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Gastrointestinal damage and metabolic disorders

Edited by
Daniele Maria-Ferreira, Marcelo Biondaro Gois and Elizabeth S. Fernandes

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Gastrointestinal damage and metabolic disorders

Topic editors

Daniele Maria-Ferreira — Instituto de Pesquisa Pelé Pequeno Príncipe, Brazil

Marcelo Biondaro Gois — Federal University of Rondonópolis, Brazil

Elizabeth S. Fernandes — Aarhus University, Denmark

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EDITED AND REVIEWED BY
Stephen J. Pandol,
Cedars Sinai Medical Center, United States

*CORRESPONDENCE
Daniele Maria-Ferreira,
daniele.ferreira@pelepequenoprincipe.org.br,
danielemariaferreira@gmail.com

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Editorial: Gastrointestinal damage and metabolic disorders

Carolina Silva Schiebel[1,2], Samilla Santos Souza Mazeti[3], Marcelo Biondaro Gois[3] and Daniele Maria-Ferreira[1,2]*

[1]Instituto de Pesquisa Pelé Pequeno Príncipe, Faculdades Pequeno Príncipe, Curitiba, Brazil, [2]Programa de Pós-graduação em Biotecnologia Aplicada à Saúde da Criança e do Adolescente, Faculdades Pequeno Príncipe, Curitiba, Brazil, [3]Faculdade de Ciências da Saúde, Universidade Federal de Rondonópolis, Rondonópolis, Brazil

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Editorial on the Research Topic
Gastrointestinal damage and metabolic disorders

Gastrointestinal diseases are characterized by physiological and/or morphological changes in the gastrointestinal tract. These include impaired motility and integrity of the mucosa, dysregulated immune responses and an imbalance of the intestinal microbiota. Regardless of the specific underlying disease, these disorders can lead to significant clinical symptoms and contribute to the development of secondary pathologies. Ten articles have been published, which have been accessed more than 20,000 times.

In a review, Nie et al. emphasize the dysfunction of the gut-organ axis as a key factor in the impairment of the intestinal barrier. Extraintestinal organ dysfunction can exacerbate intestinal permeability through inflammatory responses, metabolic changes and altered intestinal perfusion, creating a feedback loop that exacerbates intestinal and systemic damage. Early intestinal barrier dysfunction may serve as a biomarker for extraintestinal disease and aid prognosis and timely intervention. They also summarized the key molecular mechanisms, including the role of TLR4/NF-κB/MAPK in inflammation, ApoM/S1P in vascular barrier dysfunction, WNT/β-catenin in stem cell regulation, and PI3K/Akt/mTOR in autophagy and protein synthesis. Finally, they discussed how microgravity increases intestinal permeability and alters tight junctions, microbiota composition, immune responses, and secretion of protective factors such as mucin and SIgA, suggesting a significant impact on intestinal homeostasis in the space environment.

Zhang et al. performed the first scientometric analysis of refractory gastroesophageal reflux disease. Using bibliometric methods, they identified key research trends focusing on standardized diagnostic and therapeutic approaches, underlying mechanisms, new surveillance techniques, and emerging pharmacological and procedural innovations. Their analysis highlighted neuroimmune interaction as a potentially crucial factor in the pathogenesis of gastroesophageal reflux disease, pointing to a promising direction for future mechanistic studies.

In a literature review, Yang et al. emphasized the therapeutic potential of Sijunzi decoction in the treatment of various gastrointestinal diseases. Clinical

studies indicate its efficacy in functional dyspepsia, chronic gastritis, gastric cancer, irritable bowel syndrome, colon cancer and ulcerative colitis. These protective effects are associated with the regulation of gut microbiota, the attenuation of inflammation, the modulation of immune responses and the promotion of mucosal repair. However, the exact mechanisms underlying its effects are not yet fully understood.

Yeung conducted a literature review to track the development of infliximab use in Crohn's disease. He identified key milestones, including the introduction of the CDAI in 1976, early evidence of infliximab efficacy in 1995, the first randomized trial in 1997, the ACCENT I and II trials, and the European consensus guidelines. He concluded that these studies provide a solid basis for further research.

Bi et al. investigated the role of miRNA dysregulation in chronic atrophic gastritis and found significant upregulation of miR-3613-5p in gastric mucosa and serum of gastric cancer patients as well as in mucosal tissue of patients with chronic atrophic gastritis, tumor samples and gastric cancer cell lines (via GEO database analysis). In mouse models, the expression of miR-3613-5p was also increased in the gastric mucosa. Functionally, its overexpression promoted gastric cancer cell proliferation and migration *in vitro*, while silencing attenuated gastric mucosal pathology. Mechanistically, miR-3613-5p directly interferes with the 3′UTR of AQP4 and suppresses its expression. The authors concluded that miR-3613-5p contributes to the progression of chronic atrophic gastritis to gastric cancer by downregulating AQP4 and could serve as a biomarker and therapeutic target.

Using *in vivo* (mouse model of intestinal I/R injury, including C57BL/6J and vitamin D receptor knockout mice) and *in vitro* approaches (IEC-6 cells with VDR and ATF4 knockdown), Zhang et al. demonstrated that paricalcitol is a potential therapeutic agent for intestinal I/R injury. The authors showed that paricalcitol activated VDR and suppressed the ATF4–CHOP signaling pathway, thereby reducing endoplasmic reticulum stress and apoptosis and attenuating I/R-induced intestinal injury *in vivo* and H/R-induced injury *in vitro*. Moreover, VDR deficiency exacerbated I/R injury, highlighting its important protective role for intestinal epithelial cells.

Canová et al. investigated the effects of celastrol on the galaninergic system in the heart and liver of male C57BL/6J mice with diet-induced obesity and MASLD/MASH. Using a high-fat Western diet model, they showed that Celastrol was safe and effectively reduced food and energy intake, body fat, liver weight and progression from MASLD to MASH. In addition, celastrol had a positive effect on the galaninergic system, suggesting its potential as a therapeutic option for obesity and related metabolic disorders.

Guo et al. investigated the association between glucagon-like peptide-1 receptor agonists (GLP-1 RAs) and acute pancreatitis (AP) using data from the FDA Adverse Event Reporting System (FAERS) and a case series of 39 patients. Analysis of reports from January 2005 through September 2023 identified a total of 6,751 cases of suspected AP associated with GLP-1 RAs, with an average patient age of 57 years and 98.3% classified as severe. Signal detection analysis (ROR, PRR, BCPNN, MGPS) revealed a positive association between AP and all GLP-1 RAs examined, with stronger signals for exenatide and liraglutide. These results suggest that clinical vigilance is required when prescribing GLP-1 RAs given the potential risk of this serious side effect.

Sun et al. investigated the role of carbamoyl phosphate synthetase 1 (CPS1), a rate-limiting enzyme in the urea cycle, in the development *versus* persistence of glucagon-induced hyperglycemia using *in vivo*, *in vitro* and *in silico* approaches. CPS1 played a key role in mediating glucagon-induced hepatic gluconeogenesis. In addition, they showed that cynarin could be a natural inhibitor of CPS1 and has the potential of a therapeutic agent for the treatment of diabetes.

Finally, Łukawska and Mulak investigated the relationship between FGF21 serum levels, inflammatory markers and indicators of nutritional status in patients with inflammatory bowel disease (IBD). The severity of intestinal inflammation is associated with elevated FGF21 levels, which correlate negatively with indicators of nutritional status. These results suggest that dysregulation of FGF21 secretion may contribute to the multifactorial pathogenesis of malnutrition and weight loss in IBD patients.

This Research Topic has produced new and relevant findings that significantly advance current understanding in this field. We would like to thank all authors, reviewers and editors for their valuable contributions to the development and dissemination of this Research Topic.

Author contributions

CS: Writing – original draft, Writing – review and editing, Conceptualization. SS: Writing – review and editing, Writing – original draft. MG: Conceptualization, Writing – original draft, Writing – review and editing. DM-F: Writing – original draft, Writing – review and editing.

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Conflict of interest

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

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EDITED BY
Daniele Maria-Ferreira,
Instituto de Pesquisa Pelé Pequeno Príncipe, Brazil

REVIEWED BY
Beata Kasztelan-Szczerbinska,
Medical University of Lublin, Poland
Sarbjeet Makkar,
Washington University in St. Louis, United States

*CORRESPONDENCE
Agata Łukawska,
agata.lukawska55@gmail.com

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A correlation of serum fibroblast growth factor 21 level with inflammatory markers and indicators of nutritional status in patients with inflammatory bowel disease

Agata Łukawska* and Agata Mulak

Department of Gastroenterology and Hepatology, Wroclaw Medical University, Wroclaw, Poland

Background: Fibroblast growth factor 21 (FGF21) is a stress-inducible hormone that regulates nutrient and metabolic homeostasis. Inflammatory state is one of the stimulators of FGF21 secretion. The aim of the study was to assess correlations between serum FGF21 level and inflammatory markers as well as nutritional status indicators in patients with inflammatory bowel disease (IBD).

Methods: Fasting serum FGF21 level was measured using ELISA test in 105 IBD patients and 17 healthy controls. There were 31 subjects with active ulcerative colitis (UC), 16 with inactive UC, 36 with active Crohn's disease (CD), and 22 with inactive CD. Clinical and endoscopic activity of IBD was evaluated based on validated scales and indices. Fecal calprotectin, serum CRP, and selected parameters of nutritional status were tested in all patients.

Results: Serum FGF21 level was characterized by fluctuations depending on the IBD activity. FGF21 level was significantly higher in both active UC and CD compared to inactive phases of the diseases and to the controls. A correlation between FGF21 and fecal calprotectin levels was also found in UC and CD. Additionally, in CD, FGF21 level positively correlated with CRP level. In both UC and CD, a negative correlation was noted between FGF21 level and nutritional status parameters including cholesterol, protein, albumin levels, and BMI.

Conclusion: The intensity of intestinal inflammation is related to FGF21 level, which correlates negatively with nutritional status indicators in IBD. The disturbances in FGF21 secretion may contribute to the multifactorial pathogenesis of malnutrition and weight loss in IBD patients.

