The error (<10%, 10-20% or >25%) was measured as the difference between the estimated value and the reference value provided by DXA.

Sample size calculation: At present there is no data regarding the use of equations based on CT-scan images for the BC assessment in PwO therefore we were not able to calculate a sample size based on this population. Nevertheless, we took into the account the previous data reported by Mourtzakis (24) for estimating the whole-body fat mass of the subject, measured in kg, based on the fat area quantified in a CT scan image at L3 in cm^2 was also useful for subjects with a wider range of BMI values, including patients living with obesity. This study obtained the next correlation model using 51 subjects (oncological patients reported by the study with an BMI = 26.9 ± 6.2 kg/m^2):

$$FMwhole\ body\ (kg) = 0.042 \cdot FML3\ (cm2) + 11.2 \cdot r = 0.88, p < 0.001$$

Due to the high correlation obtained, and the fact that it has been further validated in other studies (Tewari et al. (39)), we assume that 51 patients should be enough to carry out this proof of concept. However, as we pretended to test the model for subjects with a wider range of BMI values (higher variability), we increased the sample size by 35%, resulting a sample size of 51 x1.35 = 68.85 patients.

Furthermore, we carried out the next calculation (based on Cohen equation) to verify that the sample-size is correct for our goal:

$$N = Z2 \cdot S2d2$$

N is the sample size; · Z is the critical value of the standard normal distribution at the 5% significance level (1.96); · S^2 is the estimated standard deviation of the estimating variable (284 cm^2 of fat tissue measured at L3); · d is the desired precision in the outcome variable (whole-body fat mass measured in kg). Based on this equation, if we expect a precision of 4 kg (d=4), the obtained N value would be also 68 subjects. We included in our study 70 subjects.

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Results

A total of 70 subjects were included in this study: 46 PwO and 24 with normal BMI. The baseline characteristics are shown in Table 1. The BMI of all the subjects, both PwO and normal BMI showed a normal distribution as reflected in the QQ plot displayed in Figure 1.

The correlations and agreement between the reference method, DXA, with the three models that were created for the study are displayed in Table 2. Multiple conclusions can be drawn from these results.

First, the correlation and agreement obtained with each model when using only 1 CT slice is very close to the one obtained when using 16 slices. This suggests that a single slice may be representative enough for the BC assessment based on CT scan images.

Secondly, it can also be observed that all models present high correlation values (higher than 0.75 in all the tests), being **FocusedON Model** the one providing the highest correlation (0.93). It should be noted that a high correlation value does not necessarily imply a good agreement between the reference method, DXA, and the tested model. In this regard, Figures 2, 3 graphically show a scatter plot with the trendlines, correlation values and confidence intervals corresponding to each model. In Figure 2, where points corresponding to **Model 1** and **Model 2** are considerably far from the tendency, reflects that these two models based on currently used equations, present bad agreement with respect to DXA in PwO. Figure 3, that corresponds to **FocusedON Model**, the points are closer to the trendline, showing that the newly proposed model presents a good agreement with respect to DXA.

Furthermore, for a better accuracy, data was confirmed by two additional statistical analyses (agreement Bland-Adman plots-Figure 4 and error grid-Figure 5). In both analysis, **FocusedON Model** still presented the best agreement with DXA in comparison with Model 1 and Model 2. The numerical values corresponding to these plots are also summarized in Table 2.

Discussion

This is the first study showing that the BC assessment based on skeletal CT images analyzed by automatic software coupled with artificial intelligence, is accurate in PwO, by comparison with DXA. Furthermore, we propose a new equation that estimates both the tissue quantity and quality, that showed higher accuracy compared with those currently used, both in PwO and subjects with normal BMI.

At present, the model most widely used in the clinical practice was developed by Mourtzakis et al. (24). This model estimates the FM and FFM using the area labelled as fat (visceral and

TABLE 1 Demographic and clinical characteristics of the subjects included in the study.

N	All	Patients with MO	Control group	p- value
	70	46	24	
Gender, females, %(n)	59 (41)	70 (32)	38 (9)	0.02
Age (years), mean (SD)	47.37 ± 12.8	43.17 ± 10.35	55.42 ± 13.37	<0.001
Weight (kg), mean (SD)	101.41 ± 23.78	114.37 ± 14.49	76.57 ± 17.5	0.00007
BMI (kg/m^2), mean (SD)	37.63 ± 9.52	43.56 ± 4.69	26.27 ± 4.86	0.00007

BMI, body mass index; SD, standard desviation.

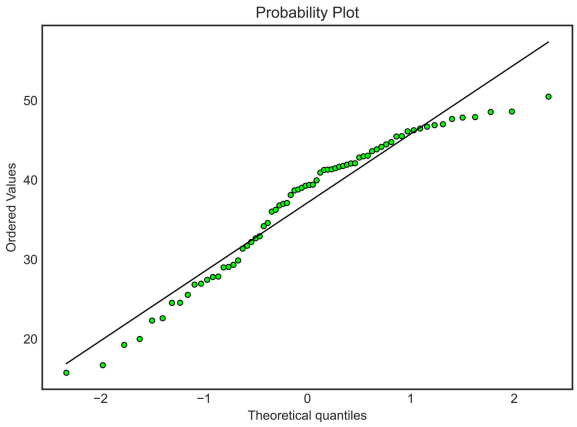

FIGURE 1
Body mass índex Quanti Quanti Plot.

TABLE 2 Correlation and agreement between the different models with DXA.

		Fat free mass				Fat Mass			
			Error (kg)				Error (kg)		
		Pearson	-1.96 SD	Mean	+1.96 SD	Pearson	-1.96 SD	Mean	+1.96 SD
1 slice	Model 1	0.75	-17.08	-2.76	11.56	0.91	-22.31	-6.03	10.25
	Model 2	0.75	-11.1	3.26	17.62	0.91	-18.56	-4.48	9.59
	FocusedON model	–	-6.77	2.42	11.61	0.93	-11.61	-2.42	6.77
16 slices	Model 1	0.76	-16.93	-2.76	11.4	0.90	-22.38	-6.07	10.23
	Model 2	0.76	-10.76	3.64	18.04	0.90	-18.46	-4.38	9.69
	FocusedON model	–	-6.9	2.23	11.36	0.93	-11.36	-2.23	6.9

-, indicates that the fat free mass has not been directly estimated by the FocusedON model, and so, the Pearson coefficient has not been calculated. Instead, the fat free mass has been calculated as the difference between patient weight and the fat mass estimated by the FocusedON model.

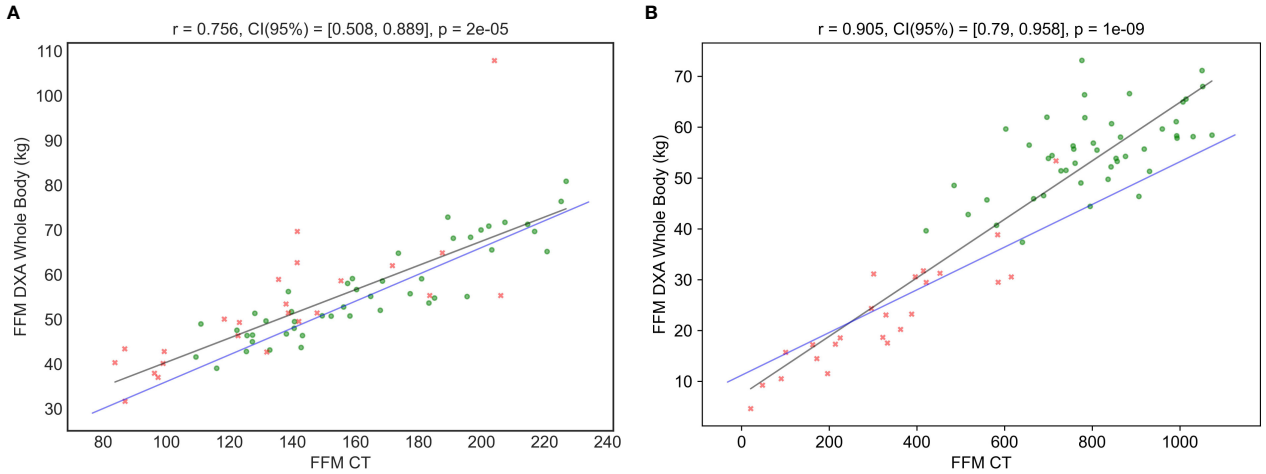

FIGURE 2
Correlation between DXA and CT in FFM **(A)** and FM **(B)** using the corresponding tissue area in cm2 as TAC Metric. Blue line represents "Model 1", and black line represents "Model 2". Red crosses represent the subjects with normal BMI and green dots the subjects with obesity.

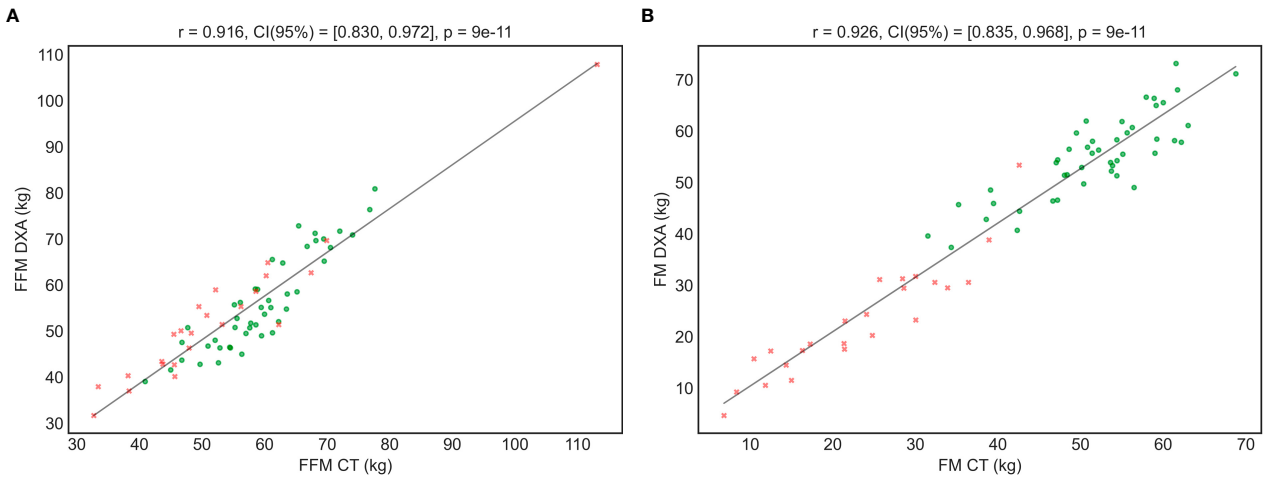

FIGURE 3
Correlation between the FFM **(A)** and FM **(B)** values measured DXA and using the FocusedON model by TC. Red crosses represent the subjects with normal weight and green dots the subjects with obesity.

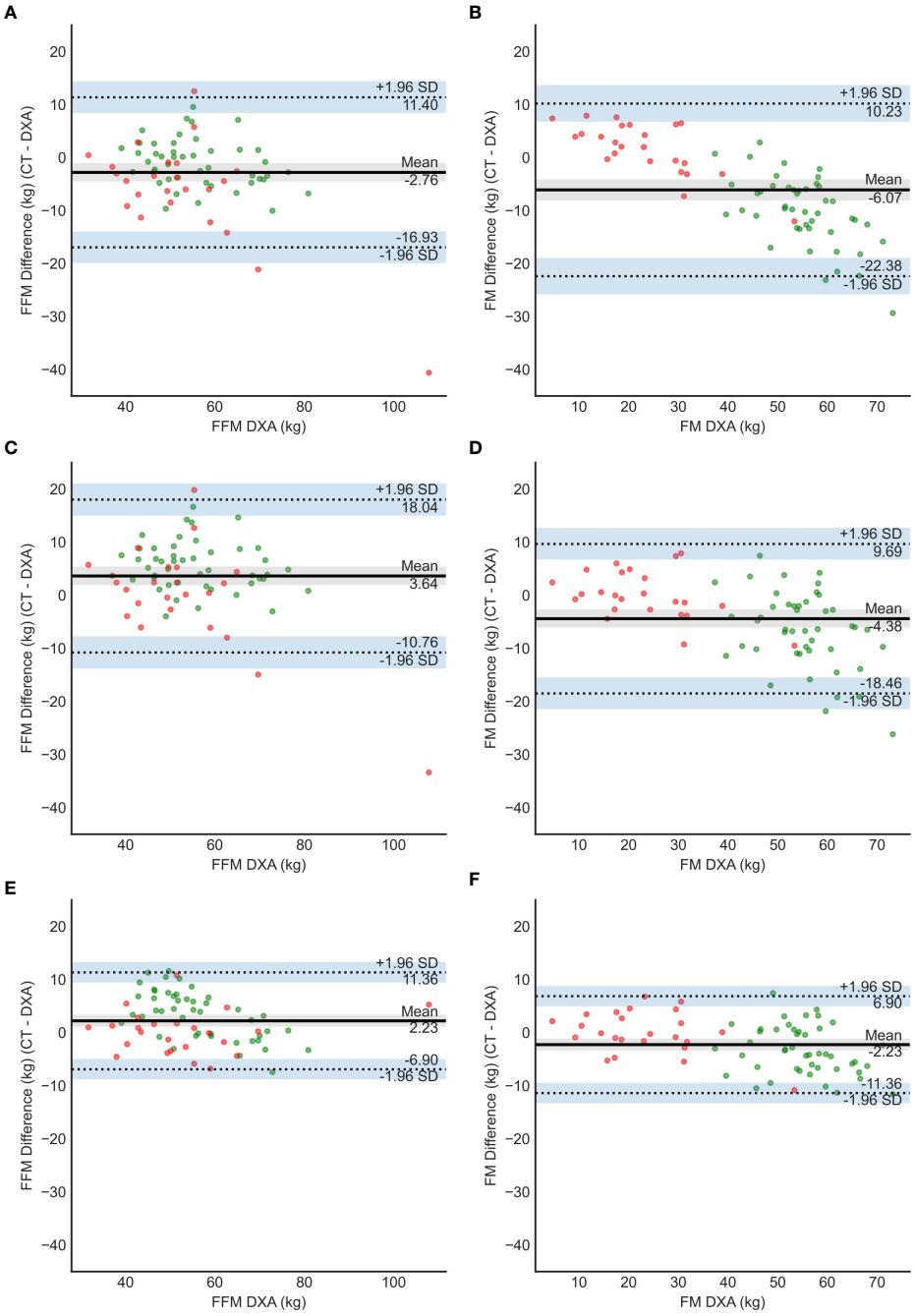

FIGURE 4
Bland – Altman plots for model 1 **(A, B)**, model 2 **(C, D)** and FocusedON model **(E, F)**. Red dots represent the subjects with normal BMI and green dots the subjects with obesity.

subcutaneous) and muscle in a CT slice at L3 vertebral level (measured in cm^2). These authors evaluated 51 patients diagnosed of lung or colorectal cancer with a BMI of 26.9 ± 6.2 kg/m^2 and obtained a high correlation with DXA, r=0.88 (MRE 3.49 ± 2.31 kg, p<0.001) for FM and r=0.94 (MRE 5.23 ± 3.54 kg, p<0.001) for FFM. Few years later Tewari et al. (39) validated the results using the same model, based on 47 patients with esophagogastric cancer with a mean BMI 27.65 ± 5.31 kg/m^2. They obtained a correlation with DXA of 0.66 (IC 9.451-0.9.964, p<0.0001) for FM and 0.76 (IC 95% -0.8621 – 0.8325, p<0.0001) for FFM. It should be noted that, to the best of our knowledge, most of the studies using this model have been performed in the oncological setting or in critical care units but not in patients with obesity (40–42).