KEYWORDS

inflammatory bowel disease, fibroblast growth factors, disease activity, fecal calprotectin, malnutrition

1 Introduction

The family of fibroblast growth factors (FGF) consists of 22 structurally related peptides with a diverse range of cellular functions (Beenken and Mohammadi, 2009; Degirolamo et al., 2016). Among them, there is a group of three factors with endocrine properties – FGF19, FGF21, and FGF23 (Łukawska and Mulak, 2022). These endocrine

FGFs are released into the bloodstream and exert their effects on distant tissues regulating multiple metabolic processes (Beenken and Mohammadi, 2009; Degirolamo et al., 2016; Łukawska and Mulak, 2022). The function of endocrine FGFs depends on the presence of their receptors and co-receptors α-Klotho or β-Klotho. The co-receptors expression in target organs determines the tissue-specific action of the FGFs (Degirolamo et al., 2016).

FGF21 is a protein produced mainly in the liver, adipose tissue, muscles, and the pancreas. FGF21 requires the presence of β-Klotho to activate appropriate receptors in target tissues. FGF21 is involved in the metabolism of lipids, carbohydrates, and proteins. It also participates in energy expenditure and body weight regulation (Dolegowska et al., 2019). The main inducers of the FGF21 expression include fasting state, overfeeding, inflammation, and physical activity (Martínez-Garza et al., 2019). The mode of FGF21 action is considered not only endocrine but also paracrine and autocrine (Martínez-Garza et al., 2019). One of the main target organs of FGF21 is white adipose tissue (WAT). FGF21 can both inhibit and stimulate lipolysis in WAT, after meals and during fasting, respectively (Xie et al., 2020). Additionally, during fasting, FGF21 stimulates gluconeogenesis, ketogenesis, and fatty acid oxidation (Gadaleta et al., 2011). Interestingly, FGF21 may cross the blood-brain barrier and exert effect at the central nervous system related to glucose homeostasis and body weight regulation (Lan et al., 2017). Moreover, FGF21 induces the production of corticotropin-releasing hormone activating the hypothalamic-pituitary-adrenal axis and increasing gluconeogenesis in the liver during prolonged starvation (BonDurant and Potthoff, 2018). The results of previous experimental and clinical studies have shown that inflammatory stimuli may also induce the FGF21 expression (Feingold et al., 2012; Gariani et al., 2013).

Inflammatory bowel disease (IBD) is a chronic recurrent immune-mediated disorder of the gastrointestinal tract that encompass ulcerative colitis (UC) and Crohn's disease (CD) (Zhang and Li, 2014). IBD is characterized by a wide spectrum of intestinal and extra-intestinal symptoms as well as systemic complications. The disease progresses with periods of flares and remissions (Torres et al., 2017; Ungaro et al., 2017). Due to intestinal inflammation, diarrhea, malabsorption, dietary limitations, and anorexia, patients with IBD are at increased risk of malnutrition, the prevalence of which among that group of patients ranges from 20% to 85% (Balestrieri et al., 2020).

The results of previous studies in animal models have suggested that FGF21 as a metabolic regulator, secreted during inflammation, may take part in the pathogenesis of IBD (Liu et al., 2017; Liu et al., 2023). It has been shown that dextran sulfate sodium-induced colitis resulted in increased expression of FGF21, while the absence of FGF21 alleviated colitis symptoms, reduced adipose tissue lipolysis and prevented weight loss (Liu et al., 2017; Liu et al., 2023). Furthermore, also in IBD patients the acute phase of the disease was found to be associated with a significant increase in serum FGF21 level (Tomasik et al., 2010; Liu et al., 2017). While low body weight in IBD has multifactorial pathogenesis, it may be hypothesized that higher FGF21 level could contribute to the state of malnutrition. Colitis-induced FGF21 expression may subsequently activate lipolysis in WAT and weight loss (Liu et al., 2017). However, the pathophysiological link between IBD and FGF21 remains to be unraveled.

The aim of the current study was to assess the correlation between serum FGF21 level and inflammatory markers such as CRP and fecal calprotectin as well as indicators of nutritional status in patients with IBD.

2 Materials and methods

2.1 Study design

This cross-sectional study was performed at the Department of Gastroenterology and Hepatology of Wroclaw Medical University (Poland) between January 2021 and March 2023. All enrolled patients underwent a detailed clinical interview to assess their symptoms, associated disorders, and current treatment. The patients underwent also routine diagnostic assessments, including a physical examination and fasting blood laboratory tests. Additionally, they provided stool samples to determine calprotectin content. In patients with clinical indications, colonoscopy and/or enterography were also carried out.

Clinical and endoscopic activity of IBD was evaluated based on validated scales and indices. The Rachmilewitz index (Rachmilewitz, 1989) and the Mayo Endoscopic Score (Rubin et al., 2019) were applied to UC patients. The Crohn's Disease Activity Index (CDAI) (Freeman, 2008) and the Simple Endoscopic Score for CD (SES-CD) (Daperno et al., 2004) were used in CD patients. Patients were categorized as being in either active or inactive phase of the disease based on the assessment of clinical, laboratory, and endoscopic criteria. Patients who fulfilled the criteria outlined as fecal calprotectin level lower than 200 μg/g, 0–4 points in the Rachmilewitz index and 0–2 points in the Mayo Endoscopic Score (for UC patients), the CDAI score lower than 200 points, and the SES-CD score lower than 7 points (for CD patients) were categorized as being in remission. All other subjects were assigned to the active phase group. CD patients with active changes in enterography were automatically included in the active phase group.

2.2 Subjects

Among 105 IBD patients there were 31 patients with active UC, 16 patients with inactive UC, 36 patients with active CD, and 22 patients with inactive CD. The control group consisted of 17 healthy volunteers without gastroenterological symptoms. To exclude undiagnosed IBD or other intestinal inflammation, fecal calprotectin test was performed in all controls.

The exclusion criteria were as follows: pancreatitis, chronic liver diseases (except single cysts and steatosis), diabetes, body mass index (BMI) $\geq$ 30 kg/m^2, treated hyperlipidemia, ischemic heart disease, chronic kidney diseases (except single cysts and kidney stones), malignancies, alcohol dependence syndrome, history of abdominal surgical procedures (except appendectomy and procedures related to IBD).

TABLE 1 Detailed characteristics of the studied patient groups.

	Active UC (Group 1)	Inactive UC (Group 2)	Active CD (Group 3)	Inactive CD (Group 4)	*p* 1 vs. 2	*p* 3 vs. 4
Group characteristics						
n	31	16	36	22		
Men, n (%)	23 (74.2)	9 (56.3)	23 (63.9)	13 (59.1)	0.211	0.715
Age, median (Q1-Q3)	36 (26–40)	33.5 (24–45.5)	31 (27–40.5)	33 (28–45)	0.955	0.386
Duration of the disease (months), median (Q1-Q3)	18 (1.5–60)	58 (13–108)	90 (20.5–162)	120 (84–192)	0.099	0.161
BMI, mean ± SD, kg/m^2	21.8 ± 3.6	22.2 ± 4.2	21.9 ± 4	22.9 ± 2.9	0.704	0.285
IBD activity						
Rachmilewitz index median (Q1-Q3)	9 (5–12)	1.5 (0–4)	–	–	<0.001	–
Mayo Endoscopic Score median (Q1-Q3)	3 (2–3)	0 (0–1)	–	–	<0.001	–
CDAI median (Q1-Q3)	–	–	266.6 (179.3–385.5)	77.9 (33.5–113.9)	–	<0.001
SES-CD mean ± SD	–	–	7.7 ± 4.8	4.2 ± 3.0	–	0.008
Treatment						
Mesalamine, n (%)	31 (100)	15 (93.8)	23 (63.9)	12 (54.5)	0.340	0.480
Steroids, n (%)	23 (77.4)	3 (18.8)	17 (47.2)	3 (13.6)	<0.001	0.008
Azathioprine, n (%)	9 (29.0)	3 (18.8)	13 (36.1)	5 (22.7)	0.505	0.285
Biological treatment, n (%)	1 (3.2)	2 (12.5)	2 (5.6)	3 (13.6)	0.264	0.357
Antibiotics, n (%)	8 (25.8)	3 (18.8)	12 (36.3)	1 (4.5)	0.725	0.009
Probiotics, n (%)	6 (19.4)	2 (12.5)	7 (19.4)	4 (18.2)	0.697	1.000

UC, ulcerative colitis; CD, Crohn's disease, BMI, body mass index; CDAI, Crohn's Disease Activity Index, SES-CD, Simple Endoscopic Score for Crohn's disease. Variables are presented as number (n) with percentage (%), mean values with standard deviation (±SD), or medians with the lower and upper quartiles (Q1-Q3).

2.3 Quantitative evaluation of serum FGF21 and fecal calprotectin levels

The fasting blood and stool samples provided by participants were stored at −80°C until the analysis. The quantitative evaluation of serum FGF21 [pg/ml] and fecal calprotectin [μg/g] levels were performed by immunoenzymatic methods: Human FGF-21 ELISA (BioVendor, Laboratorni medicina a.s., Czech Republic) and EK-CAL (Bühlmann Laboratories, Switzerland), respectively.

2.4 Statistical analysis

The obtained individual results are presented as numbers, percentage, mean values with standard deviation (±SD), or medians with the lower and upper quartiles (Q1-Q3). The normality of data was determined using the Shapiro-Wilk test. To compare quantitative variables with a normal distribution we assessed the homogeneity of variances using the Levene test, and then conducted a Student's t-test (no significant variance difference) or a *t*-test with independent variances (significant variance heterogeneity), respectively. The Mann-Whitney U test compared quantitative variables with abnormal distribution. To compare categorical variables, the assumption of expected frequencies was checked – values less than 5 in a maximum of 20% of cell fields for the chi-square test, and proceed with Pearson's chi-square test of independence or the Fisher exact test, respectively. The Spearman rank correlation coefficient and Kendall Tau correlations were calculated to test associations between variables. The statistical significance level was set at $p < 0.05$.