We tested the model proposed by Mourtzakis (24), defined as Model 1, and an adjusted version defined as Model 2 (see Methodology). Bland-Altman plots showed that both models had low agreement with DXA. Both models underestimate the FM for the subjects with obesity and overestimate it in the subjects with

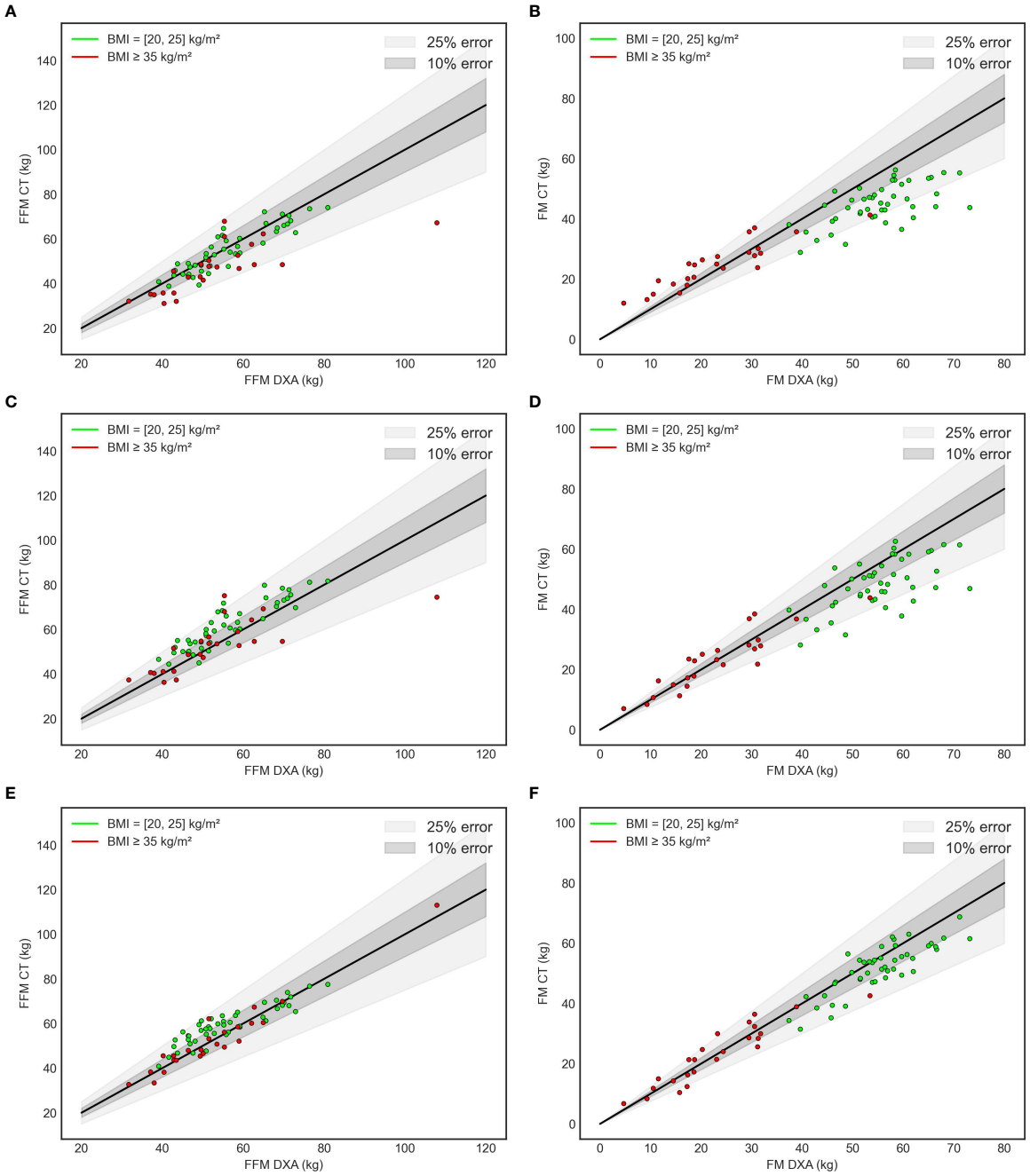

FIGURE 5
Error grid. Model 1 **(A, B)**, Model 2 **(C, D)** FocusedOn Model **(E, F)**.

normal BMI. In the case of Model 2 the errors were lower but still they remained significant. These findings suggest that these 2 models, based only in the tissue area, measured in cm^2, cannot be generalized for a wide range of BMIs to accurately estimate body FM and FFM in kg. Additionally, these models are less precise for subjects with obesity.

The CT image also provides the HU of the tissues. The HU is a quantitative measurement of radio density. The absorption/attenuation coefficient of radiation within a tissue is used during CT reconstruction to produce a grayscale image (43). The absorption of X- ray is proportional to the density of the tissue. The use of HU allows to evaluate the tissue quality and estimate its density. In this study we have created a new model that considers, for the first time, both the percentages of adipose and muscle tissue and their average HU, instead of just each tissue area in cm^2 as in the models used so far in the literature. This new model (Model 3 –

FocusedON Model) has provided results that are more accurate and the data was confirmed by three different statistical analysis methods. The correlation between Model 3 and DXA was the strongest. It also showed better agreement with DXA based on Bland-Atman plots, also without under or overestimating the FM and FFM in any of the two groups.

This study proposes a new model, FocusedON model, for estimating body composition based on CT data in PwO and normal BMI. This new model has demonstrated to be superior in accuracy to those presented in previous studies, which only take into the account the quantity of tissue measured in cm^2. Our model uses parameters of tissue quantity (%) and quality (HU) to create more accurate equations. These findings underlie the importance of revisiting the traditional equations and models where they only used CT measurements expressed in cm^2.

In addition, it should be noted that the cut-off points commonly used today for the diagnosis of sarcopenia have been developed in oncological population groups, with a small sample size that did not significantly include the overweight or people living with obesity. We have shown in our study that these models are not optimal for the assessment of PwO, so the present cut-off levels should be revaluated. Another novelty resulted from our study is that we propose a simplified method using single-slide CTscan image. Previous study recommends that multiple slices should be analyzed (44). In our study, using multiple slices in the analysis of BC was not significantly superior to use a single slice for the CT-scan image.

Nevertheless, our study has some limitations: First, patient´s weight limit: The method cannot be applied in subjects with severe obesity that cannot undergo CT-scan, such as weight > 200 kg. However, these patients represent<3% of the PwO. Small sample size, it should be noted that we performed a proof-of-concept study with a strong agreement between the model and the reference method. We consider that this apparent limitation has no significant influence on the results of the study due to several arguments: a)the sample size of our study is higher (70 subjects) than the study published by Mourtzakis et al. (24) (51 subjects) as previously explained, which is the currently used model to estimate BC based on CT-scan; b) the proposed model includes not only quantitative but also qualitative variables, allowing a better approximation to the clinical and metabolic reality of the patients. Actually, the combination of these variables will be used in future studies with a larger sample size to produce more complex and precise models (I.e., using non-linear machine learning methods.

In summary, in this proof-of-concept study we demonstrated for the first time that the equations currently used for BC assessment based on CT images are not accurate in PwO. Additionally, we proposed a new simplified model, based on single-slice approach of CT-scan image, including both quantitative and qualitative data of BC, that showed better results than the ones being widely used at present in the clinical practice. Further studies are needed to validate and refine this new methodology, which could open a new avenue in the study and management of obesity, by accurately assess the BC "at a glance" with simple and automatic methods.

Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study protocol was reviewed and approved by Comité de Ética de Investigación con Medicamentos del Hospital Universitario Vall d'Hebron, approval number PR(AG)510/2021. The study has been granted an exemption from requiring written informed consent.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Author contributions

Conceptualization: AC, FP, RS. Data curation FP, RG. Formal analysis FP, AC, RG. Funding acquisition RS. Investigation: FP, RG, DE, NR, CE, RB. Methodology FP, RG, AC. Project administration: AC, RS; Resources FP, AC. Software RG. Supervision RS, RB. Validation AC, RS. Visualization AC, RG, DE, CE, NR, RB, RS. Roles/Writing - original draft: FP, RG; Writing - review & editing AC, RS. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Role of extracellular vesicles in nonalcoholic fatty liver disease

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Background: Nonalcoholic fatty liver disease (NAFLD) is a common chronic liver disease that affects approximately one-quarter of the global population and is becoming increasingly prevalent worldwide. The lack of current noninvasive tools and efficient treatment is recognized as a significant barrier to the clinical management of these conditions. Extracellular vesicles (EVs) are nanoscale vesicles released by various cells and deliver bioactive molecules to target cells, thereby mediating various processes, including the development of NAFLD.

Scope of review: There is still a long way to actualize the application of EVs in NAFLD diagnosis and treatment. Herein, we summarize the roles of EVs in NAFLD and highlight their prospects for clinical application as a novel noninvasive diagnostic tool as well as a promising therapy for NAFLD, owing to their unique physiochemical characteristics. We summarize the literatures on the mechanisms by which EVs act as mediators of intercellular communication by regulating metabolism, insulin resistance, inflammation, immune response, intestinal microecology, and fibrosis in NAFLD. We also discuss future challenges that must be resolved to improve the therapeutic potential of EVs.

Major conclusions: The levels and contents of EVs change dynamically at different stages of diseases and this phenomenon may be exploited for establishing sensitive stage-specific markers. EVs also have high application potential as drug delivery systems with low immunogenicity and high biocompatibility and can be easily engineered. Research on the mechanisms and clinical applications of EVs in NAFLD is in its initial phase and the applicability of EVs in NAFLD diagnosis and treatment is expected to grow with technological progress.

KEYWORDS

extracellular vesicles (EV), NAFLD, diagnosis, treatment, mechanisms

1 Introduction

Nonalcoholic fatty liver disease (NAFLD) has become the most common chronic liver disorder affecting approximately 25% of the global adult population, which prevalence varies from 13.5% in Africa to 31.8% in the Middle East (1), causing a growing global burden of liver diseases (2). NAFLD encompasses a disease continuum from nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH), which is characterized by necroinflammation and faster fibrosis progression than NAFL (3). Patients with NAFLD are prone to developing cirrhosis and hepatocellular carcinoma (HCC), making NAFLD the most rapidly growing cause of liver transplantation in HCC patients, with an 11.8-fold increase during 2002-2016 (4, 5). NAFLD has become the most rapidly growing contributor to liver mortality and morbidity (6). Drugs such as glucagon-like peptide-1 (GLP-1) agonists, pioglitazone, and sodium-dependent glucose transporter 2 (SGLT2) inhibitors are currently available for the treatment of obesity and type 2 diabetes mellitus(T2DM), but there is currently no FDA-approved drug therapy for NASH (3). A healthy lifestyle and weight management remain central to the prevention and treatment of NAFLD (7).

The pathophysiology of NASH is multifactorial, involving genetic and epigenetic factors, insulin resistance (IR), adipose-derived hormones, over-nutrition, and microbiome-related factors that are not well understood, and several studies have reported that extracellular vesicles (EVs) play a significant role in the development of NAFLD (3, 8, 9). EVs act as intercellular mediators and participate in metabolic regulation, inflammatory and immune responses, intestinal microecological balance, and fibrotic processes. Therefore, understanding the mechanism of EVs is of great significance for improving the diagnosis and treatment of NAFLD. In this review, we summarize the roles of EVs in NAFLD and highlight their utility as diagnostic and therapeutic tools in NAFLD.

2 The characteristic of extracellular vesicles

An EV is a membranous vesicle derived from the cellular membrane systems of living cells with lipid bilayer membranes (10). EVs were first observed in plasma in 1967 (11). Since then, it has been established that EVs can be isolated from a variety of body fluids (12–16) and be released by almost all types of cells (17–20). Various proteins, lipids, DNA, RNA, and metabolic products, which have been proven to regulate gene expression and signaling pathways in cells, can function as cargo for EVs (21, 22). The biosynthesis of EVs may be regulated by EV cargoes bind trafficking effectors, which enrich cargoes in endosomal and plasma membrane patches, and cause the endosomal membrane to bud into the lumen of the endosome, leading to the formation of intraluminal vesicles as early endosomes mature into late endosomal multivesicular body (23). Depending on their source, size, and function, EVs can be divided into three types—exosomes, microvesicles, and apoptotic bodies (Figure 1) (24, 25). Exosomes are the smallest EVs with a diameter of 40-120 nm and are formed due to exocytosis. Microvesicles are derived from cellular budding and have a larger size of 50-1000 nm. Both exosomes and microvesicles play a role in intercellular communication. Apoptotic bodies are always derived from dead cells and are the largest of EVs with a size of 500-2000 nm. Different surface modifications of EVs, such as exosomes, can make it have different functions, so as to achieve targeted drug delivery and *in vivo* imaging and tracking. Specific membrane proteins functionalized on the surface of exosomes, such as tetraspanins proteins (CD63, CD81, CD9), lactadherin, lysosome-associated membrane protein-2B, and glycosyl phosphatidylinositol, as well as different surface modification strategies such as genetic engineering, interact with the receptor system of target cells, which is involved in the regulation of physiological functions of various organ systems (26).

3 The role of EVs in NAFLD

EVs contain specific molecules on their surface that can induce signal transduction in specific cells by recognizing target cells and binding to cell-specific receptors or fusing with the target cell membrane and transferring the cargo into their cytoplasm to regulate the physiological activities of cells (Figure 2) (21). Based on different cell sources, EVs participate not only in normal physiological processes but also in disease processes (27–29). Consequently, EVs have potential application value as diagnostic biomarkers (30). In the liver, EVs not only play an important role in mediating signal transduction in liver cells but also affect metabolic pathways in liver cells associated with apoptosis of hepatocytes, inflammation, liver fibrosis, and the development of NAFLD (Table 1) (55, 56).

3.1 Glucose and lipid metabolism

Patients with NAFLD often have glucose and lipid metabolic disorders, which have been proven to be regulated by EVs and are associated with multiple pathways (57–59). Some research report that obese individuals have higher levels of circulating EVs than normal-weight individuals (60, 61). While, the role of EVs in regulating the glucose and lipid metabolism is multifarious. The possible mechanism may be the diversity of contents carried by exosomes, including non-coding RNAs and cytokines, which play biological functions.

A recent report indicated that adipocyte-derived EVs may induce hepatitis and cirrhosis by regulating adipose tissue homeostasis, interfering with normal signaling pathways, and causing metabolic dysfunction (22). EVs play a significant role in lipid redistribution in metabolic organs such as the liver, adipose tissues, and muscles under lipid overload. The study found that EVs levels increased in response to acute lipid overload and these EVs containing let-7e-5p fuse with adipocytes to promote adipocyte regeneration by upregulating the let-7E-5p-PGC1α axis (31). In a relatively hypoxic environment, the secretion of EVs derived from

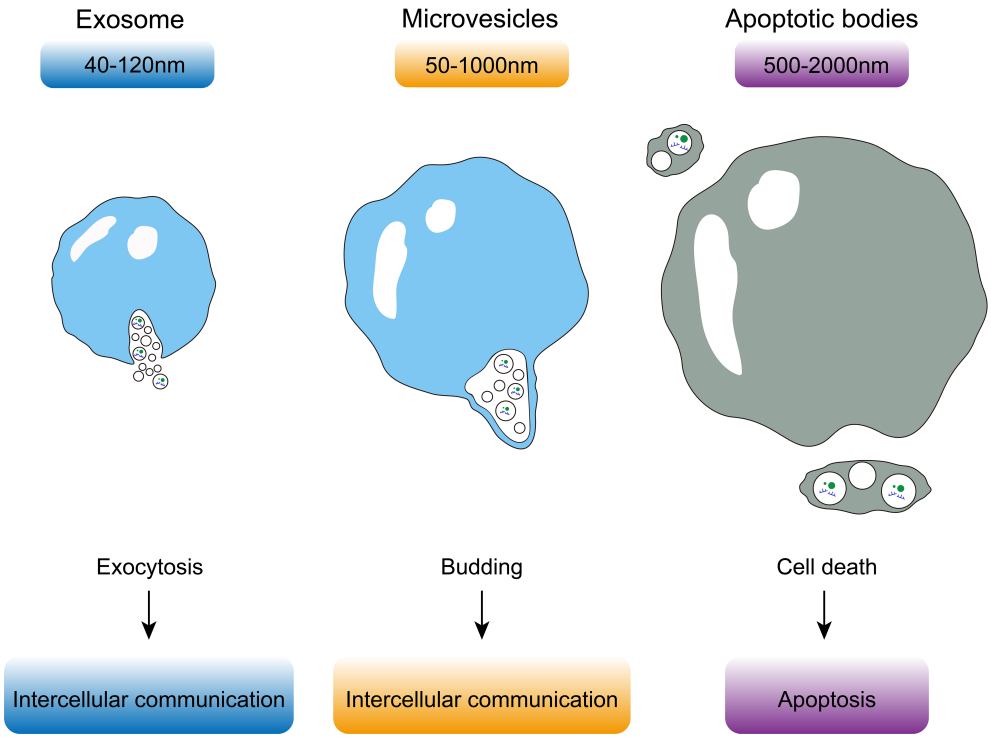

FIGURE 1
Exosomes are the smallest EVs with a diameter of 40-120 nm and are formed due to exocytosis. Microvesicles are derived from cellular budding and have a larger size of 50-1000 nm. Apoptotic bodies are always derived from dead cells and are the largest of EVs with a size of 500-2000 nm.

3T3-L1 adipocytes increased. Proteomic analysis revealed 231 protein components in these EVs, including a variety of lipogenic enzymes such as fatty acid synthase (FASN), 6-phosphate-glucose dehydrogenase (G6PD), and acetyl-CoA carboxylase (ACC), which promote fat synthesis and increase adipocyte load (32). Adipocyte-derived EVs act as adipocytokines that regulate the secretion of cytokines from adjoining cells in response to a variety of stimuli. They activate macrophages and promote the synthesis and release of macrophage colony-stimulating factor, interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), which aggravate IR, destroy gluconeogenesis in liver tissue and promote liver inflammation by adjusting the release of macrophage migration inhibitory factor (MIF), macrophage chemoattractant protein-1 (MCP-1) and IL-6 (62). EVs isolated from human adipose-derived stem cells (HASCs) generated during beige adipogenic differentiation can differentiate HASCs into beige and brown adipocytes. EVs derived from beige/brown adipocytes have beneficial effects on the browning of the white adipose tissue (22, 34). A study by Thomou et al. (63) showed that circulating EVs isolated from adipose tissue-specific miRNA knockout mice contain decreased miRNAs, and circulating miRNA levels are almost completely restored after transplantation of white/brown adipose tissue. These miRNAs play a role in improving glucose tolerance and reducing fibroblast growth factor 21(Fgf21) mRNA in hepatocytes. Another study showed that EVs containing let-7b-5p activated TGF-β-let-7b-5p signaling pathway in hepatocytes, reducing mitochondrial oxidative phosphorylation and suppressing white-to-beige fat conversion, that promoted high-fat diet (HFD)-induced steatosis and obesity (64). In summary, EVs can regulate glucose and lipid metabolism and NAFLD development by regulating gene expression or cell-specific signaling pathways. By blocking specific signaling pathways in or receptors on target cells, we can modulate the effects of EV cargo. The findings of the studies discussed above may provide new insights for the research and development of novel drugs in the future.

3.2 Insulin resistance

IR is closely related to liver steatosis and can also predict the development of NAFLD (65). Recent studies have revealed that EVs are easily internalized by cells and cause functional changes in specific tissues, regulating insulin signaling in other tissues (35). Adipocyte-derived exosomes can cause IR. The first study that identified the role of adipocyte-EVs in IR was performed in a mice model of obesity (37). EVs released by adipocytes can activate macrophages, which can induce IR through toll-like receptor 4/TIR domain-containing adaptor protein inducing interferon-β (TLR4/TRIF) pathways (37). Dang et al. (36) proposed that IR in obese individuals is highly correlated with the low expression of miR-141-

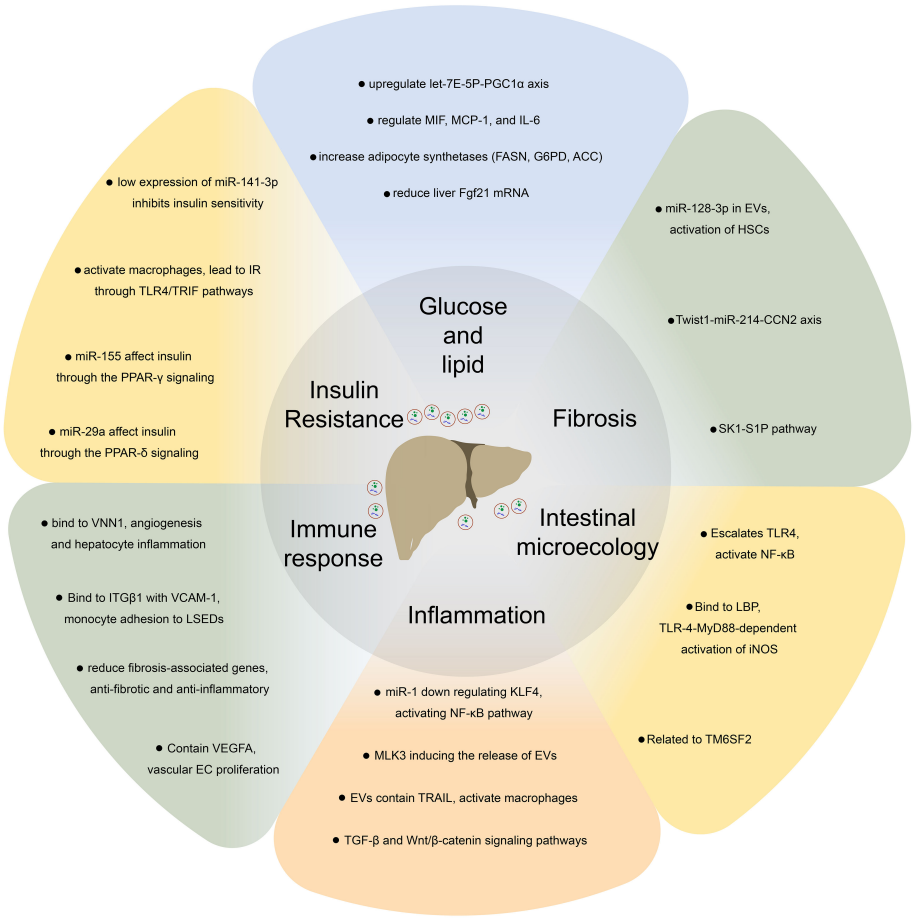

FIGURE 2
EVs regulate glucose and lipid metabolism, fibrosis, intestinal microecology, inflammation, immune response, insulin resistance and other processes by recognizing target cells and binding to cell-specific receptors or fusion with the target cell membrane to achieve substance transport and induce intracellular signal transduction, thus, participate in the pathophysiological process of non-alcoholic fatty liver disease.

3p in exosomes secreted by adipose tissues. EVs released by adipose tissue from obese mouse models can mediate crosstalk between adipose tissues and macrophages. Additionally, some studies have suggested that insulin sensitivity is related to macrophages that reside within adipose tissue (ATMs). Another study on ATMs found that miR-155 in exosomes released by ATMs was overexpressed in an obese mouse model (66). And it is reported that miR-29a was overexpressed in exosomes derived from ATMs in obese mouse models (38). miR-155 and miR-29a are key mediators s in the peroxisome proliferation-activated receptor-γ (PPAR-γ) and PPAR-δ signaling pathways, respectively. Both PPAR-γ and PPAR-δ have been identified as targets of miRNAs that regulate IR (39). The studies suggest that ATMs can impair insulin sensitivity by secreting exosomes containing specific miRNAs, leading to the inhibition of glucose uptake and directly affecting the insulin levels in an organism. Anja Fuchs et al. (67) found that systemic IR in people with obesity and NAFLD is associated with increased plasma PAI-1 concentrations and both plasma and subcutaneous abdominal adipose tissue derived exosomes. In addition, gut microbial-derived EVs can also influence glucose metabolism by regulating IR (68). It was found that fecal-derived EVs induced IR and poor glucose tolerance in high-fat diet (HFD)-fed mice compared to conventional diet-fed mice (69). In summary, EVs have been implicated in the development of IR and understanding the molecular mechanisms by which they confer IR may be effective in the prevention or treatment of NAFLD.

3.3 Immune response

The persistent inflammatory response is an important cause of the transition from simple fatty liver disease to severe liver injury, such as steatohepatitis and cirrhosis, which is related to the immune response (70). Immune regulation, including innate and adaptive immunity, is crucial to the pathogenesis of NAFLD. Innate immune cells in the liver include Kupffer cells, dendritic cells, natural killer (NK) cells, innate lymphoid cells, invariant NKT cells, and mucosal-

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TABLE 1 Mechanisms of development of nonalcoholic fatty liver disease related to extracellular vesicles.

Source of EVs	Mechanism	Pathophysiological progress		Reference
Glucose and lipid				
		Glucose metabolism	Lipid metabolism	
Hepatocytes	EVs containing let-7e-5p enhances adipocyte lipid deposition through Pgc1α.		↑	Yue Zhao, etal. (31)
Adipocytes	Hypoxic adipocyte-released exosomes were enriched in enzymes related to *de novo* lipogenesis (FASN, G6PD, ACC)		↑	Soichi Sano, etal. (32)
Body fluids	Obesity-associated exosomal miRNAs (miR-192 and miR-122) induce glucose intolerance and dyslipidemia	↑	↑	Carlos Castaño etal. (33)
HASCs	Secreted EVs during stem cell differentiation into white adipocytes or beige adipocytes can promote cell reprogramming.		↓	Youn Jae Jung, etal. (34)
IR				
		Insulin sensitivity	IR	
Plasma	IR increases the secretion of EVs, which are preferentially internalized by leukocytes, and alters leukocyte function.		↑	David W Freeman, etal. (35)
adipose tissue	Exosomes released from obesity adipose tissue containing less miR-141-3p inhibit the insulin sensitivity and glucose uptake.		↑	Shi-Ying Dang, etal. (36)
adipose tissue	The ob-EVs mediate the induction of TNF-α and IL-6 in macrophages and IR through the TLR4/TRIF pathway		↑	Zhong-bin Deng, etal. (37)
ATMs	Exosomes from ATMs in obese mice containing miR-155 cause glucose tolerance and IR by targeting PPARγ.		↑	Wei Ying, etal. (38)
Skeletal muscles	Exosomes from skeletal muscles of IUGR containing miR-29a induce IR though PPARδ/PGC-1α–dependent signals.		↑	Yuehua Zhou, etal. (39)
Immune response				
		Anti-inflammation	Pro-inflammation	
Hepatocytes	Lipotoxic hepatocyte-derived EVs are enriched with active ITGβ1, which promotes monocyte adhesion and liver inflammation in murine NASH.		↑	Qianqian Guo, etal. (40)
Hepatocytes	Lipids-induce-released hepatocyte EVs activate an inflammatory phenotype in macrophages by stimulating DR5.		↑	Petra Hirsova, etal. (41)
Hepatocytes	Cholesterol-induced lysosomal dysfunction increases the release of exosome containing miR-122-5p from hepatocytes, resulting in M1 polarization and macrophage-induced inflammation.		↑	Zhibo Zhao, etal. (42)
Hepatocytes	Steatotic hepatocyte-derived EVs promote endothelial inflammation and facilitate atherogenesis by miR-1 delivery, KLF4 suppression and NF-κB activation		↑	Fangjie Jiang, etal (43).
Neutrophils	miR-223-enriched EVs derived from neutrophils acted to inhibit hepatic inflammation and fibrosis	↑		Yong He, etal (44)
Hepatocytes	MLK3 mediates the release of CXCL10-laden EVs from lipotoxic hepatocytes, which induce macrophage chemotaxis		↑	Samar H Ibrahim, etal. (45)
Intestinal microecology				
		remission	aggravate	
Bacteria	LPS and palmitate induce the expression of TLR4 and NF-κB to promote NASH.		↑	Torfay Sharifnia, etal. (46)

(Continued)

TABLE 1 Continued

Source of EVs	Mechanism	Pathophysiological progress		Reference
Bacteria	LPS can promote the decrease of plasma adiponectin, the increase of plasma leptin levels, and greater expression of FAS and SREBP-1c mRNA in the liver		↑	Shinya Fukunishi, etal. (47)
Bacteria	The loss of functional LBP protected against early stages of NAFLD development, in part due to the protective effect of TLR-4–MyD88-dependent iNOS activation.		↑	Cheng Jun Jin, etal. (48)
Liver fibrosis				
		Anti- fibrosis	Pro-fibrosis	
EC	EC-derived SK1-containing exosomes regulate HSC signaling and migration through FN-integrin-dependent exosome adherence and dynamin-dependent exosome internalization		↑	Ruisi Wang, etal. (49)
HSCs	Cellular or exosomal Twist1 drives miR-214 expression and suppresses CCN2 production and downstream fibrogenic signaling through transcriptional activation of the DNM3os E-box	↑		Li Chen, etal. (50)
HLSC	HLSC-derived EVs attenuate liver fibrosis and inflammation		↑	Stefania Bruno, etal. (51)
Hepatocytes	Lipotoxic hepatocyte-derived EVs containing miR128-3p inhibit PPAR-γ to activate HSCs.		↑	Davide Povero, etal. (52)
PMFs	PMFs released VEGFA-containing microparticles, which activated VEGF receptor 2 in ECs and largely mediated their proangiogenic effect.		↑	Sara Lemoinne, etal. (53)
Hepatocytes	Exosomes secreted by hepatocytes exposed to FFA contribute to angiogenesis and liver damage in steatohepatitis requiring VNN1-dependent internalization		↑	Davide Povero, etal. (54)

IR, insulin resistance; IUGR, intrauterine growth retardation; EVs, extracellular vesicles; FASN, fatty acid synthase; G6PD, 6-phosphate-glucose dehydrogenase; ACC, 1.acetyl-CoA carboxylase; HASCs, human adipose-derived stem cells; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; TLR4/TRIF, toll-like receptor 4/TIR domain-containing adaptor protein inducing interferon; ATMs, adipose tissue macrophages; PPARγ, peroxisome proliferator–activated receptor γ; PPARδ, peroxisome proliferator–activated receptor δ; PGC-1α, proliferator-activated receptor-γ coactivator-1α; PMFs, portal myofibroblasts; VEGF, vascular endothelial growth factor; ECs, endothelial cells; VNN1, Vanin-1; ITGβ1, integrin β1; DR5, death receptor 5; KLF4, Kruppel like factor 4; NF-κB, Nuclear Factor-κB; HSCs, hepatic stellate cells; HLSC, human liver stem cells; MLK3, mixed lineage kinase 3; CXCL10, (C-X-C motif) ligand 10; SK1, sphingosine kinase 1; FN, fibronectin; LBP, lipopolysaccharide-binding protein; iNOS, inducible nitric oxide synthase.

associated invariant T cells, which form the first line of defense against invading organisms and environmental challenges. The hepatic innate immune response plays a prominent role in the progression of liver disease; therefore, it is an important driving force in NAFLD (71). Increasing evidence suggests the role of lymphocyte-mediated adaptive immunity as a factor promoting liver inflammation, including the role of B cells and $CD4^+$T and $CD8^+$T cells in sustaining NASH progression (72).

3.3.1 Innate immunity

Patients with NAFLD show increased levels of EVs derived from macrophages and NK cells. The levels of EVs derived from immune cells can be used to assess the extent of chronic liver disease, which is related to the enhancement of innate immune function during the development of NAFLD (73). EVs promote pathological angiogenesis and fibrosis in NASH by transporting a variety of mediators including growth factors, hedgehog molecules, proteins, and miRNAs (74). A study by Pover et al. (54) showed that under saturated lipotoxicity, the caspase8-caspase3-ROCK1 pathway in hepatocytes is activated, releasing EVs containing a large amount of Vanin-1, which can reinforce the internalization of EVs, initiate the migration of endothelial cells (ECs), and promote the generation of new small blood vessels, resulting in hepatocyte inflammation. Lemoinne et al. (53) showed that EVs carrying vascular endothelial growth factor A (VEGF-A) can be released by activated portal myofibroblasts and bind to VEGF-A receptors on vascular EC to promote vascular ECs and ductal hyperplasia. Vascular ECs co-cultured with steatotic hepatocytes or treated with steatotic hepatocyte-derived EVs decreased Kruppel-like factor 4 release, which activated the intracellular NF-κB pathway and significantly increased pro-inflammatory factor release (43).

Lipotoxic liver cells can release EVs that contain various macrophage chemokines and active mediators. Protein mass spectrometry of EVs showed that EVs contain many damage-associated molecular patterns, which can activate the inflammatory response in mammals (75). Lipid molecules can promote NAFLD by activating cytokines in hepatocytes. To activate the death receptor 5 (DR5) of hepatocytes through non-ligand-dependent pathways, the secretion of EVs in hepatocytes increase. TNF-related apoptosis-inducing ligand (TRAIL) on the surface of the EVs activates DR5-RIP1-NF-κB signaling pathway in macrophages to increase the secretion of IL-1β and IL-6 (41), which aggravates inflammation in hepatocytes. EVs carrying miR-122-5p secreted by hepatocytes can stimulate the secretion of pro-inflammatory factors and polarize hepatic macrophages into the M1 phenotype (42). Studies also have reported that EVs can

mediated the macrophages, which is supposed to play an important role in the regulation of fibrosis (76, 77). Several studies have demonstrated that NAFLD are associated with exosomes derived from or transferred to macrophages (78). For example, exosomal miRNA-411-5p derived from M2 macrophages plays an inhibitory role in HSCs activation during NASH progression by inhibiting its target gene CAMSAP1 (79). Furthermore, the exosomes released by lipotoxic hepatocytes can be ingested by macrophages, resulting in activation of M1 macrophages and hepatic inflammation by regulating the Rictor/Akt/FoxO1 signaling pathway (80). Hepatocyte-derived EVs can also promote monocyte adhesion via an integrin β1-dependent mechanism to induce an inflammatory response (40). Besides, under the inflammation or mechanical stimulation, the hepatic stellate cells (HSCs) activated and participate in the formation of liver fibrosis through the proliferation and secretion of the extracellular matrix. One possible mechanism for this transformation is through the upregulation and release of miR128-3p by EVs under lipotoxicity caused by increased free fatty acids in hepatocytes (64). These hepatocyte-derived EVs are internalized by HSCs and inhibit PPAR-γ in quiescent HSCs to facilitate phenotypic conversion (52). When HSCs were exposed to miR128-3p-deficient EVs, a higher PPAR-γ level and reduced proliferation and migration were observed. EVs containing connective tissue growth factor or miR214 promote the phenotypic transformation of activated HSCs (81). This is a possible mechanism of the translation from NAFLD to liver fibrosis and NASH. However, some studies suggest that EVs derived from hepatocytes can significantly downregulate the expression of genes related to fibrosis and have anti-inflammatory and anti-fibrotic effects. Neutrophil-derived miR-223 with high apolipoprotein E expression can be taken up by hepatocytes to limit the progression of steatosis to NASH (44). Fibrosis-related genes were significantly downregulated in immune-deficient NASH mice (methionine-choline-deficient diet-induced) that were treated with human hepatocyte-derived EVs (51). EVs from human liver stem cells are believed to slow down the symptoms of fibrosis and inflammation by regulating gene expression in liver cells. Therefore, the role of EVs in innate immunity appears to be dynamic and must be further investigated.

3.3.2 Adaptive immunity

The current concept is that innate immunity represents a key element in development of NAFLD, however, adaptive immunity is increasingly being recognized as an additional factor of NAFLD (72). NASH is characterized by increased levels of liver and circulating IFN-γ-producing $CD4^+$ T cells (82). $CD4^+$ T cells can differentiate into T helper 17 cells that release IL-17. IL-17 can promote M1-type macrophage polarization and exacerbate the liver inflammatory response to accelerate NAFLD progression (83). Mice lacking $CD8^+$ T cells and NKT cells are protected from steatosis and NASH when fed with a choline-deficient HFD, which is associated with reduced production of LIGHT by $CD8^+$T cells and NKT cells (84). Adaptive immunity and innate immunity are not completely independent, and there is an interplay between the two. Sun et al. (85) showed that OX40 was a key regulator of intrahepatic innate and adaptive immunity and mediated two-way signals and promotes both pro-inflammatory monocytes and macrophages, as well as T cell function, resulting in the development of NASH. By promoting NK cell activation, lymphocytes stimulate the secretion of IL-15 and IL-18 by macrophages, thereby modulating the progression of steatohepatitis and fibrogenesis (86). In conclusion, adaptive immune responses are crucial in the progression of NAFLD. EVs have been proven to be key factors in mediating adaptive immune responses by playing roles in antigen presentation, T-cell activation, T-cell polarization to regulatory T-cells, and immune suppression (87). Therefore, EVs play a role in NAFLD through the modulation of adaptive immunity.