3 Results

3.1 Group characteristics

The characteristics of the studied groups of patients are presented in Table 1. The whole group of IBD patients included 68 males (65%) and 37 females (35%), at median age of 33 (27–41) years. There were no significant differences between the patient groups with respect to gender and age. The median duration of the disease was 63 (13–132) months. Of note, subjects with active UC were characterized by the shortest median disease duration amounting to 18 months and 39% of those patients were diagnosed with IBD within 1 year preceding the study. The control group (n = 17) included 9 males and 8 females, at median age of 28 (27–30) years and mean BMI of 22.4 ± 2.8 kg/m^2.

TABLE 2 Laboratory test results in the groups of studied patients.

	Active UC (Group 1)	Inactive UC (Group 2)	Active CD (Group 3)	Inactive CD (Group 4)	*p* 1 vs. 2	*p* 3 vs. 4
Fecal calprotectin [μg/mL], mean ± SD	1528.7 ± 673.5	78.9 ± 61.2	1266.7 ± 733.9	74.9 ± 41.9	<0.001	<0.001
CRP [mg/L], median (Q1-Q3)	16.6 (5.2–46.8)	1.8 (0.9–6.9)	7.9 (4.3–24.6)	2.1 (1.2–7.0)	<0.001	0.001
Total cholesterol [mg/dL], mean ± SD	143.5 ± 39.3	194.1 ± 34.2	148.1 ± 37.3	160.9 ± 54.9	<0.001	0.046
LDL [mg/dL], mean ± SD	85.1 ± 27.7	115.4 ± 36.3	79.1 ± 30.6	97.5 ± 35.9	0.002	0.042
HDL [mg/dL], mean ± SD	41.3 ± 13.9	53.3 ± 15.0	49.2 ± 13.7	55.0 ± 15.4	0.009	0.142
Triglycerides [mg/dL], median (Q1-Q3)	104.5 (67–125)	88.5 (65–122)	91.0 (69–108)	102.5 (72–122)	0.308	0.380
Total protein [g/dL], mean ± SD	6.1 ± 0.9	7.2 ± 0.7	6.7 ± 0.7	7.12 ± 0.4	<0.001	0.022
Albumin [mg/dL], mean ± SD	3.5 ± 0.6	4.3 ± 0.5	3.8 ± 0.5	4.2 ± 0.4	<0.001	0.003
Hemoglobin [g/dL], mean ± SD	11.6 ± 1.6	14.1 ± 1.9	12.3 ± 2.3	13.6 ± 2.1	<0.001	0.035
Iron [μg/dL], median (Q1-Q3)	31 (17–50)	85 (68–114)	36 (17–84)	70 (25–96)	<0.001	0.194
Ferritin [μg/L], median (Q1-Q3)	54.6 (10.6–249.8)	90.9 (51.2–182.5)	46.8 (20.9–158.8)	23.7 (15.5–67.9)	0.541	0.067
Transferrin [g/L], median (Q1-Q3)	2.4 (1.6–2.7)	2.4 (2.2–2.7)	2.3 (2.0–2.8)	2.6 (2.5–2.9)	0.189	0.011
Vitamin D [ng/mL], median (Q1-Q3)	20.7 (10.0–26.2)	27.9 (17.3–42.0)	24.8 (16.9–33.3)	29.3 (15.5–33.5)	0.103	0.684

UC, ulcerative colitis; CD, Crohn's disease, LDL, low-density lipoprotein; HDL, high density lipoprotein.
Variables are presented as mean values with standard deviation (SD), or medians with the lower and upper quartiles (Q1-Q3).

The results of the evaluation of clinical and endoscopic IBD activity based on the applied scales and indices are presented in Table 1.

In most cases, the patients in an active phase of IBD were administered steroids. Three patients with inactive UC and 3 with inactive CD were still on steroids while tapering their dose. They were admitted to the hospital for check-ups after an exacerbation of the disease which had occurred 2–3 months earlier.

The results of blood and stool tests in IBD patients including the evaluation of serum CRP and fecal calprotectin are presented in Table 2. Mean fecal calprotectin in the control group amounted to 15.3 ± 10.3 μg/g.

3.2 Serum FGF21 level

Analyzing the serum FGF21 level in IBD patients, a clear tendency for higher values in active IBD phase was observed. The median serum FGF21 level was higher in active UC than in inactive UC, as well as in the control group. However, there was no significant difference in FGF21 level between patients with inactive UC and the controls. The same fluctuation depending on disease activity was observed in the group of CD patients (Figure 1). An elevated concentration of FGF21 (>300 pg/mL) was detected in 39 patients that accounted for 37% of all IBD patients. Most of those subjects (32/39, 82%) were in the active phase. The increased FGF21 level was found in 48% of patients with active UC and 47% of patients with active CD. FGF21 values below the normal range were not detected in any of the studied IBD patients.

3.3 Nutrient deficiencies in IBD patients

Evaluating the nutritional status, underweight (BMI <18.5 kg/m^2) was diagnosed in 17% of the IBD patients. Weight loss exceeding 5% of body mass within 1 month was documented in 21% of the subjects. Selected nutrient deficiencies detected in the studied patients are presented in Table 3. Almost 30% of subjects with active UC and active CD had low cholesterol level. Almost 40% of patients with active UC and 17% of patients with active CD had hypoproteinemia. More than 50% of patients with active UC and almost 30% of patients with active CD had hypoalbuminemia. Iron deficiency and anemia was also commonly observed. Hemoglobin levels lower than 11.9 g/dL in women and lower than 12.9 g/dL in men were reported in 81% of patients with active UC, 25% with inactive UC, 44% with active CD, and 32% with inactive CD. Vitamin D deficiency was confirmed in 64% of the patients, with a deep deficiency (<10 ng/mL) identified in 14% of them. Nutritional deficiencies were more often observed in patients with active UC than those with inactive UC, except for low vitamin D levels. In contrast, these deficiencies occurred at similar rates for patients with CD regardless of whether the disease was active or inactive, except for low albumin levels, which were more prevalent in active CD.

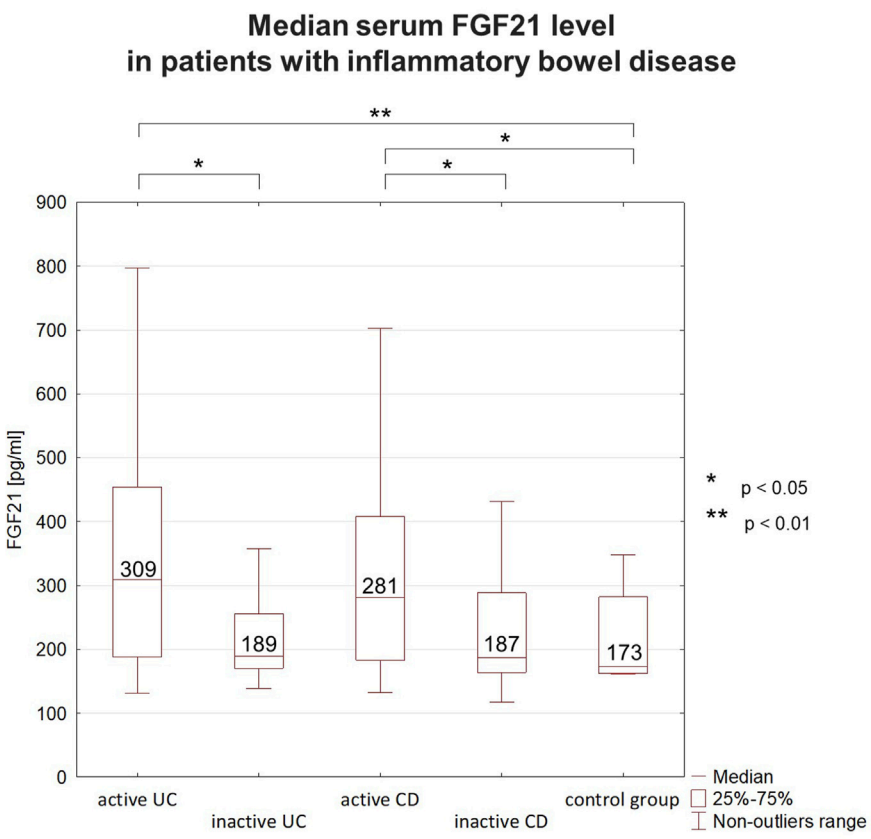

FIGURE 1
Median serum FGF21 levels in patients with inflammatory bowel disease (IBD). In IBD patients FGF21 level fluctuated depending on the disease activity. Higher concentrations were observed during flares than during remission. FGF21 concentrations during remission were comparable to those in the control group. FGF21 - fibroblast growth factor 21, UC–ulcerative colitis, CD–Crohn's disease.

TABLE 3 Nutrient deficiencies in the studied IBD patients.

	Active UC (Group 1)	Inactive UC (Group 2)	Active CD (Group 3)	Inactive CD (Group 4)	*p* 1 vs. 2	*p* 3 vs. 4
n	31	16	36	22	-	-
Hypocholesterolemia, n (%)	9 (29.0)	0 (0.0)	10 (27.8)	3 (13.6)	0.019	0.332
Hypoproteinemia, n (%)	12 (38.7)	1 (6.3)	6 (16.7)	0 (0.0)	0.036	0.073
Hypoalbuminemia, n (%)	16 (51.6)	1 (6.3)	10 (27.8)	1 (4.6)	0.003	0.039
Iron deficiency, n (%)	19 (61.3)	3 (18.8)	23 (63.9)	12 (54.6)	0.007	0.480
Vitamin D deficiency, n (%)	24 (77.4)	8 (50.0)	24 (66.7)	11 (50.0)	0.056	0.208

Hypocholesterolemia (total cholesterol level <125 mg/dL), hypoproteinemia (total protein level <6 g/dL), hypoalbuminemia (albumin level <3.5 mg/dL), iron deficiency (ferritin level <30 µg/L in inactive phases and <100 µg/L in active phases), and vitamin D deficiency (25(OH)2D3 level <30 ng/mL).
UC, ulcerative colitis; CD, Crohn's disease.