3.4 Inflammation

Recently, many studies have found that EV levels significantly increase in NASH mice models (54, 75). HFD promotes the release of EVs, and the number of EVs increases in a time-dependent manner (88). MiR-1 in hepatocyte-derived EVs is an important factor in the promotion of endothelial inflammation. EVs aggravate not only endothelial inflammation but also atherosclerosis by delivering miR-1, which induces the inhibition of Kruppel like factor 4 (KLF4) and activation of the NF-κB pathway (43). In addition to promoting inflammation in endothelial cells, EVs also mediate inflammation through macrophages. Several studies have shown that the aggregation of Kupffer cells is closely related to hepatocyte-derived EV levels (75, 89–91), suggesting that EVs mediate the inflammatory response in liver damage by inducing chemotaxis of macrophages. In lipotoxic hepatocytes, the activated mixed lineage kinase 3 pathway promotes EV secretion by upregulating c-Jun N-terminal kinase. The secreted EVs further mediate chemotaxis of macrophages by releasing C-X-C motif ligand 10 (CXCL10) via binding to C-X-C receptor-3 (CXCR-3) and promoting macrophage-associated hepatic inflammation (45). Garcia-Martinez, et al. (92) found higher levels of Mitochondrial DNA (mtDNA) in EVs of mice and patients with NASH, with concurrent increase in hepatocyte-specific marker that activate toll-like receptor 9 (TLR9). TLR9 can mediate inflammation, thereby contributing to the transition from simple steatosis to steatohepatitis. Another study has shown that the mechanism of released EVs is related to the activation of the DR5 signaling pathway and the activation of macrophages by TRAIL of the released EVs to promote a metabolic response (41). Ferrante et al. (93) analyzed EVs shed by adipocytes from obese people and confirmed that adipocyte-derived EVs participate in transforming growth factor (TGF)-β and Wnt/β-catenin signaling pathways through miRNAs, which promote inflammation and fibrosis. In

summary, the lipotoxicity in hepatocytes promotes the release of EVs, and increased EVs mediate the inflammatory response by enabling intercellular interaction.

3.5 Intestinal microecology

Intestinal microorganisms produce a variety of proteins and bile acids, participate in bidirectional communication along the enterohepatic axis, and regulate intestinal microecology. Damage to gut microflora balance, such as changes in intestinal microflora composition and intestinal bacterial metabolites, plays an important role in regulating the development of NAFLD (58). Many bacteria-derived molecules, including nucleic acids, proteins, polysaccharides, and glycolipids, exist in microbe-derived EVs (21). These EVs not only support the survival of bacteria by delivering virulence factors and nutrients but also participate in the regulation of multiple signaling pathways in host cells (94). They influence NAFLD by regulating glucose and fat metabolism, immune responses, and redox balance (95).

Bacterial EVs can trigger multiple metabolic cascades and immune responses (95). Bacteria-derived EVs contain and transfer lipopolysaccharide (LPS), enter hepatocytes via the biliary tract, portal vein, and enterohepatic axis, and aggravate NAFLD. These EVs can induce liver inflammation by activating the TLR4-TRIF-GBPs signaling pathway (96) or delivering LPS into the cytosol of host cells to activate caspase-11, which regulates the immune response (97). Compared to patients with NAFLD, patients with NASH show a significant increase in LPS and free fatty acid (FFA), as well as an increase in TLR4 mRNA and interferon regulatory factor 3 (IRF-3) in the myeloid differentiation factor 88-independent signaling pathway. In addition, when using small interfering RNA-mediated TLR4 inhibitors, the inductive effect of LPS on NF-κB was weakened, suggesting that LPS can affect the TLR4-mediated NF-κB signaling pathway (46). TLR4 activates downstream signaling pathways that stimulate the release of cytokines and chemokines, leading to liver damage (47).

LPS-binding protein (LBP) and CD14 also participate in recognizing LPS, which is increased in NASH and NAFLD patients. LBP knockout in mice and subsequent prevention of LPS and TLR4 binding improved lipid metabolism in mice, protecting them from developing NAFLD under HFD conditions (48), suggesting that LBP is a crucial factor in NAFLD development. LBP and LPS levels have been shown to be associated with the development of NASH and fibrosis (98). Short RNA (sRNA) from bacteria-derived EVs can participate in regulating the innate immune response in host animals (99). Thus, damage to intestinal microecology can promote NAFLD by regulating intestinal bacteria-derived EVs containing sRNA or LPS.

As discussed above, gut microbiota-derived EVs may affect NAFLD through different mechanisms. Therefore, augmentation of beneficial gut microbes is a potential therapeutic approach. Previous studies have found that probiotics, prebiotics and other products can improve the condition of NAFLD patients (100). For example, ingestion of Lactobacillus acidophilus La5 and Bifidobacterium lactis Bb12 improved liver enzyme, serum total cholesterol, and LDL cholesterol levels in patients with NAFLD (101). Prebiotics significantly reduced TNF-α, CRP, liver enzymes, and steatosis in patients (102). Considering the potential benefits of probiotic transplantation and prebiotics in the treatment of NAFLD, combined with the role of EVs in NAFLD, we believe that this will be a direction of great research potential in the future, but further in-depth research is still needed.

3.6 Liver fibrosis

In addition to promoting inflammation, EV levels can influence liver fibrosis. Studies have shown that EVs can induce the activation of HSCs (52, 56, 103) and transmit information between liver cells and HSCs (104). Many studies have confirmed that HSC activation and proliferation are closely related to liver fibrosis (105). A study showed that miR-128-3p in EVs plays a crucial role in HSC activation, indicating that hepatocyte-derived EVs can mediate HSC activation through endocytosis (52). Additionally, HSCs can deliver connective tissue growth factor (CCN2) via the secretion of EVs. Besides CCN2, Twist1 and miR-214, which comprise the Twist1-miR-214-CCN2 axis in HSCs, also mediate fibrosis through delivery by EVs (50). Moreover, the migration of HSCs is affected by EC-derived EVs containing sphingosine kinase 1 (SK1), which mediates HSC chemotaxis through the SK1-S1P pathway (49). Besides, Studies have reported that PTEN has been proved to play an important role in the fibrosis in kidney (106) and liver (107) and highly related to exosome. For example, lipotoxic hepatocytes exosome transplantation aggravated the degree of PTEN-induced expression of putative protein kinase 1 (PINK1) mediated mitophagy suppression, steatohepatitis, lipidosis, and fibrosis in the livers of NAFLD mice with cirrhosis (108). Research have found that transfer of circDIDO1 mediated by MSC-isolated exosomes can suppress HSC activation through the miR-141-3p/PTEN/AKT pathway to suppress the proliferation, reduce pro-fibrotic markers, and induce apoptosis as well as cell cycle arrest in HSCs (109). The lipotoxic hepatocyte-derived exosomal miR-1297 could promote the activation and proliferation of HSCs through the PTEN/PI3K/AKT signaling pathway, accelerating the progression of NAFLD (110). In conclusion, as mediators of communication between cells, EVs play a significant role in the development of liver fibrosis by interacting with HSCs in different ways, such as by regulating specific signaling pathways.

4 Applications of EVs in NAFLD

We summarized the ways in which EVs play an important role in glucose and lipid metabolism, insulin residence, immune response, inflammation, intestinal microecology, and fibrosis in NAFLD. Several studies investigating the biological mechanisms of EVs have addressed their utility in the diagnosis and treatment of complex pathologies. Owing to the complex cargo and delivery

functions of EVs, they can be used as part of a multicomponent diagnostic strategy for disease detection and as a targeting vehicle for disease therapy (111). Herein, we discuss the potential diagnostic and therapeutic applications of EVs.

4.1 Diagnostic utility of EVs in NAFLD

There is currently no reliable method to diagnose or stage NAFLD except via invasive liver biopsy. Some studies have shown that the components in circulating EVs, such as RNAs and proteins, provide new evidence for the diagnosis of NAFLD and NASH (112), suggesting the potential of liquid biopsy as a noninvasive and accurate approach to diagnose and monitor NAFLD (113, 114). Therefore, EVs as biomarkers can be measured in body fluids and may be a promising noninvasive method for diagnosing NAFLD, overcoming some limitations of surgical biopsy (25, 112). For example, miR-135a-3p-enriched EVs have been proven to be an accurate and sensitive biomarker in NAFLD. It has been shown that the amount of circulating EVs was significantly increased after 8 weeks L-amino acid defined diet, and miRNA-122 and miR-192 are enriched in circulating EVs in NAFLD (115, 116). Therefore, the EV levels change at the early stage of NAFLD, and can be traced to identify the latent development of potential fatty liver disease at an early stage; this may be valuable for the early diagnosis of NAFLD. The contents in EVs also change dynamically at different stages during the progression of NAFLD (25) and can be exploited for identifying biomarkers for sensitively monitoring the progression of NAFLD (117). Newman et al. (118) found a stable predictive performance for total cell-free RNA and EV derived miR-128-3p in health people, NAFL and NASH patients. Therefore, EV-derived miRNA biomarkers can robustly distinguish patients with NAFL and NASH and show the severity of NAFLD.

In addition to NAFLD, EVs have been used as biomarkers in liquid biopsies for cancer diagnosis, monitoring, and prognosis (119, 120). The development of engineered EVs as individualized imaging diagnostic reagents and for facilitating targeted therapy has been proposed (121). Many exosome sensing technologies including exosome chips, EV array, and proteomic platforms, are designed to detect EVs in cancers, and CD26, CD81, and CD10 have been proposed as markers for the detection of hepatic damage associated with liver cancer (120, 122–124).

In recent years, researchers have also found that the composition of circulating exosome content in peripheral blood may be significantly changed in obese patients after bariatric surgery, and the content of circulating exosomes may be used as a serological marker to evaluate the prognosis of bariatric surgery (125–127). For example, it is reported that the microRNA content of circulating adipocyte-derived exosomes isolated from the peripheral blood are significantly modified following gastric bypass bariatric surgery and these changes are correlated to improvements in IR post-surgery (128).Another study found that total circulating EVs and hepatocyte-derived EVs are elevated in NAFLD and decrease following NAFLD resolution due to weight loss surgery, which may be new biomarkers for NAFLD resolution and response to weight loss surgery (129). In conclusion, the changes in the types and quantities of peripheral exosome contents may be used as a new indicator to evaluate the efficacy of preoperative and postoperative bariatric surgery.

4.2 Therapeutic utility of EVs in NAFLD

EVs have potential benefits as key mediators of cell therapy because of their advantageous features of product stability, immune tolerability, effectiveness in systemic delivery, and efficacy enhancement (130).

Currently, many studies have explored the therapeutic application of EVs (131). These include the use of mesenchymal stem cell (MSC)-derived EVs in the treatment of SARS-CoV-2-associated pneumonia (132) and the use of ticagrelor to decrease the release of procoagulant EVs from activated platelets to treat patients with myocardial infarction (133). EV-based antitumor and antibacterial vaccines have shown good safety and tolerance in patients with advanced melanoma and non-small cell lung cancer (134). Some EV cargos alleviate NAFLD.

After treatment with MSC exosomes, the levels of blood glucose and insulin, volume of visceral fat, number of lipid droplets, ballooning degeneration in liver tissue, and NAFLD activity score decreased in NASH mice. MSC exosomes can alleviate fatty liver in NASH mice and promote M2 polarization of macrophages (our unpublished data). A melanocorticosterone type 4 receptor knockout NASH mouse model challenged with LPS showed that treatment with MSC-derived EVs had anti-inflammatory and anti-fibrotic effects (135). Many studies indicate that the development of drugs to inhibit the expression of certain genes or signaling pathways with EVs participation may prevent lipid deposition and fibrosis (70, 136, 137). For example, the ROCK1 inhibitor fasudil can effectively block lipotoxicity-induced EV release in mouse models and prevent NASH progression *in vivo* (41).

As natural carriers of functional small RNA and proteins, EVs also have high application potential as drug delivery systems with low immunogenicity and high biocompatibility for chemotherapy (138, 139). In addition, EVs can be engineered to enhance bioactivity and targeting ability, avoid undesired and unnecessary cell toxicity, and enhance therapeutic effects (140). Zhang et al. found that compared with chemotherapy alone, umbilical cord-derived macrophage exosomes loaded with cisplatin significantly increased cytotoxicity in drug-resistant ovarian cancer cells (A2780/DDP and A2780 cells) (141), and TNF-α-loaded EV-based vehicles enhanced cancer-targeting under a magnetic field and suppressed tumor growth in murine melanoma subcutaneous models (142). Studies have also shown that exosomes loaded with doxorubicin have the same efficacy as doxorubicin and prevent cardiotoxicity (143). Therefore, EVs are capable of safe and efficient drug delivery and provide a viable alternative to conventional drug delivery in NAFLD.

Synthetic exosome mimics have been fabricated as therapeutic tools for drug delivery and have been reported to have therapeutic

effects (144). However, most of these studies are in the laboratory research stage; therefore, it is also necessary to establish reliable assays to assess the therapeutic potential of EVs and further develop them into formal potency tests for promoting the clinical applicability of EVs (145).

5 Limitation

The limitations of this study include three aspects below. Firstly, most of our research results are from the laboratory, clinical research data is insufficient. Secondly, current standards of EV detection methods are not consolidated, so it is necessary to further test and standardize the detection technology. Thirdly, current studies are limited to published articles, while ongoing studies are not included. So, it is supposed to track the updated research results.

6 Conclusion

EVs contain various biological molecules, including proteins, nucleic acids, and lipids; they play an important role in intercellular communication in various biological processes, including the development and progression of diseases such as NAFLD. EVs participate in different signaling pathways to regulate the initiation and progression of NAFLD. As natural carriers of biological molecules, EVs have potential advantages in the treatment of NAFLD. Circulating EVs have been considered potential diagnostic and prognostic biomarkers fin NAFLD. Exploring the precise mechanism of EVs in NAFLD will help us identify new biomarkers. Research on the mechanisms and clinical applications of EVs in NAFLD is in its initial phase and the applicability of EVs in NAFLD diagnosis and treatment is expected to grow with technological progress.

Author contributions

YX, J-CC, and WJ are responsible for collecting and sorting the literature and writing the paper. Y-HL, YH, C-HL, EC and HT are responsible for supplementing, revising and improving content. HZ and DW are responsible for guidance and proofreading. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Cordyceps cicadae polysaccharides alleviate hyperglycemia by regulating gut microbiota and its mmetabolites in high-fat diet/streptozocin-induced diabetic mice

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Introduction: The polysaccharides found in *Cordyceps cicadae* (*C. cicadae*) have received increasing academic attention owing to their wide variety of therapeutic activities.

Methods: This study evaluated the hypoglycemic, antioxidant, and anti-inflammatory effects of polysaccharides from *C. cicadae* (CH-P). In addition, 16s rDNA sequencing and untargeted metabolomics analysis by liquid chromatography-mass spectrometry (LC-MS) were used to estimate the changes and regulatory relationships between gut microbiota and its metabolites. The fecal microbiota transplantation (FMT) was used to verify the therapeutic effects of microbial remodeling.

Results: The results showed that CH-P treatment displayed hypoglycemic, antioxidant, and anti-inflammatory effects and alleviated tissue damage induced by diabetes. The CH-P treatment significantly reduced the *Firmicutes/Bacteroidetes* ratio and increased the abundance of *Bacteroides, Odoribacter, Alloprevotella, Parabacteroides, Mucispirillum,* and significantly decreased the abundance of *Helicobacter* and *Lactobacillus* compared to the diabetic group. The alterations in the metabolic pathways were mostly related to amino acid biosynthesis and metabolic pathways (particularly those involving tryptophan) according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. Correlation analysis showed that *Bacteroides, Odoribacter, Alloprevotella, Parabacteroides,* and *Mucispirillum* were positively correlated with indole and its derivatives, such as 5-hydroxyindole-3-acetic acid. Indole intervention significantly improved hyperglycemic symptoms and insulin sensitivity, and increased the secretion of glucagon-like peptide-1 (GLP-1) in diabetic mice. FMT reduced blood glucose levels, improved glucose tolerance, and increased insulin sensitivity in diabetic mice. However, FMT did not significantly improve GLP-1 levels.

Discussion: This indicates that *C. cicadae* polysaccharides alleviate hyperglycemia by regulating the production of metabolites other than indole and its derivatives by gut microbiota. This study provides an important reference for the development of novel natural products.

KEYWORDS

***Cordyceps cicadae*, polysaccharides, hypoglycemic, gut microbiota, metabolites**

1. Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disease characterized by insulin resistance and relative insulin insufficiency, and it is one of the most prevalent worldwide public health challenges (1). The number of T2DM and T2DM-related complications continues to increase, despite great efforts to combat the disease. Currently, many drugs have been used to treat T2DM, including sulfonylureas, biguanides, α-glucosidase inhibitors, and others. However, there are adverse effects associated with these drugs, including hypoglycemia, liver damage, gastrointestinal symptoms, and weight gain. Therefore, there is an urgent need to develop more effective and safer drugs to treat T2DM.

Many natural products are considered acceptable and alternative therapeutic options for treating diabetes and its related complications because of their efficacy, multiple beneficial effects, and minimal toxicity or side effects (2). *Cordyceps cicadae* (*C. cicadae*) is an intriguing entomogenous fungus that belongs to the *Claviciptaceae* family. It parasitizes on the larvae of cicada and has been used as a tonic food and herbal medicine to treat palpitations, infantile convulsions, chronic kidney diseases, heart palpitations, dizziness, and eye disease for hundreds of years (3, 4). Futhermore, *C. cicadae* has multiple effects, such as antioxidant, anti-inflammatory, antitumor effects, and blood glucose regulation (5, 6). Polysaccharides are one of the major bioactive ingredients found in *C. cicadae* that have received increasing academic attention owing to the wide variety of therapeutic activities (7). Crude polysaccharides from *C. cicadae* significant reduced the hyperglycemia and improved the hyperlipidemia of diabetic rats induced by alloxan monohydrate (8). *Cordyceps cicadae* polysaccharides also improved insulin resistance and glucose tolerance in rats with diabetic nephropathy (9). Therefore, *C. cicadae* polysaccharides may serve as potential drugs to prevent and treat T2DM. However, the underlying mechanisms of *C. cicadae* polysaccharides in hyperglycemia and diabetes requires further exploration.

An increasing number of studies have shown that polysaccharides can exert therapeutic effects in the treatment of obesity and diabetes by regulating intestinal microbial disorders. High-molecular-weight polysaccharides from *Ganoderma lucidum* reduce body weight, inflammation, insulin resistance, and reverse gut dysbiosis in high-fat diet (HFD)-induced obese mice (10). Polysaccharides from *Hirsutella sinensis* reduce obesity, inflammation, and diabetes-related symptoms by modulating the gut microbiota composition (11). Apple polysaccharides exert health benefits by inhibiting gut dysbiosis and chronic inflammation, and modulating gut permeability in HFD-induced dysbiosis rats (12). Monosaccharides and short-chain fatty acids (SCFAs) that are beneficial for host and intestinal health by modulating obesity, diabetes, and other metabolic diseases are produced by the hydrolysis and fermentation of polysaccharides by intestinal microorganisms (13). Nevertheless, the beneficial effects of *C. cicadae* polysaccharides on gut microbiota dysbiosis and their mechanisms of improvement in diabetes are not fully clarified. This study investigated the hypoglycemic, antioxidant, and anti-inflammatory effects of *C. cicadae* polysaccharides. The abundance and changes in the gut microbiota were examined using 16S rDNA gene sequencing. Nontargeted metabolite analysis was conducted using a liquid chromatography-mass spectrometry (LC–MS) platform (Waters, UPLC; Thermo, Q Exactive). This study provides an important reference to develop novel fungal polysaccharide drugs to prevent diabetes and its associated complications.

2. Materials and methods

2.1. Polysaccharides preparation from *Cordyceps cicadae*

Hot water extraction and ethanol precipitation were used to extract crude polysaccharides from *C. cicadae*, which stored in our laboratory. Briefly, dried *C. cicadae* powder was dissolved in distilled water according to the solid–liquid ratio at 1:30 and was reflux extracted at 90°C for 3 h. The extract was collected after filtration with the 0.22 μm filter paper (Whatman, China) and concentrated using a rotary evaporator (SENCO, China) at 65°C under a vacuum. The concentrate was precipitated by adding anhydrous ethanol at a ratio of 1:4 (v/v) and incubated overnight at 4°C. Precipitates were obtained by centrifugation at 5,000 × *g* for 10 min at 4°C and dissolved in distilled water. The pigments in the crude polysaccharides were decolorized using activated carbon in a water bath at 60°C for 2 h, and the proteins were removed using the Sevag method. The solution was dialyzed in dialysis bag (3,500 Da m_w cut-off) for 48 h at 4°C using distilled water, and the crude polysaccharides (CH-P) were obtained after freeze-drying. Since the polysaccharides characterization of *C. cicadae*, including chemical composition, molecular weight, and characteristic groups, were detail described in many studies (9, 14–16), the hypoglycemic activity of crude polysaccharides by regulating gut microbiota and its metabolites was investigated in this study.

2.2. Animals treatment

Male SPF grade C57/BL6J mice (20.0 ± 2.0 g) were purchased from Pengyue Experimental Animal Breeding Co., Ltd. (Jinan, China). A mouse diabetes model was established as described in our previous study (17). Briefly, the mice were acclimatized for 1 week under a 12-h light/dark cycle at 23 ± 2°C and a relative humidity level of 50 ± 5%. The mice had access to food and water *ad libitum*. The mice were randomly divided into normal control (Con, $n = 5$) and diabetic ($n = 20$) groups. The Con group was fed a maintenance diet throughout the experiment. The diabetic group ($n = 20$) was fed a high-fat diet (66.5% maintenance diet, 10% lard, 20% sucrose, 2.5% cholesterol, and 1% sodium cholate) for 4 weeks, followed by a single intraperitoneal injection of 100 mg/kg streptozotocin (STZ, sigma-aldrich, United States) after fasting overnight. The Con group was injected with an equal volume of citrate buffer (0.1 mol/L, pH 4.4). Mice with fasting blood glucose levels higher than 11.1 mmol/L 1 week after STZ injection were considered diabetic.

Diabetic mice were randomly divided into five groups: (1) diabetic control group (Dia, $n = 5$) treated with saline; (2) metformin positive group (Met, $n = 5$) treated with metformin at dose of 300 mg/kg; and (3) diabetic group were orally administered CH-P at doses of 200 mg/kg (CH-P-200), 400 mg/kg (CH-P-400), and 800 mg/kg (CH-P-800) body weight for 3 weeks. All animals were treated once a day. Food intake, water intake, and body weight were measured daily, and blood glucose levels were measured once a week throughout the study. The

oral glucose tolerance test (OGTT) was performed as previously described in our study (18).

A homeostatic model assessment was used to assess the changes in insulin sensitivity (HOMA-IS). The area under the curve (AUC) and HOMA-IS were calculated using the following formulas:

$$\mathrm{AUC}\left(\frac{\mathrm{min}\cdot\mathrm{mmol}}{\mathrm{L}}\right)=\frac{1}{2}\times\left[\mathrm{BG}\left(0\,\mathrm{min}\right)+\mathrm{BG}\left(120\,\mathrm{min}\right)\right]+\left[\mathrm{BG}\left(90\,\mathrm{min}\right)+\mathrm{BG}\left(60\,\mathrm{min}\right)-\mathrm{BG}\left(30\,\mathrm{min}\right)\right]\times 30\,\mathrm{min} \quad (1)$$

$$\mathrm{HOMA}-\mathrm{IS}=1/\left(\mathrm{FBG}*\mathrm{FINS}\right) \quad (2)$$

The mice were sacrificed at the end of the experiment and blood and tissues samples were collected for further analysis.

2.3. Biochemical parameters analysis and histological analysis

Blood samples were incubated for 30 min at 37°C, and then centrifuged at 3,000 × *g* for 15 min at 4°C to obtain serum for biochemical analysis. Commercially available kits were used to measure lipid indicators (total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C)); antioxidant parameters (malondialdehyde (MDA) and total superoxide dismutase (T-SOD) activity); and inflammatory factors (TNF-α, IL-6, and IL-1β) according to the manufacturer's instructions.

Tissues from pancreas, liver, heart, kidney, adipose, and aorta were fixed using 4% paraformaldehyde (biosharp, China), then dehydrated in a graded series of ethanol (70, 80, 90, 95, and 100%) and embedded in paraffin. The tissues were sectioned to a thickness of 5 μm using a slicer (Leica, German) for staining with hematoxylin–eosin (H&E).

2.4. Gut microbiota analysis

Total DNA was extracted from fecal samples using an AxyPrep DNA recovery kit (Axygen, United States) according to the manufacturer's instructions. The quality of the extracted DNA was determined by 1% agarose gel electrophoresis, and the concentration and purity of DNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, United States). The V3-V4 variable region of the 16S rDNA gene was amplified by PCR using the following primers: 357F (5′-ACTCCTACGGRAGGC AGCAG-3′) and 806 R (5′-GGACTACHVGGGTWTCTAAT-3′). The amplicon was sequenced using the NGS Illumina (Illumina, United States) platform and used to construct sequencing libraries. All sequences were divided into operational taxonomic units (OTUs) based on their similarity levels, and the OTUs were statistically analyzed at a similarity level of 97%. The representative OTU sequence was compared with the database for species annotation using Mothur (classify.seqs) software. Alpha diversity was estimated using the Chao and Shannon indices to evaluate species richness and species diversity of the samples. β diversity was evaluated by principal coordinates analysis-based OTU (PCoA) developed by Jaccard and Bray-Curtis to reveal the aggregation and dispersion of samples. Linear discriminant analysis Effect Size (LEfSe) was performed to generate a cladogram to identify different biomarkers in the gut microbiota [linear discriminant analysis (LDA) > 3, $p < 0.05$]. The metabolic pathways involved in the changes of the metabolites were determined using KEGG pathway enrichment analysis.

2.5. Untargeted metabolomics analysis by LC-MS

50 mg fecal samples were weighed and fully mixed with 800 μL 80% methanol. The samples were grinded with high-throughput tissue grinder (SCIENTZ-48, China) at 65 Hz for 180 s, and ultrasound-treated (PS-80A, China) at 4°C for 30 min. The samples were then incubated at −40°C for 1 h, vortexed for 30 s, and centrifuged at 12,000 × *g* for 15 min at 4°C to collect the supernatant. Two hundred microliters supernatant was mixed with 5 μL dichlorophenylalanine (0.14 mg/mL, aladdin, China) for further analysis. Untargeted metabolomics was analyzed using an LC–MS platform (Waters, UPLC; Thermo Fisher Scientific, Q Exactive). Mobile phase A was 0.05% v/v formic acid (aladdin, China) and mobile phase B was acetonitrile (sigma-aldrich, United States). The injection volume was 5 μL and the automatic injector temperature was 4°C. The ACQUITY HSS T3 column (2.1 mm × 100 mm, 1.8 μm, Waters) temperature was set to 40°C at a flow rate of 0.300 mL/min. Data were monitored in positive ion mode (POS) and negative ion mode (NEG). Quality control (QC) samples were prepared by mixing equal amounts of fecal samples to be tested. All original LC–MS data were imported into Compound Discoverer 3.1 data processing software to conduct spectrum processing and database searching to obtain the qualitative and quantitative results of the metabolites. Multivariate statistical analyses [including principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA)] were used to reveal differences in metabolites between the different groups. Hierarchical clustering and metabolite correlation analyses were used to reveal the relationships between samples and metabolites. The biological significance of the metabolites was explained through functional analysis of the metabolic pathways. Hierarchical clustering analysis was used to interpret the correlation between gut microorganisms and metabolites.

2.6. Indole intervention and fecal microbiota transplantation

Fecal samples were collected from the C-P-800 group into sterile frozen pipes using a sterile toothpick, quickly snap-frozen in liquid nitrogen, and stored at −80°C until further processing. 300 mg fresh fecal pellets were dissolved and suspended in 2 mL of normal saline. Homogenized fecal mixtures were centrifuged at 2,000 × *g* for 1 min at 25°C, and the supernatants were transferred to new tubes that were immediately administered to the mice by oral gavage. The diabetic mice were divided into a diabetic control group (F-DG, $n = 5$), an indole intervention group (I-TG, $n = 5$), and an FMT treatment group (F-TG, $n = 5$). Healthy C57/BL6J mice were randomly selected for the normal control group (F-NG; $n = 5$). Mice in the F-TG group were

treated with 300 μL fecal suspensions collected from CH-P treatment groups and the indole intervention group was treated with a dose of 50 mg/kg by oral gavage daily for 4 weeks. F-NG and F-DG mice received equivalent volumes of normal saline.

2.7. Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism version 8.0.2. Differences between groups were evaluated using One-way ANOVA. Differences between samples were considered statistically significant at $p < 0.05$.

3. Results

3.1. CH-P treatment improves diabetes symptoms in mice

Body weight, food intake, and water intake of diabetic mice significantly decreased, which was positively correlated with the concentration of polysaccharides purified from *C. cicadae* compared with the diabetic control group after 4 weeks of treatment (Supplementary Figures 1A–C). Moreover, *C. cicadae* polysaccharides significant reduction in blood glucose and insulin levels in a dose-dependent by decreased by 39.92 and 31.27% at a dosage of 800 mg/kg, respectively, and it also significantly improved the HOMA-IS relative to the diabetic control (9.63 ± 1.03 vs. 20.08 ± 1.66, $p < 0.001$; Figures 1A–C). The OGTT results showed that the changing trend in blood glucose was the same in all mice. However, the blood glucose tolerance level significantly improved in the CH-P treatment group compared with that in the diabetic control group. The area under the curve (AUC) values in the CH-P treatment group decreased in a dose-dependent manner, and exhibited up to a 35.56% decrease compared to the untreated diabetic mice (Figure 1D). Dyslipidemia is one of the main risk factors for diabetes and its associated complications. HbA1c, TG, TC, and LDL-C levels significantly increased, whereas HDL-C levels significantly decreased in diabetic mice compared with the control group. The CH-P treatment reversed these index values in a dose-dependent manner; there was a significant decrease of 39.76% HbA1c, 43.36% TG, 27.63% TC, and an increase of 28.10% HDL-C at a dose of 800 mg/kg compared with the diabetic control, respectively (Figures 1E–I). Overall, the therapeutic effect of *C. cicadae* polysaccharides at the dose of 800 mg/kg is better than that of metformin, but it was lower at the dose of 400 mg/kg than that of metformin.

3.2. Treatment with CH-P alleviated tissue damage in diabetes

Long-term hyperglycemia in diabetes can lead to chronic injury and tissue dysfunction. The endocrine pancreas plays a central role in controlling blood glucose levels and regulating energy metabolism (19). The islets of diabetic mice showed contraction and distortion compared to the control group. The islet boundaries were blurred and the number of islet cells significantly decreased. Treatment with CH-P significantly increased the number of islets and islet cells. In addition, CH-P treatment made the boundaries of the islets clearer, and the shape and size of the islets were significantly restored (Figure 2A). Adipose tissue is classified as white, brown, or beige fat based on its anatomical location and metabolic functions. It is a major metabolic and endocrine organ that plays a key role in glucose and energy homeostasis (20). White adipose tissue is the main tissue for energy storage, and excessive white adipose tissue (WAT) is associated with hyperglycemia and insulin resistance. The functions of brown adipose tissue include energy consumption and heat production, which play important roles in the regulation of energy metabolism (21). This study showed that CH-P treatment significantly reversed the enlargement of white adipocyte tissue and adipose tissue increases associated with diabetes. In addition, CH-P treatment significantly increased the number of brown fat cells in diabetic mice, decreased the outline and boundaries of adipose cells, and decreased the degree of cell swelling (Figures 2B,C). The liver is an important metabolic organ that plays an important role in the maintenance of blood glucose homeostasis. Glucose metabolism disorders in the liver are involved in the development of T2DM (22). Diabetic mice show significant adipose vacuoles and cellular swelling. The group treated with CH-P showed significantly improved hepatocyte hypertrophy and lipid accumulation compared to those in diabetic mice (Figure 2D). Diabetic nephropathy is a serious complication of diabetes (23). CH-P treatment also improved renal lesions in diabetic mice, significantly reduced glomerular volume, and alleviated mesangial hyperplasia compared to diabetic mice (Figure 2E). Chronic hyperglycemia and diabetes lead to impaired cardiac function and an increased risk of arrhythmia (23). The cardiac tissue of diabetic mice exhibited myocardial hypertrophy with varying degrees of interstitial edema and histiocytosis and displayed inflammation and fibrosis of the endocardium or pericardium. CH-P treatment ameliorated myocardial hypertrophy and relieved tissue inflammation and fibrosis (Figure 2F). T2DM can induce ultrastructural damage to the tunica intima and tunica media of the aorta (24). The tunica media of the aortae was significantly thickened in diabetic mice and CH-P treatment significantly reversed the pathological damage (Figure 2G).

3.3. The effect of *Cordyceps cicadae* polysaccharides on antioxidant and anti-inflammation profiles

Oxidative stress is the main factor in hyperglycemia-induced tissue injury and is responsible for events related to the early development of T2DM (25). The total antioxidant capacity of diabetic mice significantly decreases, which is consistent with the results of this study. However, CH-P treatment significantly decreased the MDA content and increased the T-SOD activity in the serum of diabetic mice in a dose-dependent manner; there was a 24.16% decrease in MDA and a 37.38% increase in T-SOD levels at a dose of 800 mg/kg relative to diabetic mice (Figures 3A,B).