3.4 The analysis of correlation between serum FGF21 level and studied variables

There was no significant correlation of serum FGF21 level with the clinical and endoscopic disease activity indices (Table 4).

A significant positive correlation between FGF21 and fecal calprotectin levels were identified in both UC and CD, along with a correlation between FGF21 and CRP levels in CD patients, but not in UC patients (Table 4).

Negative correlations were observed between FGF21 level and total cholesterol, protein, and albumin levels in both UC and CD patients. Moreover, in UC patients, negative correlations of FGF21 level with hemoglobin level and BMI were noticed, and a positive correlation between FGF21 and triglyceride levels. In CD

TABLE 4 The analysis of correlations between serum FGF21 level and the studied variables.

Variable	UC		CD	
	Correlation coefficient	*p*	Correlation coefficient	*p*
Rachmilewitz index	0.19	0.203	–	–
Mayo Endoscopic Score*	0.20	0.080	–	–
CDAI	–	–	0.25	0.057
SES-CD	–	–	0.20	0.216
Fecal calprotectin	0.53	0.001	0.29	0.047
CRP	0.21	0.165	0.27	0.036
Total cholesterol	−0.33	0.028	−0.27	0.035
Triglycerides	0.38	0.009	0.07	0.615
Total protein	−0.43	0.003	−0.33	0.011
Albumin	−0.40	0.006	−0.37	0.004
Iron	−0.14	0.337	−0.39	0.002
Hemoglobin	−0.37	0.010	−0.21	0.113
Vitamin D	−0.21	0.175	−0.32	0.014
BMI	−0.29	0.047	−0.22	0.091

UC, ulcerative colitis; CD, Crohn's disease, CDAI, Crohn's Disease Activity Index, SES-CD, Simple Endoscopic Score for Crohn's disease, BMI, body mass index.
The Spearman rank correlation coefficient and Kendall Tau Correlations (*) were calculated to test associations between variables.

patients negative correlations of FGF21 level with iron and vitamin D levels were detected (Table 4).

4 Discussion

In this study, we have confirmed that serum FGF21 levels are higher in patients with active IBD, both UC and CD, compared to patients with inactive IBD and healthy controls. The available data on FGF21 level fluctuations in adult IBD patients are scarce. In one study higher plasma FGF21 levels in IBD patients compared to healthy controls were reported; however, the investigated group of patients was not divided into subjects with active and inactive disease (Liu et al., 2017). In another study conducted in children with IBD, serum FGF21 level was higher in the disease flare with a subsequent significant decrease after treatment (Tomasik et al., 2010). Additional compelling evidence regarding the role of FGF21 in intestinal inflammation comes from studies in animal models which show that chemically induced colitis is one of the factors stimulating the secretion of FGF21 (Liu et al., 2017; Liu et al., 2023; Al-Aqil et al., 2018). In an experimental IBD model, Liu et al. (2023) demonstrated that endogenous FGF21 was increased in dextran sulfate sodium-induced colitis, which contributed to the progression of the disease and a significant loss of body weight. The abovementioned study has also shed light on one of the potential mechanisms contributing to the FGF21 action. In FGF21 knockout mice, FGF21 depletion attenuated the severity of chemically induced acute colitis by enhancing the activation of the interleukin 22-STAT3 signaling pathway in intestinal epithelial cells (Liu et al., 2023).

The identification of FGF receptors and β-Klotho co-receptors within intestinal tissues supports the hypothesis that FGF21 plays a role in intestinal pathophysiology of IBD (Danopoulos et al., 2017; Aaldijk et al., 2023). Indeed, an immunohistochemistry analysis revealed that in the intestines of UC patients there is increased FGF21 expression located in the extra-epithelial compartment, while increased β-Klotho co-receptor expression is observed mainly on the surface of the intestinal epithelium (Muise et al., 2008; Rydén, 2009).

The current results not only confirm previous observations, but further indicate that FGF21 level correlates with the intensity of intestinal inflammation reflected by fecal calprotectin level. Moreover, the association between FGF21 and CRP levels is also present in CD patients. The fact that no correlation was found between FGF21 and CRP levels in UC patients may be related to quite wide range of CRP values in that group, particularly in active phase of disease. Additionally, it has been suggested that in UC, fecal calprotectin level has a better significance for detecting colitis compared to CRP (Anindita et al., 2023). Noteworthy, other researchers (Gariani et al., 2013) demonstrated a correlation between FGF21 level and CRP level in patients with systemic inflammatory response syndrome and had even proposed FGF21 as a non-specific marker for systemic inflammation.

A novel aspect of the current research is related to the evaluation of potential correlation between serum FGF21 level and validated clinical and endoscopic disease activity scales and indices in IBD. However, despite the observed fluctuations in FGF21 level in active *versus* inactive IBD phases, as well as the presence of correlation between FGF21 and inflammatory markers, no significant association of FGF21 level with validated disease activity scales and indices were found. Nevertheless, FGF21 as an inflammatory marker doesn't have to correlate directly with more complex scales and indices of clinical or endoscopic activity of the diseases.

Given the role of FGF21 as an endocrine metabolic regulator that is expressed in many metabolically active tissues such as the liver and

WAT as well as its concomitant involvement in intestinal inflammation, one of the aims of the current study was to assess the relation between FGF21 level and nutritional status parameters in IBD patients. Among the studied IBD patients, 17% of subjects were underweight. A significant percentage of patients were characterized by hypocholesterolemia, hypoproteinemia, hypoalbuminemia, iron deficiency anemia and vitamin D deficiency. The observed deficiencies in our study were more prevalent compared to some previous reports (Casanova et al., 2017; Prieto et al., 2021). Particularly frequently nutritional disturbances were detected in patients with active UC. To some extend it could be related to the relatively short duration of the disease (many patients in that group were only recently diagnosed). In fact, according to the available data, malnutrition is more prevalent among patients with a recently diagnosed IBD (Gold et al., 2023). Furthermore, patients with UC are more prone to develop nutritional deficiencies during active phase of the disease, whereas subjects with CD typically develop features of malnutrition gradually over an extended period of time (Balestrieri et al., 2020). Regarding a high rate of vitamin D deficiency in the studied population (amounting to 64% of the subjects), it was comparable to the results of previous studies performed in IBD patients in Poland (Krela-Kaźmierczak et al., 2015; Tulewicz-Marti et al., 2022). Importantly, vitamin D3 deficiency affected a large percentage of IBD patients in remission.

While analyzing the associations between FGF21 level and selected nutritional status parameters, we found that FGF21 level negatively correlated with BMI, total cholesterol, total protein, albumin, iron, hemoglobin and vitamin D levels. A positive correlation of FGF21 and triglyceride levels may be associated with increased lipolysis activity.

The spectrum of nutritional disturbances in IBD patients is wide. On one hand, malnutrition is a major complication of IBD and it is primarily responsible for chronic weight loss (Scaldaferri et al., 2017). On the other hand, numerous recent studies have linked IBD to metabolic syndrome, which includes diabetes, obesity, and dyslipidemia, as they share some common pathophysiological links including inflammation, adipose tissue dysregulation, and gut dysbiosis (Michalak et al., 2016; Szilagyi, 2020; Verdugo-Meza et al., 2020). Experimental and clinical evidence supports parallels between metabolic nature of gut inflammation in IBD and the inflammatory state in metabolic diseases (Adolph et al., 2022). Specifically, the role of adipose tissue in the development of metabolic syndrome and IBD has been extensively studied (Choe et al., 2016). Noteworthy, in various metabolic disorders such as obesity, hyperlipidemia, diabetes, and metabolic dysfunction-associated steatotic liver disease, elevated serum FGF21 level were also recorded (Yang et al., 2023). However, in these conditions the FGF21 resistance seems to occur (Aaldijk et al., 2023).

A recently published meta-analysis of clinical trials demonstrated that treatment with FGF21 analogues significantly reduces total cholesterol level and contributes to weight loss (Carbonetti et al., 2023). Additionally, several other studies propose FGF21 as potential therapeutic agent with anti-inflammatory effect (Feingold et al., 2012; Singhal et al., 2016; Wang et al., 2018). Contrary, an increase in FGF21 level during the active phase of IBD exerts negative metabolic effects contributing to malnutrition and weight loss and exacerbating inflammation. This discrepancy may be explained by the possible dual action of FGF21. In fact, FGF21 has the capability to either suppress or promote lipolysis in WAT, depending on whether the person is during fasting or in the postprandial state, respectively (Xie et al., 2020). Similarly, pro- or anti-inflammatory action of FGF21 may depend on the given pathophysiological state such as acute *versus* chronic inflammation. In experimental studies, it has been shown that overexpression of FGF21 may reduce hepatic cholesterol production (Huang et al., 2017), increase the liver production of bile salts from cholesterol (Al-Aqil et al., 2018), induce the lipolysis of adipose tissue (Liu et al., 2017), reduce muscle mass and strength (Oost et al., 2019). Therefore, modulation of FGF21 signaling pathway could emerge as a target in IBD and related metabolic disorders. However, the effects of exogenous FGF21 treatment on acute and chronic colitis and colitis recovery have not been adequately examined so far.

Among limitations of the study is its cross-sectional character. Furthermore, the effects of used medications may constitute confounding factors affecting the results. For example, steroid therapy leads to enhanced FGF21 expression in the liver (Vispute et al., 2017; Al-Aqil et al., 2018). However, since many studies have confirmed that inflammatory stimuli are inducers of FGF21 expression, the use of steroids most likely only contributes to the FGF21 level increase, but is not solely responsible for the effect. Additionally, to further explore the association between FGF21 and nutritional status, it would be useful to perform more detailed analysis including the evaluation of fat mass index, fat-free mass index or muscle strength. It might be important given that some IBD patients with sarcopenia have normal BMI values (Scaldaferri et al., 2017; Balestrieri et al., 2020). The novelty of the study is related to the pioneer report on FGF21 level fluctuations in adult IBD patients in active and inactive phases of the disease as well as on the correlation of FGF21 level with inflammatory markers and nutritional status parameters.