Chronic inflammation is closely related to liver insulin resistance, and the progression of T2DM is often accompanied by chronic low-grade inflammation (26). The levels of circulating inflammatory markers such as proinflammatory cytokines significantly increased in diabetes and play an important role in the occurrence and development of T2DM (27). The effects of CH-P on serum

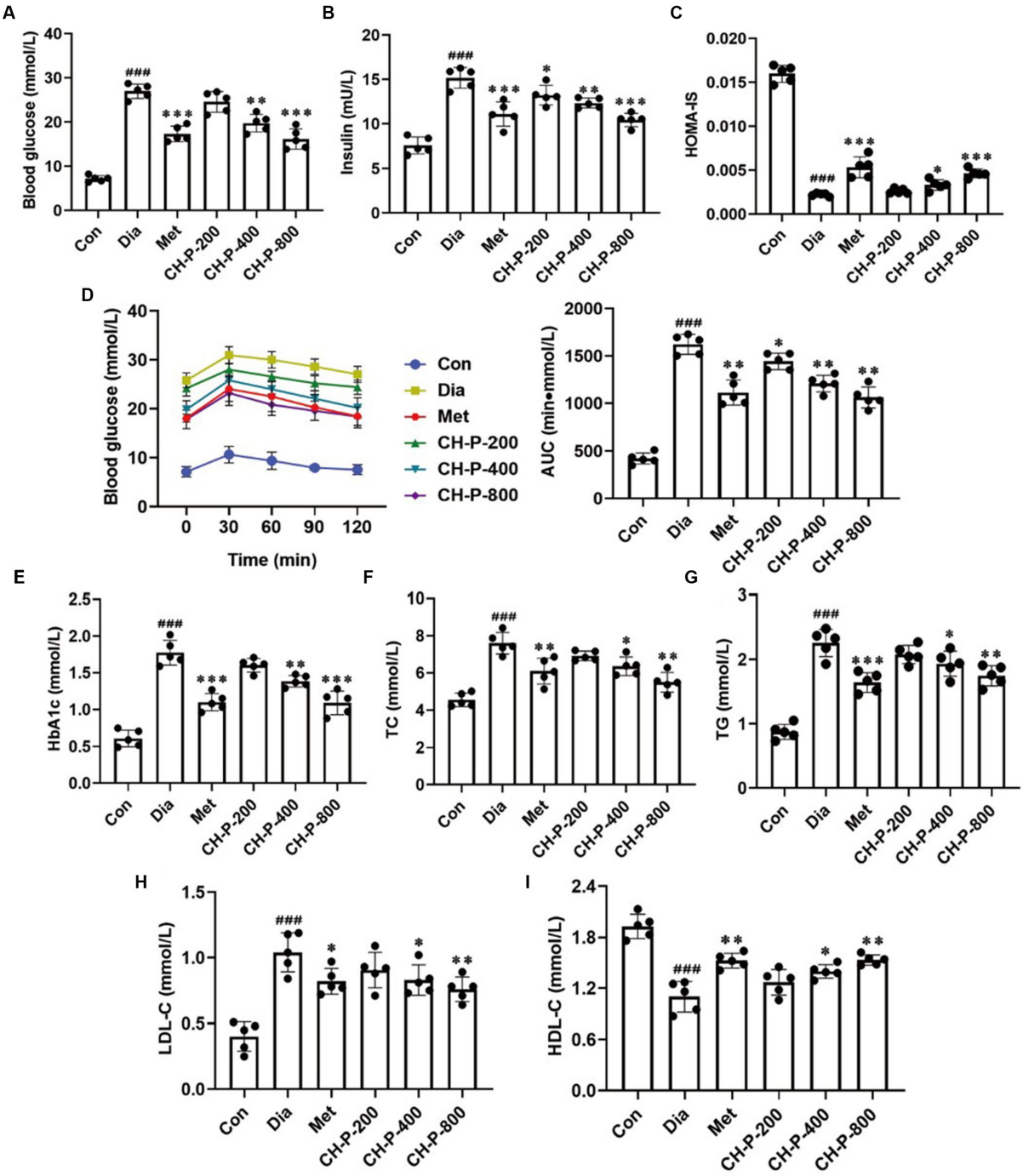

FIGURE 1
Analysis of CH-P treatment on hyperglycemia and dyslipidemia. **(A)** Blood glucose. **(B)** Insulin. **(C)** Homeostatic model assessment of insulin sensitivity (HOMA-IS). **(D)** Oral glucose tolerance test (OGTT) and area under the curve (AUC). **(E)** HbA1c. **(F)** Total cholesterol (TC). **(G)** Total triglycerides (TG). **(H)** Low-density lipoprotein-cholesterol (LDL-C). **(I)** High-density lipoprotein-cholesterol (HDL-C). $^{###}p<0.001$, vs. Con; $^{*}p<0.05$, vs. Dia; $^{**}p<0.01$, vs. Dia; $^{***}p<0.001$, vs. Dia.

pro-inflammatory cytokine levels were shown in Figure 3. CH-P treatment markedly reduced the levels of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 in a dose-dependent manner compared to the high levels of pro-inflammatory cytokines in the diabetic control group. TNF-α, IL-1β, and IL-6 decreased by 26.09, 20.13, and 22.85%, respectively at a dose of 800 mg/kg CH-P compared with the diabetic control (Figures 3C–E).

3.4. The gut microbiota composition changes following treatment with *Cordyceps cicadae* polysaccharide

Perturbations in the gut microbiota can influence the development of some metabolic disorders, (including obesity and diabetes) through different metabolic and immune pathways,

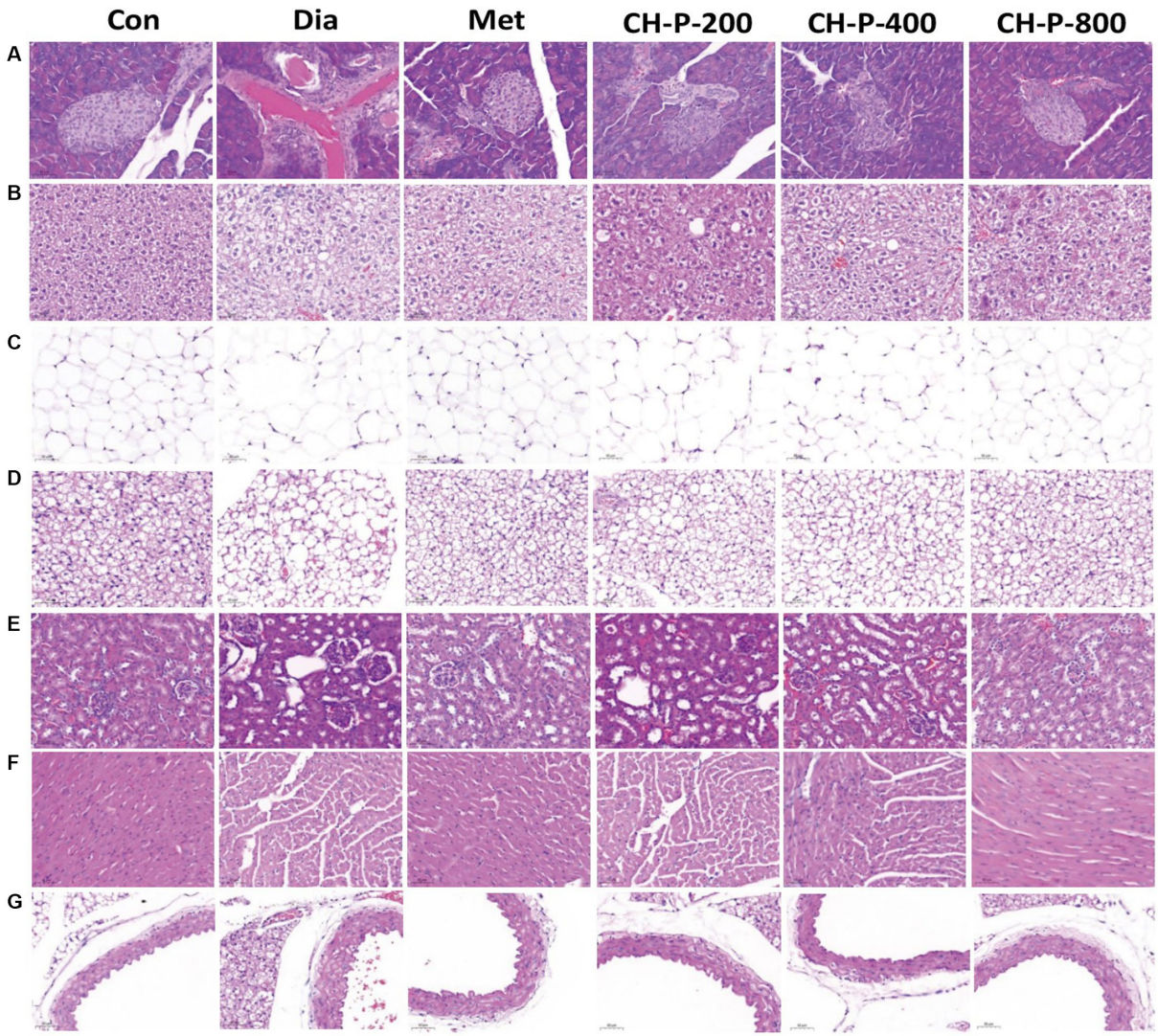

FIGURE 2
CH-P treatment recovers tissue damage caused by diabetes. **(A)** Pancreas. **(B)** Liver. **(C)** White fat. **(D)** Brown fat. **(E)** Kidney. **(F)** Heart. **(G)** Aorta. Bars represents 50μm.

including anti-inflammatory status, abnormal glucose metabolism, and insulin resistance (28, 29). 16S rDNA sequencing was used to detect the fecal microbiota composition and changes in mice to explore whether CH-P exerts a hypoglycemic effect by modulating intestinal microorganisms. The alpha diversity significantly increased in the CH-P treatment group compared to the diabetic group according to the Chao (376.6 ± 34.52 vs. 329.2 ± 20.83, $p < 0.05$) and Shannon indices (4.38 ± 0.19 vs. 3.72 ± 0.61, $p < 0.05$; Figure 4A). This suggests that the CH-P treatment boosts species richness and species evenness of gut microbiota in diabetic mice. Moreover, PCoA based on Jaccard and Bray-Curtis showed that the microbial communities of the two groups were clearly separated and clustered (Figure 4B). This indicated that CH-P treatment significantly influenced the gut microbiota composition. To investigate the changes in fecal microflora in response to CH-P treatment, the gut microbiota was analyzed at different taxonomic levels. The relative abundance of phyla levels of *Firmicutes*, *Bacteroidetes*, *Proteobacteria,* and *Desulfobacterota* was primarily dominated in both groups, and more than 90% of the total bacteria were comprised of *Firmicutes* and *Bacteroidetes* (Figure 4C). The *Firmicutes*/*Bacteroidetes* ratio was significantly reduced in the CH-P treatment group compared to the diabetic group (Figure 4D). An increased ratio of *Firmicutes*/*Bacteroidetes* and changes in several bacterial species can promote the development of obesity in dietary and genetic models of obesity in mice (30). These results suggest that CH-P has a beneficial effect on the health of intestinal microbiota. The top 20 gut genera microbiota were analyzed to determine the relative abundance differences between the two groups. The CH-P treatment significantly increased the abundance of *Bacteroides*, *Odoribacter*, *Alloprevotella*, *Parabacteroides*, and *Mucispirillum*, and significantly decreased the abundance of *Helicobacter* and *Lactobacillus* compared to the diabetic group (Figure 4E). The relative abundance of class, order, family, and species levels were showed in Supplementary Figure 2.

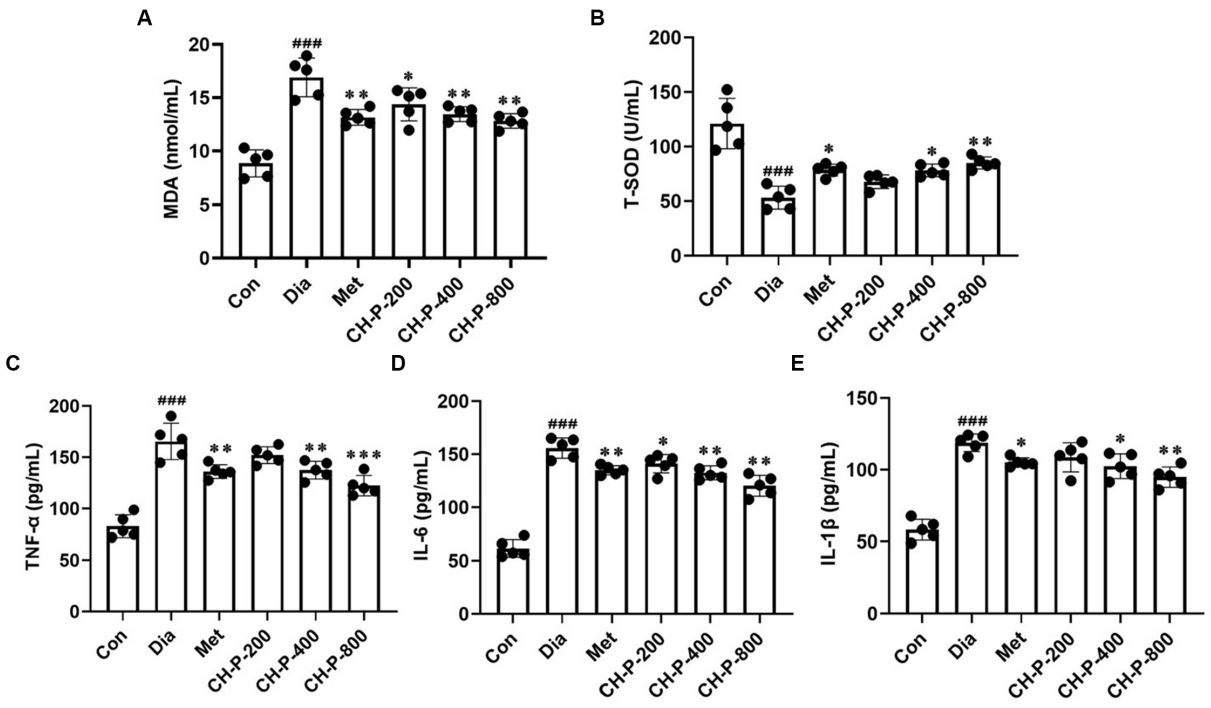

FIGURE 3
Analysis of the antioxidant and anti-inflammatory activities of CH-P. **(A)** MDA content. **(B)** T-SOD activity. **(C)** TNF-α. **(D)** IL-1β. **(E)** IL-6. ###$p<0.001$, vs. Con; *$p<0.05$, vs. Dia; **$p<0.01$, vs. Dia; ***$p<0.001$, vs. Dia.

Linear discriminant analysis effect size analysis was performed to generate a cladogram to identify different biomarkers in the gut microbiota composition of the two groups. There were distinguishing components at different taxon levels, including 12 species enriched in the CH-P group and nine species enriched in diabetic mice (Figure 5). The decreased abundance in *Firmicutes* was primarily related to a decrease in *Clostridiales*, *Lachnospiraceae*, *Ruminococcus*, and *Streptococcus hyointestinalis* in CH-P-treated mice. Meanwhile, the increased abundance of *Bacteroidetes* was mainly attributed to an increase in *Parabacteroides* and *Bacteroides vulgatus*. Moreover, the CH-P treatment showed desirable effects in restoring other key bacterial species in diabetic mice including *Mucispirillum* (which significantly increased from the phylum to the genus level) and *Marvinbryantia* (Figure 5).

3.5. The effect of CH-P treatment on the metabolism of diabetic mice

The gut microbiota exerts important and diverse effects on host physiology by generating metabolites with health benefits (31). Therefore, an untargeted gas chromatography–mass spectrometry (GC–MS)-based metabolomic analysis was performed to study the effect of CH-P on the metabolic profiles of mouse fecal samples. A total of 6,242 (POS) and 4,760 (NEG) metabolites were detected (Figure 6A; Supplementary Figure 3A). The quality control (QC) samples clustered in the PCA plots demonstrate the stability and repeatability of the acquisition method (Figure 6B; Supplementary Figure 3B). The PCA plots showed that the metabolomics in the diabetic and CH-P groups showed a clear separation trend (Figure 6B; Supplementary Figure 3B). Partial least-squares discriminant analysis (PLS-DA) showed that the CH-P treatment significantly altered the metabolic profiles of diabetic mice. The permutation test of the PLS-DA model showed good stability and no overfitting (Figure 6C; Supplementary Figure 3C). The upregulated and downregulated metabolomics revealed that there were 38 significantly changed metabolites (25 POS and 13 NEG) between the diabetic and CH-P groups visualized by the heat maps (Figure 6D; Supplementary Figure 3D).

The metabolic pathways involving the altered metabolites were determined using KEGG pathway enrichment analysis, and the top 20 metabolic pathways are shown in Figure 7A and Supplementary Figure 4A. Interestingly, pathway enrichment analysis indicated that major alterations in metabolic pathways were related to amino acid biosynthesis and metabolic pathways, including arginine biosynthesis; tryptophan metabolism; valine, leucine, and isoleucine biosynthesis; and phenylalanine, tyrosine, and tryptophan biosynthesis. Therefore, we speculated that CH-P could improve diabetes by regulating the production of metabolites related to amino acid biosynthesis and metabolism, particularly tryptophan biosynthesis and the metabolic pathways of gut microorganisms (Figure 7A). Indole and its derivatives (such as 5-hydroxyindole) are core intermediates of tryptophan metabolism (32). CH-P treatment significantly improved the relative abundance of indole, 5-hydroxyindole, and 5-hydroxyindole-3 acetic acid compared to that of diabetic mice (Figures 7B–D). Differential metabolite correlation analysis was performed to analyze the correlations between individual metabolites. The metabolites correlated well with each other and the relationships between various metabolites are shown in Figure 7E and Supplementary Figure 4B. The abundance of indole and its derivatives

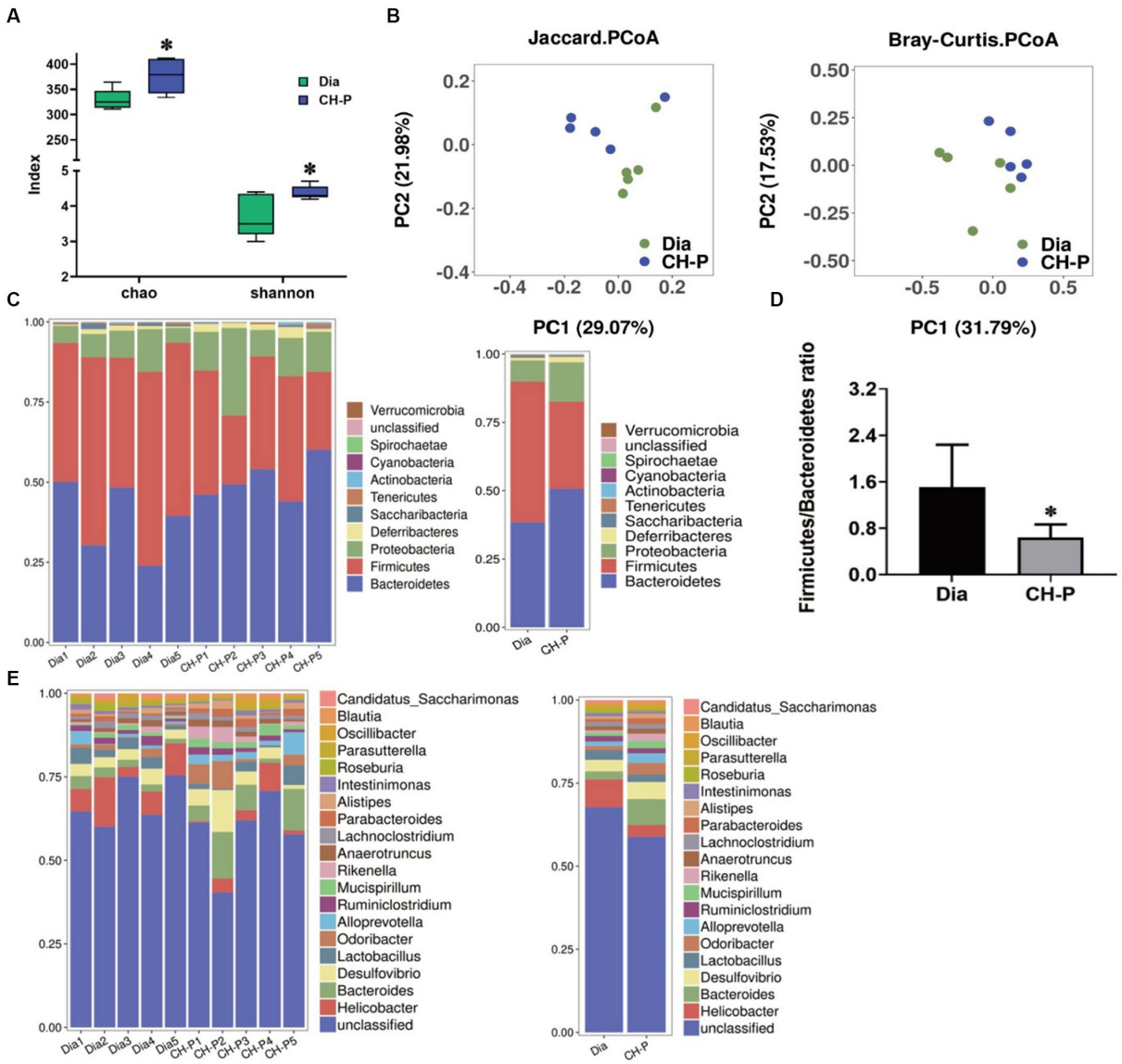

FIGURE 4
Gut microbiota diversity and composition analysis. **(A)** The alpha diversity indexes of gut microbiota for richness (Chao index) and evenness (Shannon index) between the two groups. **(B)** Principal component analysis of the gut microbiota between the diabetic and CH-P treatment group based on Jaccard and Bray-Curtis. **(C)** Relative abundance of gut microbiota at the phylum level. **(D)** The ratio of Firmicutes/Bacteroidetes. **(E)** Relative abundance of fecal microbiota at the genus level. *p<0.05 indicated significant differences.

were significantly positively correlated. This discovery may provide a new theory for the therapeutic mechanisms of polysaccharides from *C. cicadae*.