In conclusion, our results show that FGF21 level correlates directly with the intensity of intestinal inflammation and inversely with nutritional status of IBD patients. Therefore, the multifactorial pathogenesis of malnutrition and weight loss in IBD patients may be related to disturbances in FGF21 level. Further studies are warranted to clarify the exact mechanism of complex action of FGF21 within the gut-liver axis to unravel potential new therapeutic targets in IBD and related metabolic disturbances.

Data availability statement

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

Ethics statement

The studies involving humans were approved by the Ethics Committee at the Wroclaw Medical University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

AL: Data curation, Formal Analysis, Investigation, Methodology, Visualization, Writing–original draft. AM: Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Validation, Writing–review and editing.

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Conflict of interest

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

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EDITED BY
Thomas Brzozowski,
Jagiellonian University Medical College, Poland

REVIEWED BY
Daniele Maria-Ferreira,
Instituto de Pesquisa Pelé Pequeno Príncipe, Brazil
M. Ahmed,
Phcog.Net, India

*CORRESPONDENCE
Sheng-Quan Fang,
fsq20032003@163.com

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A scientometrics analysis and visualization of refractory gastroesophageal reflux disease

Nan Zhang, Ming Han, Qin-Wei Zheng, Meng-Yuan Zhang, Wen-Lan Zhi, Jing-Jing Li, Lin-Xuan Cui, Jin-Li Tian, Yi Wang and Sheng-Quan Fang*

Department of Gastroenterology, Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai, China

Background: Refractory gastroesophageal reflux disease (refractory GERD) is a heterogeneous disease characterized by unresponsiveness or poor efficacy to proton-pump inhibitors (PPIs). This chronic disorder substantially weakens patients' mental wellbeing and quality of life, increasing the financial burden on society. Multiple articles have been reported in this area. However, literature involving scientometric analysis of refractory GERD is absent. Therefore, it is necessary to understand the evolution of research themes and the main hotspots of refractory GERD through bibliometric methods.

Methods: All documents related to refractory GERD based on the WOS Core Collection from January 2000 to November 2023 were selected for analysis. Citespace V 6.1 R6, VOSviewer V 1.6.20, and Scimago Graphica V 1.0.38 were used to perform bibliometric analysis.

Results: We collected a total of 241 research articles from 36 countries and 322 institutions, contributed by over 1,000 authors. Over the last 20 years, the number of articles in this field has increased year by year, and since 2011, the number of publications has increased dramatically, with 85.89% of the papers. These countries are led by the United States and Japan. *GUT* had the highest number of citations and *DIGESTION* had the highest number of publications. Research on standardized diagnosis and management, mechanisms, novel monitoring methods, and innovative drugs and procedures for refractory GERD are the main topics and hotspots in this field. This study also found that neuroimmune interaction is closely related to refractory GERD, which may be a new direction for future mechanism research.

Conclusion: Our study is the first bibliometric analysis of the global literature on refractory GERD. This research provides valuable insights for researchers, enabling them to quickly understand the research frontier and hot topics of this field.

KEYWORDS

refractory GERD, scientometrics analysis, visualization, Citespace, research hotspots

1 Introduction

Refractory gastroesophageal reflux disease (refractory GERD) is a heterogeneous disease characterized by unresponsiveness or poor efficacy to proton-pump inhibitors (PPIs). The latest update of the Lyon Consensus 2.0 identifies heartburn, oesophageal chest pain, and regurgitation as typical symptoms, and atypical manifestations such as belching and chronic cough also exhibit a potential pathophysiological association with this condition (Gyawali et al., 2023). As a distinct subtype of gastroesophageal reflux disease, the persistence of symptoms and the variability in treatment efficacy pose challenges for clinicians in daily management and therapy (Armstrong et al., 2022). Consequently, the diagnosis and treatment of refractory patients have become prominent areas of challenge and difficulty within the field of gastrointestinal diseases.

One study reported that the global prevalence of GERD in 2020 was about 13.98% (Nirwan et al., 2020). According to the World Population Prospects issued by the United Nations in 2022, it is estimated that over 1 billion individuals will be affected by GERD, with approximately 13.2%–54.1% of them being non-responsive or inadequately responsive to long-term PPIs treatment (Chey et al., 2010; Katz et al., 2013a; Kahrilas et al., 2013; Delshad et al., 2020). In the United States and the United Kingdom, patients with refractory GERD have significantly more visits to primary care facilities and emergency departments, significantly reducing patient productivity and sleep quality (Toghanian et al., 2011; Kahrilas et al., 2013; Wang HM. et al., 2023). Compared to patients who responded positively to PPIs therapy, those with refractory responses incurred an average of $7,000 to $10,000 higher in associated healthcare costs and were also more susceptible to gastrointestinal bleeding, dysphagia, and other related conditions (Gerson et al., 2011; Howden et al., 2021). In addition, long-term PPIs use as well as referrals for further treatment not only directly impose financial burdens on patients but also exert a considerable negative impact on their mental health and overall quality of life while simultaneously imposing additional financial strains on society.

With the growing attention in this field, researchers have made more in-depth explorations in this field every year to have a more thorough understanding of this disease. Simultaneously, numerous related articles have been published, posing a challenge on how to efficiently comprehend research topics and identify potential directions in this field. Bibliometric methods offer unique advantages in addressing this issue effectively. However, no bibliometric analysis of refractory GERD has been reported so far.

Here, we employed bibliometric methods to analyze the research status and development history of refractory GERD from 2000 to 2023. Through manual classification and summarization, we demonstrate scientific collaboration among institutions and regions while identifying key research areas such as standardized diagnosis and management, novel mechanisms and detection methods, as well as the development of new drugs and surgical procedures. These findings highlight the focal points of research in this field along with future trends.

2 Data and methods

2.1 Data sources and search strategies

Web of Science (WOS) is a database and research tool with comprehensive literature content and strong influence, which can help us quickly locate high-impact papers, understand the latest progress in the field, and find breakthroughs in research ideas (Li et al., 2018). Therefore, the WOS Core Collection was used as the source of literature data in this study. The search time span was from January 2000 to November 2023, and the search strategy was as follows: TIm = (Refractory OR resistant OR "no respon*" OR "not respond" OR "Non-Responsive" OR Ineffective OR "No effect") AND TI=("Gastroesophageal reflux disease" OR GERD OR "Gastric esophageal reflux" OR GORD OR "Non*erosive Reflux Disease" OR NERD OR "Endoscopy-negative reflux" OR "Endoscopy normal reflux" OR Heartburn OR "Reflux Esophag*" OR "Erosive Reflux" OR "Erosive Esophag*" OR "Oesophageal reflux" OR "Barrett's esophag*" OR "Gastric Acid Reflux" OR "Acid Reflux" OR Reflux OR "Proton Pump Inhibitor" OR PPI). The search time was 10 December 2023, and a total of 842 records were retrieved.

2.2 Data extraction and collection

The following document types were excluded from this study: Proceeding Paper or Meeting Abstract or Letter or Book Chapters or Early Access or Editorial Material or Reprint or Meeting Summary, only articles and reviews in English were retained. Although TI can accurately search the literature, there are still some articles that are not related to refractory GERD. After screening and checking by two researchers, the literature whose content did not conform to the research theme was excluded. A total of 601 articles were excluded, and 241 publications were included for bibliometric analysis. Figure 1 shows the flowchart of the literature-screening process and research framework.

2.3 Bibliometric analysis

The data were imported into the corresponding analysis software for bibliometric and visualization analysis. The software includes Citespace V 6.1 R6, VOSviewer V 1.6.20, and Scimago Graphica V 1.0.38. These tools excel at presenting the intricate and extensive development of a specific field through visualization techniques, enabling quick identification of key information and pivotal moments to predict scientific hotspots and frontiers. In this study, Citespace V 6.1R6 made the keyword cluster map, Timeline view of keywords map, and analyzed the burst citations. VOSviewer 1.6.20 was used to analyze institutions, collaborative relationships, and co-cited references. Scimago Graphica V 1.0.38 shows the network of cooperation between countries. Microsoft Excel 2021 was used for the top 10 cited references, the top 10 cited authors, and the top 15 journals by number of publications. Complementary information and visual images were integrated across different software platforms to

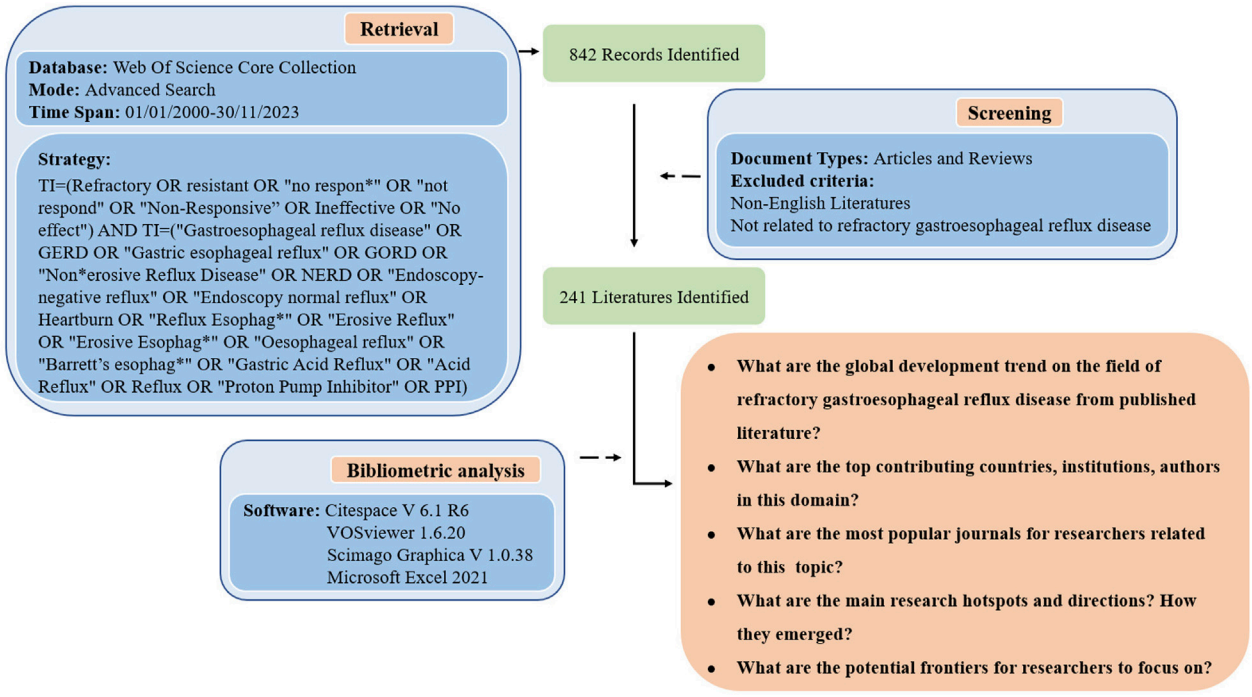

FIGURE 1
Flowchart of the literature-screening process and research framework.

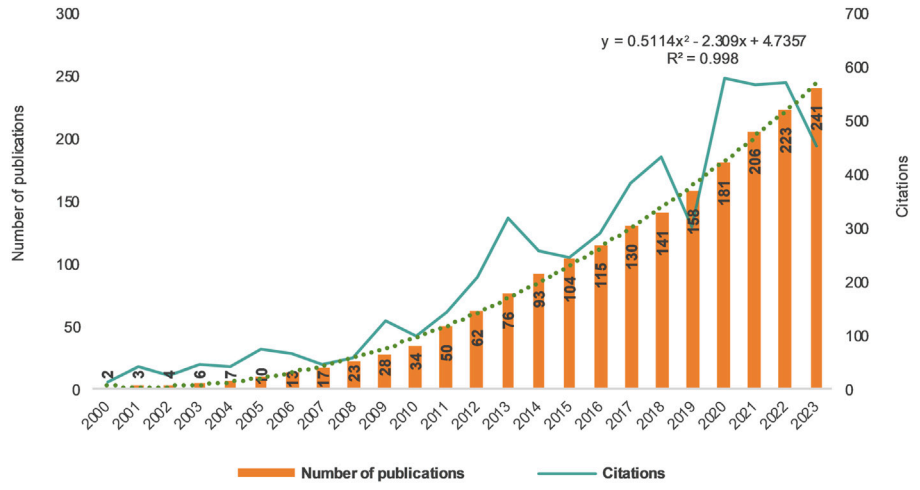

FIGURE 2
Trends in the growth of the publications and numbers of cited articles worldwide from 2000 to 2023.

comprehensively and scientifically analyze the literature data. The data underwent preprocessing before conducting analysis. Nonsensical keywords (e.g., "disease," "health," "esophagus") were excluded. Keywords with similar concepts but varying expressions or spellings were combined for analysis purposes (e.g., "gastroesophageal reflux disease," "GERD," "GORD"; "pH/mii," "impedance-pH monitoring").

3 Results

3.1 Annual publication and citation trends

This study examined articles on refractory GERD for 24 years, from 2000 to 2023. In the initial 11-year period, only 14.11% of all articles were published, whereas in the subsequent 13 years from

TABLE 1 Top 10 countries/regions by number of publications.

Rank	Country	Publications	Centrality	Citations	TLS
1	United States	74 (30.71%)	0.40	2,011	43
2	Japan	58 (24.07%)	0.09	1,036	13
3	China	36 (14.84%)	0.04	380	19
4	Italy	22 (9.13%)	0.02	652	14
4	Belgium	12 (4.98%)	0.17	572	10
4	Netherlands	12 (4.98%)	0.36	920	19
5	France	10 (4.15%)	0.00	395	8
5	Canada	9 (3.73%)	0.01	455	10
6	United Kingdom	9 (3.73%)	0.09	390	14
7	South korea	6 (2.49%)	0.10	162	13

TLS: total link strength.

2011 until 2023, the number of articles accounted for 85.89%. Figure 2 illustrates that a turning point occurred in this field in 2011 when the number of publications rapidly increased from 6 to 17. The quantity of publications has reached a new peak, rising from 2 in 2000 to 24 in 2021. Since 2019, there has been consistent annual publication exceeding 17 articles and reaching its highest citation count at an impressive figure of 581. The percentage of review articles and articles was 11.62% and 88.38% respectively. By employing curve regression modeling, this study generated a polynomial function growth curve representing the yearly expansion of literature and found it to be highly consistent with the actual growth trend observed within these publications (R^2 = 0.998), thus confirming the annual progression pattern concerning articles and research on refractory GERD.

3.2 Analysis of countries or regions and institutions

In the past, 322 institutions from 36 countries and regions have conducted research and published articles in this field. Table 1 presents the 10 countries that have contributed the most papers, with the United States, Japan, and China leading with 74, 58, and 36 articles respectively. These publications account for a significant portion of the total articles (69.71%) and highlight their prominent role in the development of refractory GERD. Figure 3 visualize the cooperative relationships between countries. By comparing the centrality, it can be found that United States and Netherlands have the highest centrality, which is 0.4 and 0.36 respectively, indicating that these two countries have closer cooperation with other countries in this field, while other countries have less communication with each other. Table 2 describes the top 10 institutions with the largest number of papers published, and Japanese institutions account for the largest proportion, with 7 institutions. In addition, the single institution with the highest citation is the University of Arizona in the United States, with a citation frequency of 465 times, which represents the institution has a certain influence in this academic field.

3.3 Analysis of authors

A total of 1,265 authors have contributed to the field of research Figure 4. The author cooperation network depicts different author groups or teams using distinct colors. Node size represents the number of papers, while line thickness indicates the strength of collaborative relationships among authors (Chen, 2006). Table 3 presents the top 10 authors by number of publications and their number of citations and H-index. Notably, Japanese scholars have made remarkable contributions to the research on refractory GERD. Among them, Professor Iwakiri and Katsuhiko stand out as not only having the highest number of publications, but also the closest cooperation with other authors. It is worth mentioning that Iwakiri, Katsuhiko, Danie Sifrim, Ronnie Fass, Nakagawa Kenichiro, Koike Tomoyuki, Kinoshita Yoshikazu, Higuchi Kazuhide, Hoshikawa Yoshimasa eight authors may be important leaders in the collaborative project between institutions. Furthermore, it is noteworthy that Professor Ronnie Fass holds the highest H-index value. The author with the highest number of citations is Professor Danie Sifrim. These findings underscore their significant academic stature and potential pivotal role in pioneering breakthroughs related to refractory GERD.

3.4 Analysis of journals and literature citation

The top 10 journals with the highest number of citations and their number of publications, impact factors, and Journal Citation Reports (JCR) are presented in Table 4, which can provide a reference for the quality assessment and selection of journals in this research area. The most cited journals are *GUT* and GASTROENTEROLOGY. Although 70% of the top 10 journals are in the JCR Q1 region, there are still many articles on refractory GERD published in journals with low impact factors, suggesting that more in-depth and high-quality research should be carried out. In terms of article quantity, *DIGESTION* emerges as the most popular journal among authors, with a total of 17 publications.

Table 5 shows the top 15 cited articles among the 241 articles (Fass et al., 2000; Klinkenberg-Knol et al., 2000; Koek et al., 2003;

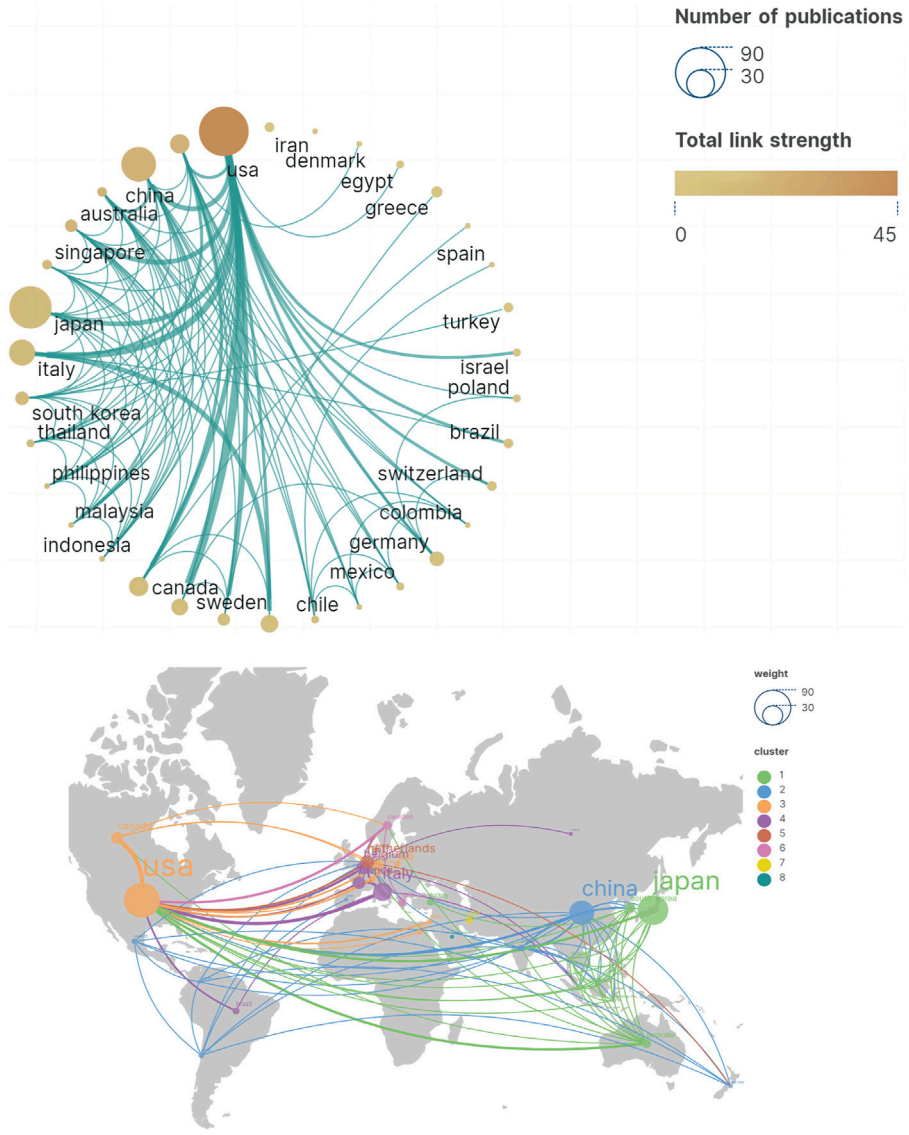

FIGURE 3
Cooperation map of countries/regions in refractory GERD.