3.6. Potential relationship analysis between gut microbiota and metabolites in diabetic mice

Spearman's correlation analysis was used to explore the potential relationship between gut microbiota and fecal metabolites. *Bacteroides*, *Odoribacter*, *Alloprevotella*, and *Mucispirillum* were positively correlated with 5-hydroxyindole-3-acetic acid, while *Alloprevotella* was negatively correlated with glycerol 3-phosphate and oleamide. *Parabacteroides* was positively correlated with indole and N, N-dimethylsphingosine, and negatively correlated with cholest-4,6-dien-3-one, cytosine, and 16-hydroxyhexadecanoic acid. Interestingly, indole is an important component involved in tryptophan metabolism and phenylalanine, tyrosine, and tryptophan biosynthesis, while 5-hydroxyindole-3-acetic acid is involved in tryptophan metabolism (Figure 8). These results were consistent with those of the KEGG pathway enrichment analysis. Together, the results showed that CH-P treatment can improve diabetes by affecting the abundance of gut microorganisms such as *Bacteroides*, *Odoribacter*, *Alloprevotella*, *Paraberoides*, and *Mucispirillum*. The gut microbiota can produce metabolites such as indole and 5-hydroxyindole-3-acetic acid to participate in tryptophan metabolism and phenylalanine, tyrosine, and tryptophan biosynthesis, and play a role in treating diabetes.

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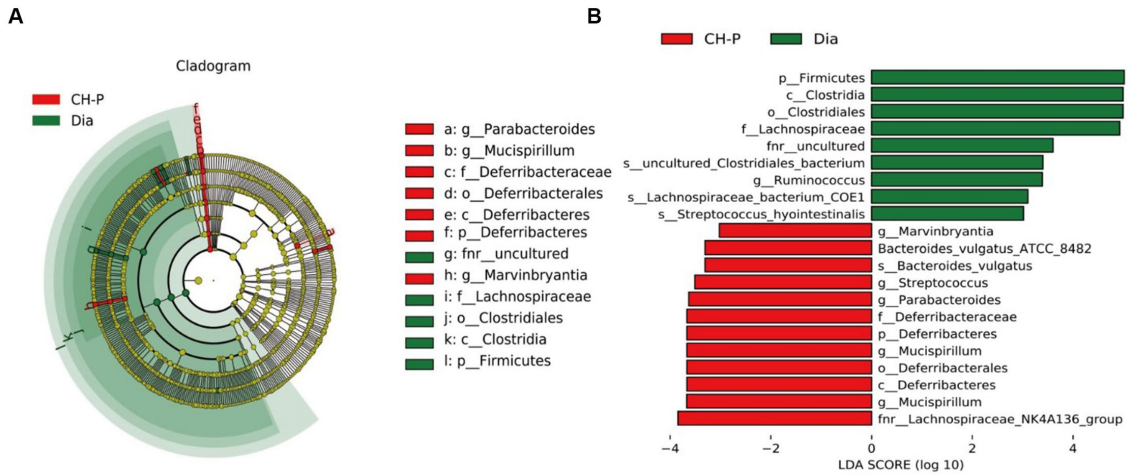

FIGURE 5
Linear discriminant analysis Effect Size (LEfSe). **(A)** Cladogram indicating the phylogenetic distribution of microbiota correlated with the diabetic or CH-P treatment groups. **(B)** The difference in abundance between the diabetic and CH-P treatment groups.

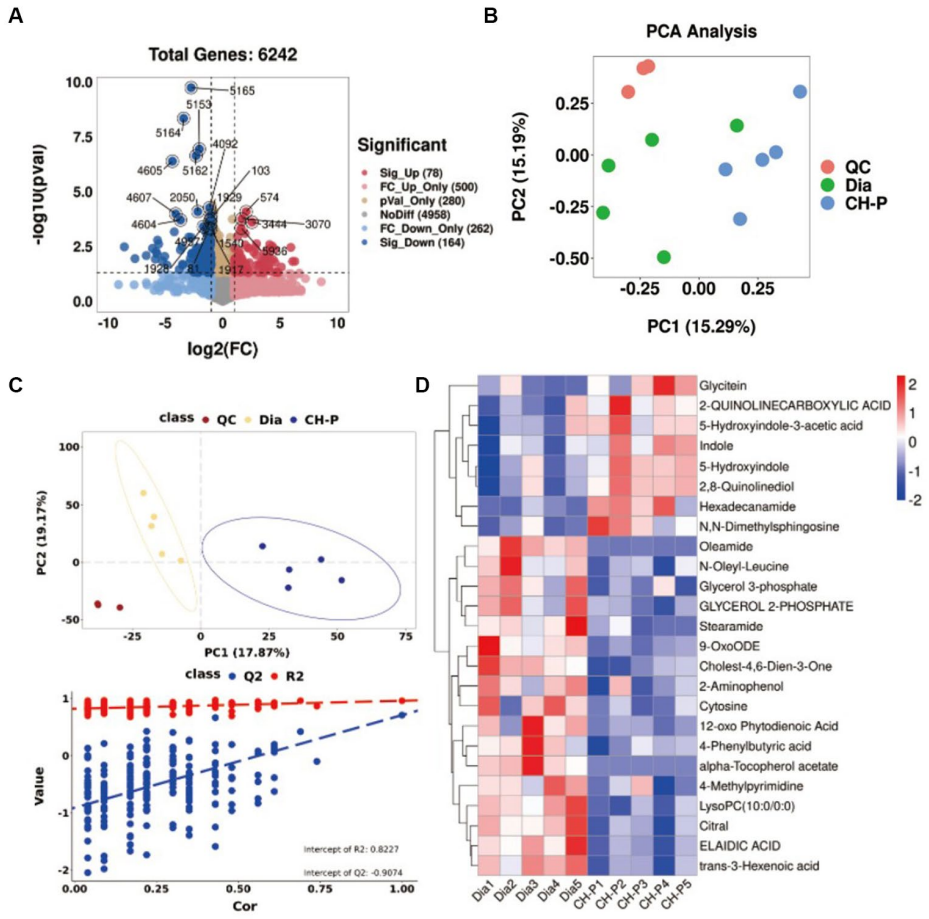

FIGURE 6
Untargeted metabolomics analysis of the metabolites in the diabetic and CH-P treatment groups using the POS model. **(A)** Principal component analysis score plot showing comparisons of the metabolomic profiles in both groups. **(B)** Volcano plot showing the differentially accumulated and significantly changed metabolites. **(C)** The PLS-DA score plot comparing the metabolome profiles in both groups and statistical validation of the PLS-DA model by permutation testing. **(D)** Hierarchical clustering heatmap of metabolites showing metabolites that significantly differ in abundance between both groups.

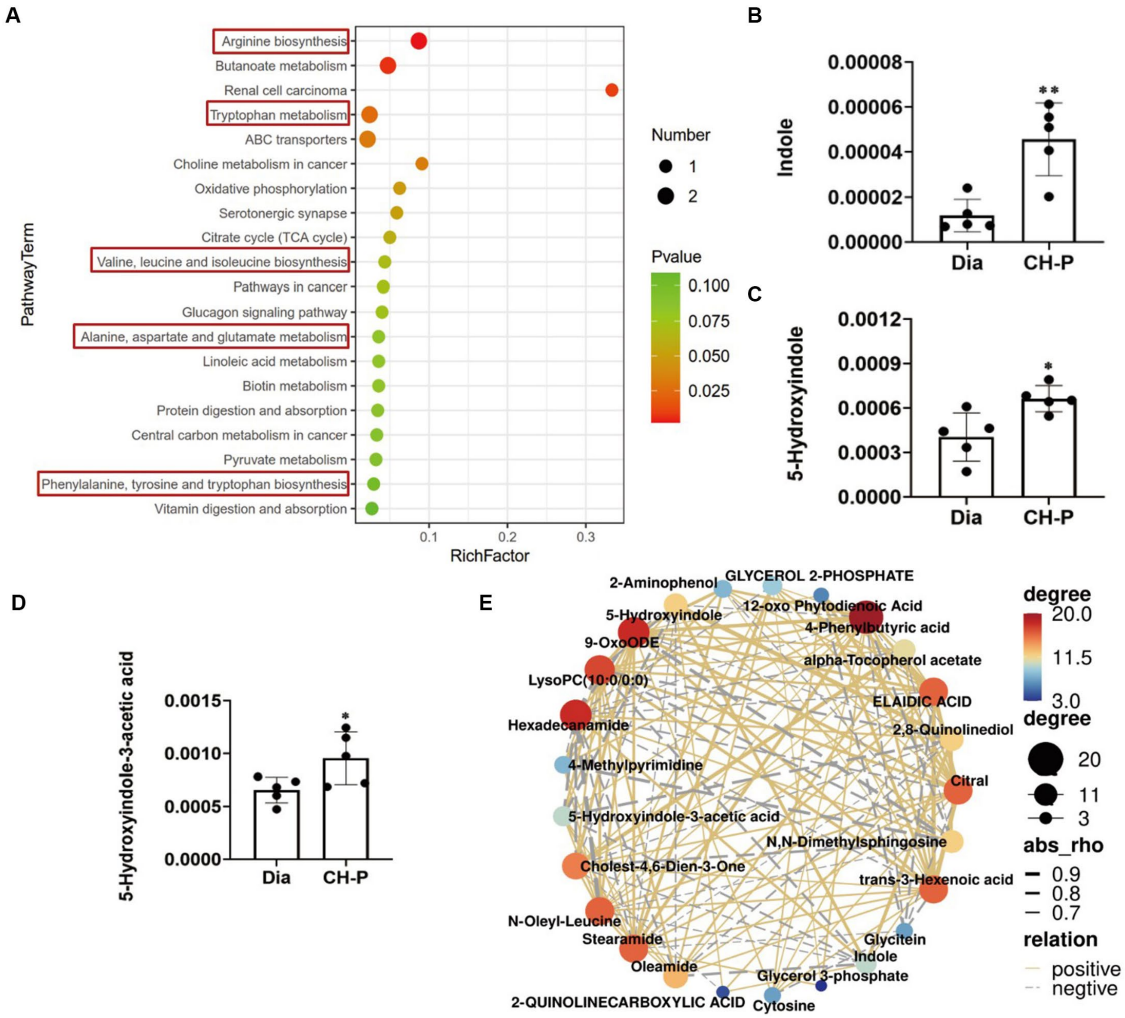

FIGURE 7
Metabolic pathways involving the differential metabolites and their correlation. **(A)** Pathway enrichment analysis of the differential metabolites. **(B)** Comparison of the relative abundance of indole in diabetic and CH-P treatment groups. **(C)** Comparison of the relative abundance of 5-hydroxyindole in diabetic and CH-P treatment groups. **(D)** Comparison of the relative abundance of 5-hydroxyindole-3-acetic acid in diabetic and CH-P treatment groups. **(E)** Correlation analysis of the differential metabolites using the POS model. *p<0.05, vs. Dia; **p<0.01, vs. Dia.

3.7. Effects of indole intervention and FMT on alleviating diabetes

Fecal microbiota transplantation experiments were performed by gavaging fecal suspensions from diabetic or CH-P-treated mice into reconstructed diabetic mice to further evaluate whether the gut microbiota and their metabolites exert hypoglycemic effects. Indole intervention was also performed to verify the effects of the differential metabolites. There was no significant difference in body weight; however, the water intake, urinary output, or blood glucose AUC of F-TG and I-TG mice significantly decreased compared to those of F-DG mice (Figures 9A,B,D,F,H). Moreover, they significantly improved insulin sensitivity compared to F-DG mice. However, indole treatment significantly reduced food intake and insulin concentration (Figures 9C,E). KEGG pathway analysis showed that the gut microorganisms that play a hypoglycemic role in the CH-P treatment group may produce metabolites through amino acid biosynthesis and metabolic pathways, especially tryptophan biosynthesis and metabolism (Figure 7A). Tryptophan is an essential aromatic amino acid that can be decomposed into indole and its derivatives, which have various beneficial effects on diabetes via gut microbiota (33). Interestingly, CH-P treatment significantly and positively correlated with the indole, 5-hydroxyindole, and 5-hydroxyindole-3-acetic acid metabolites. Indole modulates glucagon-like peptide-1 (GLP-1) to regulate postprandial glucose levels by stimulating insulin secretion and inhibiting glucagon secretion (34, 35). This study found that GLP-1 secretion significantly increased in I-TG mice compared to that in the F-DG group (Figure 9I). GLP-1 expression did not significantly increase in the F-TG mice; however, it showed an upward trend. This indicates that the polysaccharides from *C. cicadae* may alleviate hyperglycemia by regulating the production of metabolites other than indole and its derivatives by the gut microbiota.

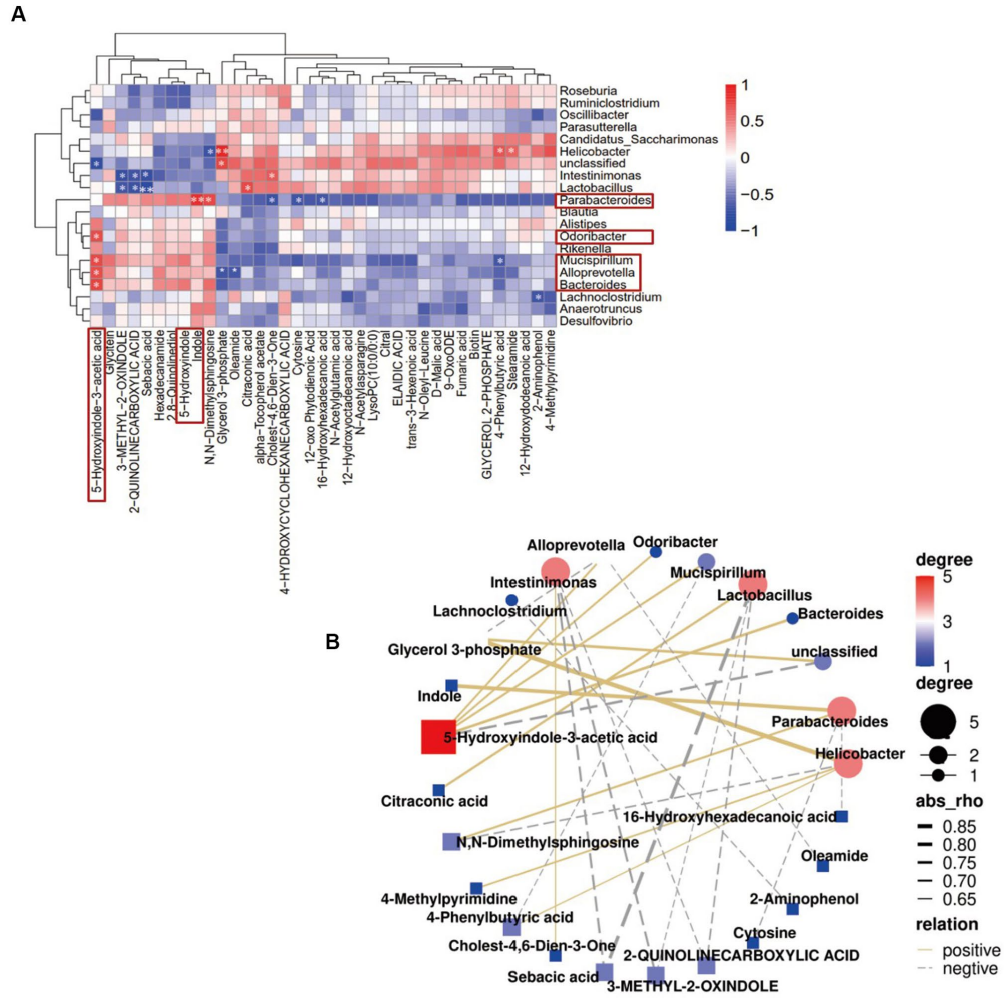

FIGURE 8
Correlation analysis of dominant gut microbiota genera with the differential metabolites. **(A)** Hierarchical clustering heatmap showing the correlation between dominant gut microbiota genera and the differential metabolites. The color intensity shows the degree of correlation (red and blue represents a positive and negative correlation, respectively). *p<0.05 and **p<0.01 indicated significant correlations. **(B)** Network map illustrating interactions among the dominant gut microbiota genera and the differential metabolites.