Hemmink et al., 2008a; Fass and Sifrim, 2009a; Slaughter et al., 2011; Vela et al., 2011; Sifrim and Zerbib, 2012a; Tominaga et al., 2012; Noar et al., 2014; Penagini et al., 2015a; Fock et al., 2016; Hoshino et al., 2017a; Frazzoni et al., 2017; Spechler et al., 2019; Delshad et al., 2020). Among them, the article published by Klinkenberg-Knol (Klinkenberg-Knol et al., 2000) in 2000 was cited the most times, reaching 390 times. This study followed patients with refractory reflux esophagitis (RE) for an average duration of 6.5 years, providing substantial clinical evidence supporting the long-term efficacy and safety of omeprazole in treating this disease. It may have played a pivotal role in promoting the widespread clinical application of omeprazole for this condition. Among these top 15 cited articles, two were published within the past 5 years. One was a randomized controlled trial (Spechler et al., 2019) comparing surgery and medicine as treatment options for this disease, published in the NEW ENGLAND JOURNAL OF MEDICINE in 2019, with a total citation count of 121. Additionally, there was a large-scale cohort study published by Sean (Delshad et al., 2020) in GASTROENTEROLOGY in 2020 that revealed crucial epidemiological characteristics associated with refractory GERD.

3.5 Analysis of references with citation bursts

Figure 5 shows the top 25 references with the strongest citation bursts from 2000 to 2023 (Charbel et al., 2005; Mainie et al., 2006; Vakil et al., 2006; Zerbib et al., 2006; Kahrilas et al., 2008a; Hemmink et al., 2008b; Dent et al., 2008; Fass and Sifrim, 2009b; Sweis et al., 2010; Frazzoni et al., 2011a; Sifrim and Zerbib, 2012b; Kahrilas et al., 2012; Kohata et al., 2012; Katz et al., 2013b; Kahrilas et al., 2014; Herregods et al., 2015; Kahrilas et al., 2015; Sakurai et al., 2015; Ashida et al., 2016; Aziz et al., 2016; Iwakiri et al., 2016; Scarpellini et al., 2016; Hoshino et al., 2017b; Gyawali et al., 2018a; Tack and

TABLE 2 Top 10 institutions by number of publications.

Rank	Organization	Publications	Citations	TLS
1	Nippon Medical School (Japan)	14	213	23
2	Gunma University Hospital (Japan)	9	223	58
3	Osaka City University (Japan)	8	230	44
3	Northwestern University (United States)	8	211	43
3	Tohoku University (Japan)	8	208	34
4	University of Arizona (United States)	7	465	80
4	Queen Mary University of London (United Kingdom)	7	374	23
4	Osaka Medical College (Japan)	7	173	54
4	Shimane University (Japan)	7	170	57
5	University of Padua (Italy)	6	193	26
5	University of Pisa (Italy)	6	193	23
5	Hamamatsu University School of Medicine (Japan)	6	132	31

TLS: total link strength.

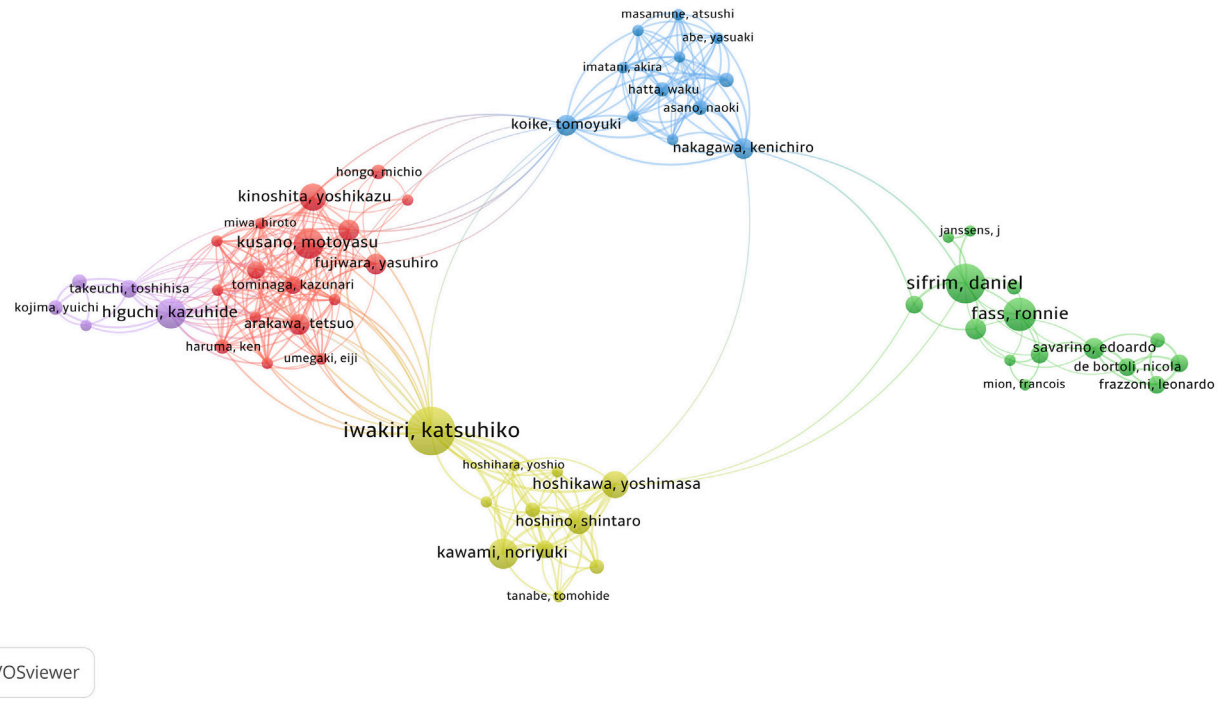

FIGURE 4
Cooperation map of authors in refractory GERD.

Pandolfino, 2018; Zerbib et al., 2021a). Among these, 12 were reviews or consensus documents that guided subsequent research endeavors. Burst literature is identified using Professor Kleinberg's burst detection algorithm, which highlights references that have been frequently cited in a short period and labeled as hotspots (Chen et al., 2012). The red line segment on the right corresponds to the burst time interval of each reference, while strength indicates its intensity. The Lyon consensus, issued by global gastroenterologists in 2018, had the highest outbreak intensity (Gyawali et al., 2018b) and continues to be widely recognized and frequently cited by peers.

3.6 Analysis of co-occurrence keywords

Keywords play a crucial role in quickly identifying the literature's topic and effectively extracting vital information

TABLE 3 The top 10 productive authors.

Rank	Authors	Country	Publications	Citations	H-index	TLS
1	Iwakiri, Katsuhiko	Japan	15	267	25	110
2	Sifrim, Daniel	United Kingdom	10	596	36	31
3	Fass, Ronnie	United States	10	499	62	13
4	Kusano, Motoyasu	Japan	9	223	28	53
4	Higuchi, Kazuhide	Japan	9	184	45	58
4	Kawami, Noriyuki	Japan	9	157	12	47
5	Kinoshita, Yoshikazu	Japan	8	181	43	46
5	Hoshikawa, Yoshimasa	Japan	8	130	9	47
6	Hoshino, Shintaro	Japan	7	104	20	44
7	Arakawa, Tetsuo	Japan	6	166	48	45

TLS: total link strength.

TABLE 4 The top 10 most cited journals.

Rank	Journal	Citations	Publications	If	H-index	JCR
1	*Gut*	742	5	24.5	262	Q1
2	*Gastroenterology*	513	5	29.4	368	Q1
3	*American Journal of Gastroenterology*	419	9	10.2	234	Q1
4	*Alimentary Pharmacology & Therapeutics*	384	12	7.6	159	Q1
5	*Neurogastroenterology and Motility*	288	10	3.5	93	Q2
6	*Digestion*	255	17	3.2	71	Q3
7	*Surgical Endoscopy and Other Interventional Techniques*	239	12	3.1	141	Q1
8	*Journal of Gastroenterology*	216	7	6.3	99	Q1
9	*Clinical Gastroenterology and Hepatology*	176	4	12.6	151	Q1
10	*Digestive Diseases and Sciences*	150	11	3.1	113	Q3

TLS: Total link strength IF: impact factor (Journal Citation Reports 2022).

(Dotsika and Watkins, 2017). Keywords co-occurrence, an essential component of bibliometric analysis, aids in speculating potential research hotspots. In this study, we extracted 74 keywords with the highest co-occurrence and presented them as a network in Figure 6. Employing a clustering algorithm, we divided these keywords into four clusters distinguished by colors: red, yellow, blue, and green. The red cluster pertains to refractory GERD, focusing on its management and diagnosis. Key terms include "management," "diagnosis," "guidelines," and "prevalence." The green cluster primarily explores the mechanism behind refractory GERD using Keywords such as "mechanism," "acid reflux," "esophageal sphincter relaxation," and "GABA(B) agonist baclofen." This module aims to identify possible causes for this challenging condition. The yellow cluster delves into drugs that suppress gastric acid like PPIs, omeprazole, rabeprazole, potassium-competitive acid blockers (P-CABs), and vonoprazan. This module mainly discusses the application and development of drugs represented by PPIs. The blue cluster mainly deals with refractory symptoms and detection methods. The main keywords are "refractory symptoms," "non-erosive reflux disease," "impedance-ph monitoring," and "high-resolution manometry." This cluster discusses the characteristics of refractory GERD and the application of new detection methods such as esophageal impedance-pH monitoring and high-resolution manometry in this field. The topics corresponding to the four clusters covered the mainstream academic literature on physiology and pathology, diagnosis, detection methods, and treatment of refractory GERD.