4. Discussion

Type 2 diabetes mellitus is a metabolic disease characterized by hyperglycemia resulting from insulin resistance or deficiency (8). Controlling blood glucose levels is an effective method to treat and prevent diabetes and various complications associated with diabetes. Naturally, active polysaccharides were proposed as alternative therapeutic agents for T2DM. Polysaccharides from *S. thunbergii* have many pharmacological effects, including antioxidant and hypoglycemic properties (36). *Ganoderma atrum* polysaccharides display hypoglycemic activity by reducing fasting blood glucose levels, increasing endothelium-dependent aortic relaxation, and improving PI3K, AKT, eNOS, and NO levels in diabetic rats (37). This study found that CH-P significantly reduced blood glucose levels, and increased insulin sensitivity in diabetic mice (Figures 1A–C). In addition, CH-P improved oral glucose tolerance in diabetic mice and alleviated hyperglycemia-induced tissue damage (Figures 1D, 2).

Dyslipidemia is a common feature of diabetes and manifests as elevated plasma levels of TC, TG, and LDL-C and/or decreased HDL-C levels (38). Current evidence supports the beneficial effects of polysaccharides on blood lipid levels in T2DM patients. Administration of *Acanthopanax senticosus* polysaccharides improves hyperglycemia by decreasing TC, TG, and LDL levels in alloxan-induced diabetic mice (39). Polysaccharide-simulated hydrolysates obtained from the dried fruiting body of *Auricularia auricular* significantly decrease serum TG and LDL-C levels in diabetic rats (40). Similarly, this study showed that CH-P significantly reduced TC, TG, and LDL-C levels and increased HDL-C levels in diabetic mice (Figures 1E–I). This indicated that CH-P could be used as an alternative therapeutic agent to alleviate cardiovascular complications.

Oxidative stress is implicated in the development of impaired glucose tolerance, insulin resistance, and islet cell dysfunction in T2DM (41). Many studies demonstrate that fungal polysaccharides have remarkable antioxidant effects. *Tricholoma mongolicum Imai* polysaccharides display strong antioxidant activity by scavenging DPPH and hydroxyl radicals (•OH) radicals *in vitro*. *Auricularia auricular* polysaccharides significantly protect against exhaustive swimming exercise-induced oxidative stress by regulating the levels

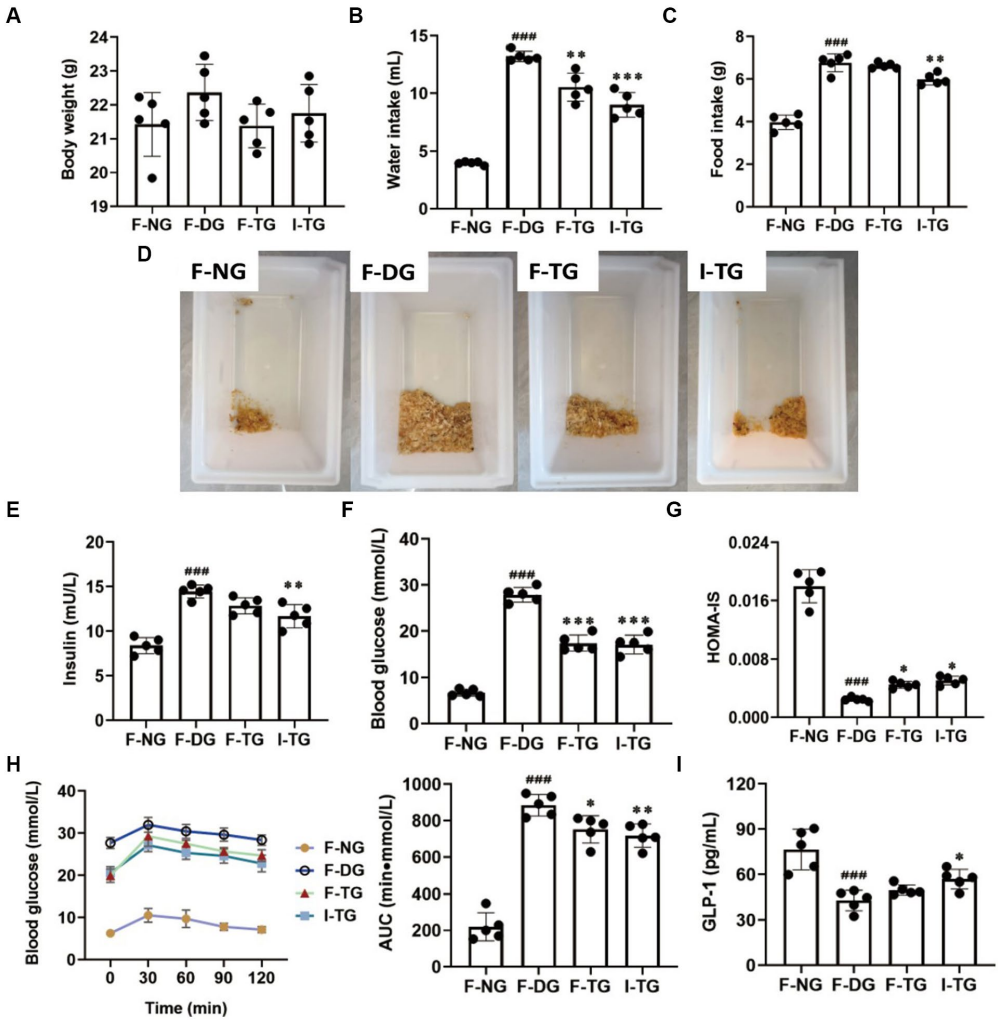

FIGURE 9
Effects analysis of indole intervention and FMT on alleviating diabetes. **(A)** Body weight. **(B)** Water intake. **(C)** Food intake. **(D)** Urination analysis. **(E)** Insulin. **(F)** Blood glucose. **(G)** HOMA-IS. **(H)** OGTT and AUC. **(I)** GLP-1. $^{###}p<0.001$, vs. F-NG; $^{*}p<0.05$, vs. F-DG; $^{**}p<0.01$, vs. F-DG; $^{***}p<0.001$, vs. F-DG.

of SOD, MDA, glutathione peroxidase, and catalase in mice (42). Moreover, polysaccharide from seeds of *Plantago asiatica* L. improved the antioxidant capacity by increasing SCFAs-producing strain (43). Evidence indicates gut microbiota enhanced the antioxidant capacity by increasing the high-antioxidant molecules, reactive sulfur species, such as hydrogen sulfide and cysteine persulfide (44). This study found that CH-P significantly reduced serum MDA content and increased SOD activity in diabetic mice (Figures 3A,B), indicated that CH-P may exert antioxidant effects f by regulating gut microbial composition.

Chronic inflammation significantly contributes to the development of T2DM by promoting insulin resistance and β-cell failure (45, 46). Adolescents with T2DM have significantly higher concentrations of hsCRP, TNF-α, and IL-1β inflammatory markers (47). Decreased insulin sensitivity in T2DM is related to inflammatory mediators such as TNF-α, IL-1β, IL-6, IL-8, and MCP-1 (48). Inflammatory markers are considered therapeutic parameters for the development of T2DM and its complications, and targeted modulation of the inflammatory system is proposed as a therapeutic strategy for T2DM (49). Fungal polysaccharides are currently regarded as a powerful active ingredient to regulate inflammation (50, 51). This study showed that CH-P significantly reduced the levels of TNF-α, IL-1β, and IL-6 in serum (Figures 3C–E). This shows that CH-P can alleviate diabetes by reducing inflammatory factors.

An aberrant gut microbiota composition is highly correlated with obesity and insulin resistance, which play important roles in the onset and development of T2DM (52). *Enterobacter cloacae* is the causative gut bacterium that induces obesity and insulin resistance in animals (53). The percentage of butyrate-producing bacteria (such as *Roseburia* and *Faecalibacterium prauznitzii*) is significantly reduced in the gut microbiota of patients with T2DM (54). However, there are increased levels of *Lactobacillus* and some opportunistic pathogens such as *Desulfovibrio* sp. and *Clostridium* (55, 56). Therefore, regulating the composition of intestinal microorganisms may be a new strategy to improve T2DM. *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* are the three major phyla responsible for degrading complex nondigestible polysaccharides (57). *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and

Desulfobacterota phyla were the dominant bacterial communities in the CH-P treatment group and the diabetic model group (Figure 4C). CH-P treatment significantly reduced the *Firmicutes*/*Bacteroidetes* ratio (Figure 4D). This is consistent with previous studies (58, 59) and indicated that CH-P regulated the abundance and composition of intestinal microorganisms to improve diabetes. The decreased abundance in *Firmicutes* was primarily related to a decrease in *Clostridiales*, *Lachnospiraceae*, *Ruminococcus*, and *Streptococcus hyointestinalis* in CH-P-treated mice. Meanwhile, the increased abundance of *Bacteroidetes* was mainly attributed to an increase in *Parabacteroides* and *Bacteroides vulgatus*. Moreover, the CH-P treatment showed desirable effects in restoring other key bacterial species in diabetic mice including *Mucispirillum* and *Marvinbryantia* (Figure 5). In addition, CH-P treatment significantly increased the abundance of *Bacteroides*, *Odoribacter*, *Alloprevotella*, and *Parabacteroides* genera and significantly decreased the abundance of *Helicobacter* and *Lactobacillus* (Figure 4E). *Bacteroides* is a common SCFA-producing gut microbiota that benefits humans by maintaining an anaerobic intestinal luminal environment (60). An increased abundance of SCFA-producing- and anti-inflammatory bacterium *Alloprevotella* improves the symptoms of type 2 diabetic rats (61). *Parabacteroides distasonis* alleviates obesity and metabolic dysfunction by producing succinate and secondary bile acids (62). Intermittent fasting increases the levels of butyrate-producing *Odoribacter* to alleviate diabetes-induced cognitive impairment (63). These results indicate that CH-P can improve diabetes by changing the abundance and composition of intestinal microorganisms. However, the function and mechanism of these bacteria require further study.

Disease states can be ameliorated or prevented by reshaping the gut microbiota. In other words, inoculation of the host with intestinal microbiota can lead to durable metabolic changes with therapeutic utility (64). Many interventional studies show that the intake of intestinal probiotics improves intestinal microecological disorders and relieves the symptoms of patients with diabetes. Administration of polysaccharides from *Enteromorpha prolifera* ameliorates HFD-induced gut dysbiosis by modulating the composition of gut microbiota, such as *Bacteroides*, *Parabacteroides*, *Alloprevotella*, *Ruminococcus*, and gut barrier-protective *Akkermansia muciniphila*, which resulted in an enrichment of the related metabolites of SCFA, and a reduction in circulating lipopolysaccharide (LPS) levels (65). *Parabacteroides distasonis* alleviates obesity and metabolic dysfunction by producing succinate and secondary bile acids. Carnosic acid exerts anti-inflammatory effects against colitis by altering the gut microbiota and correlated metabolites (66). Specific gut microbes can transform some amino acids into various bioactive metabolites (29). This study showed that the alterations in the metabolic pathways were mostly related to amino acid biosynthesis and metabolic pathways, particularly those involving tryptophan. Tryptophan metabolism plays a role in the gastrointestinal tract through three major pathways: (1) Tryptophan is directly decomposed by microorganisms into small molecule ligands that can bind to aryl hydrocarbon receptors or other receptors; (2) Kynurenine pathway; and (3) Serotonin pathway (33). Tryptophan can be metabolized to indole by several bacterial species, such as *Bacteroides thetaiotaomicron*, *B. ovatus*, *Clostridium limosum*, and *C. bifermentans* (32). Indole, as an interspecies signaling molecule, plays important modulate roles in bacterial pathogenesis and eukaryotic immunity, such as antibiotic resistance, oxidative stress, intestinal inflammation, and hormone secretion (67). Moreover, indole and its derivatives such as indole-3-propionic acid (IPA) have various beneficial effects on diabetes. Indole modulates the secretion of glucagon-like peptide-1 (GLP-1) from intestinal enteroendocrine L-cells (34). The main role of GLP-1 is to stimulate insulin secretion and inhibit glucagon secretion, thereby improving postprandial glucose fluctuations (35). This study showed that CH-P treatment significantly increased the metabolites of indole, 5-hydroxyindole and 5-hydroxyindole-3-acetic acid (Figures 6D, 7). Spearman's correlation analysis showed that *Bacteroides*, *Odoribacter*, *Alloprevotella*, and *Mucispirillum* were positively correlated with 5-hydroxyindole-3-acetic acid, while *Parabacteroides* was positively correlated with indole and N, N-dimethylsphingosine. Indole is an important component involved in tryptophan metabolism and phenylalanine, tyrosine, and tryptophan biosynthesis, while 5-hydroxyindole-3-acetic acid is involved in tryptophan metabolism (Figure 8). Together, the results showed that CH-P treatment can improve diabetes by affecting the abundance of gut microorganisms such as *Bacteroides*, *Odoribacter*, *Alloprevotella*, *Paraberoides*, and *Mucispirillum*. The gut microbiota can produce metabolites such as indole and 5-hydroxyindole-3-acetic acid, and modulated GLP-1 secretion to improve diabetes. Therefore, this study verified the hypoglycemic effect of indole. Indole significantly improved the hyperglycemic symptoms and insulin sensitivity of mice, including decreased water intake, food intake, urinary output, insulin level, blood glucose, and glucose tolerance compared to F-DG (Figures 9B–H). More importantly, indole significantly increased GLP-1 secretion (Figure 9I). This confirmed that gut microbiota produces metabolites such as indole and its derivatives and modulates the secretion of GLP-1 to alleviate the effects of diabetes.

Fecal microbiota transplantation improves insulin resistance and pancreatic islet β-cell function by reconstructing the gut microbiota (68). Moreover, it alters the susceptibility of obese rats to T2DM (69). This study used FMT to reconstruct the gut microbiota in high-fat diet/STZ-induced diabetic mice. FMT successfully reduced blood glucose levels and improved glucose tolerance in diabetic mice (Figures 9F,H). The insulin sensitivity in the F-TG group increased after T2DM mice reconstructed their microbiota by administering the feces of CH-P-treated mice (Figure 9G). Similarly, a clinical trial showed that obese patients receiving FMT from lean, healthy individuals showed a positive change in insulin sensitivity (70). However, FMT did not significantly improve GLP-1 levels (Figure 9I). This indicates that *C. cicadae* polysaccharides alleviated hyperglycemia by regulating the production of metabolites other than indole and its derivatives by gut microbiota. However, CH-P treatment increased the levels of SCFA-producing bacteria (*Bacteroides*, *Odoribacter*, *Alloprevotella*, and *Parabacteroides*). Further investigation is required to determine whether CH-P plays a therapeutic role in diabetes through the production of SCFA. In addition, this work did not analyze the structural characteristics of CH-P, which requires further in-depth explore in the future.

In addition to a variety of pharmacological activities, *C. cicadae* also have the advantages of low toxicity, low price and easy artificial cultivation. At present, a wide variety of functional products and health care products are produced from *C. cicadae*, covering health food, cosmetics, biological agriculture, and pharmaceutical fields (71). However, researches on *C. cicadae* are limited to preliminary

pharmacochemical and pharmacological studies. Therefore, the further exploration of *C. cicadae* polysaccharide in this study is expected to expand its application scope and better play its health care effect.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found at: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA954535.

Ethics statement

All animal experimental protocols were reviewed and approved by the Experimental Animal Ethics Committee of Xuzhou Medical University (approval number: L20210226457).

Author contributions

AC contributed to conception and design of the study. YW, ZN, JL, YS, YY, and WL performed the experiments. SZ and TF performed the data analysis. ZN wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1203430/full#supplementary-material

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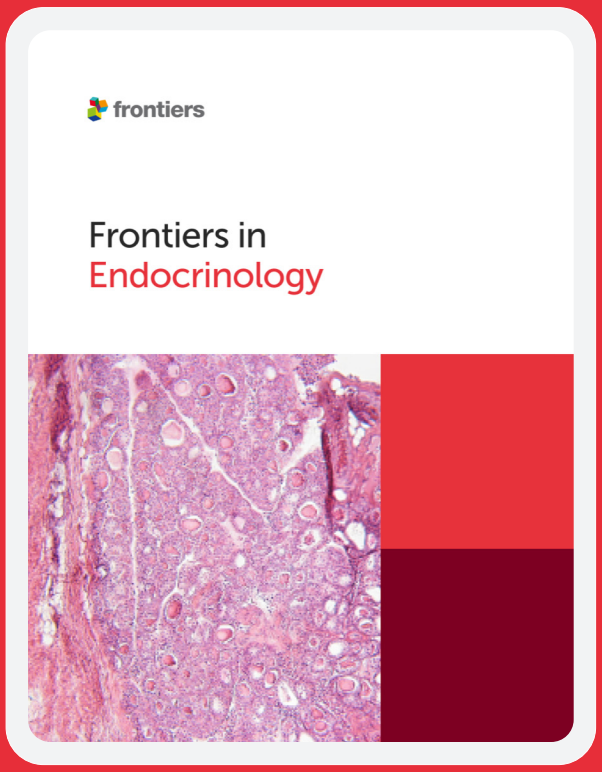

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