Furthermore, Figure 7 illustrates the temporal evolution of keywords throughout the study period using years as a reference point. It is evident that post-2005, researchers shifted their focus toward understanding the mechanism and identification of this disease. Keywords included "gastric emptying," "eosinophilic esophagitis," "hypersensitive esophagus," and "functional heartburn." Around 2010, researchers developed an interest in exploring the nature of refluxate in refractory gerd with subject words such as "nonacid reflux," "acid reflux,"

TABLE 5 The top 15 cited articles related to refractory gastroesophageal reflux disease.

Rank	Title	Year	Journal	If	JCR	Types of research	Total Citations
1	Long-term omeprazole treatment in resistant gastroesophageal reflux disease: Efficacy, safety, and influence on gastric mucosa	2000	*Gastroenterology*	29.4	Q1	Clinical Trial	390
2	Diagnosis and management of patients with reflux symptoms refractory to proton pump inhibitors	2012	*Gut*	24.5	Q1	Review	224
3	Management of heartburn not responding to proton pump inhibitors	2009	*Gut*	24.5	Q1	Review	219
4	Effect of the GABA(B) agonist baclofen in patients with symptoms and duodeno-gastro-oesophagealreflux refractory to proton pump inhibitors	2003	*Gut*	24.5	Q1	Clinical Trial	190
5	Esophageal pH-Impedance Monitoring in Patients With Therapy-Resistant Reflux Symptoms: "On" or"Off "Proton Pump Inhibitor?	2008	*American Journalof Gastroenterology*	10.2	Q1	RCT	154
6	Randomized Trial of Medical versus Surgical Treatment for Refractory Heartburn	2019	*New England Journal of Medicine*	158.5	Q1	RCT	121
7	Asia-Pacific consensus on the management of gastro-oesophageal reflux disease: an update focusingon refractory reflux disease and Barrett's oesophagus	2016	*Gut*	24.5	Q1	Practice Guideline	109
8	Caution About Overinterpretation of Symptom Indexes in Reflux Monitoring for Refractory Gastroesophageal Reflux Disease	2011	*Clinical Gastroenterology and Hepatology*	12.6	Q1	Cross-Sectional Study	107
9	Prevalence of Gastroesophageal Reflux Disease and Proton Pump Inhibitor-Refractory Symptoms	2020	*Gastroenterology*	29.4	Q1	Cohort Study	88
10	The added diagnostic value of postreflux swallow-induced peristaltic wave index and nocturnal baseline impedance in refractory reflux disease studied with on-therapy impedance-pH monitoring	2017	*Neurogastroenterologyand Motility*	3.5	Q2	Cohort Study	87
11	Refractory Heartburn: Comparison of Intercellular Space Diameter in Documented GERD vs. Functional Heartburn	2011	*American Journal of Gastroenterology*	10.2	Q1	Comparative Study	70
12	Rikkunshito improves symptoms in PPI-refractory GERD patients: a prospective, randomized, multicenter trial in Japan	2012	*Journal of Gastroenterology*	6.3	Q1	RCT	70
13	Omeprazole 40 mg once a day is equally effective as lansoprazole 30 mg twice a day in symptom control of patients with gastro-oesophageal reflux disease (GERD) who are resistant to conventional-dose lansoprazole therapy - a prospective, randomized, multi-centre study	2000	*Alimentary Pharmacology & Therapeutics*	7.6	Q1	RCT	69
14	Long-term maintenance effect of radiofrequency energy delivery for refractory GERD: a decade later	2014	*Surgical Endoscopy and Other Interventional Techniques*	3.1	Q1	Clinical Trial	68
15	Efficacy of Vonoprazan for Proton Pump Inhibitor-Resistant Reflux Esophagitis	2017	*Digestion*	3.2	Q3	Clinical Trial	66

TLS: Total link strength IF: impact factor (Journal Citation Reports 2022).

and "weakly acidic reflux." By 2015, attention was directed towards investigating the role of esophageal motility disorders with keywords like "esophageal motility" and "proximal reflux."

4 Discussion

This work utilized a diverse range of bibliometric tools to analyze refractory GERD across five dimensions: publication

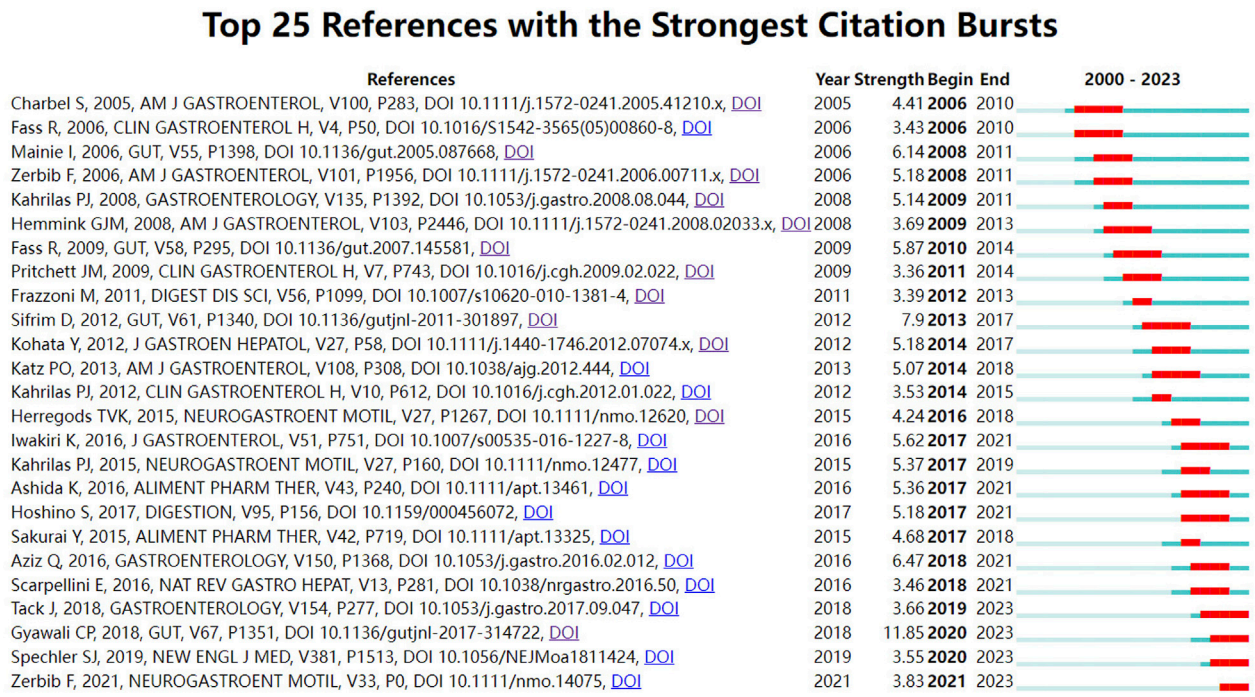

FIGURE 5
References with the strongest citation bursts.

trends, country and author contributions, core literature analysis, hot topics investigation, and frontier direction exploration. The main conclusions are as follows.

4.1 General information

In the more than 20 years since 2000, clinicians and researchers have shown significant interest in refractory GERD. Consequently, related research has been carried out rapidly in different regions, and related articles have been increasing year by year.

For the surge in publications on refractory GERD since 2011, we analyzed several reasons. First, epidemiological surveys published in 2009–2010 revealed that the persistence of reflux symptoms after taking PPIs was very common, ranging from 17% to 32%. Moreover, this incidence was observed to be increasing over time, prompting researchers to direct their attention towards this concerning phenomenon that posed challenges for both patients and physicians (El-Serag et al., 2010a; Chey et al., 2010). Secondly, the International High Resolution Esophageal Manometry Group published the first edition of the classification standard for esophageal motility disorders in 2009, which was subsequently named Chicago Classification (CC), heralding a groundbreaking advancement in esophageal manometry technology. Peter J invented pressure topography plots based on traditional conventional manometric recordings, which are superior in defining the spatial characteristics of esophageal constriction segments and in determining pressure changes (Kahrilas, 2010). As a result, HRMs that incorporate this technology can accurately identify patients with more subtle esophageal motility disorders and categorize them according to classification criteria as distal esophageal spasm, vigorous achalasia, functional obstruction, and subtypes of nutcracker esophagus. This change has transformed esophageal manometry data from crude to refined and the interpretation of results from complex to intuitive. The new technique facilitates clinicians to detect anatomical defects of the esophagus as well as other dysfunctions, thus distinguishing truly refractory patients, which has a significant impact on the rigorous screening of eligible cases for subsequent clinical studies and convincing statistics (Kahrilas et al., 2008b). Meanwhile, the method developed by L. B. Gerson et al., in 2011 offers a novel approach to assess clinical features, severity, and predict the necessity of additional anti-reflux treatment. This advancement holds significant potential for enhancing the management of refractory GERD in primary care and community settings (Gerson et al., 2011). As a result, on the one hand, there is a growing awareness among individuals regarding the limitations of PPIs therapy, and an increasing number of clinicians are reporting refractory patients who exhibit resistance to acid suppressors. On the other hand, newly discovered detection and evaluation methods have provided directions for subsequent research, thereby prompting researchers to undertake numerous in-depth studies on the etiology of refractory cases. It is highly plausible that these factors have contributed to the surge in publications on refractory GERD since 2011.

The United States, being the country with the highest number of publications in this field, has played a leading role in the development of research on refractory GERD. This finding may be attributed to researchers' attention to the high incidence and substantial medical burden associated with this condition in the United States (Wahlqvist et al., 2006; El-Serag, 2007; El-Serag et al., 2010b; Delshad et al., 2020; Nirwan et al., 2020; Howden et al., 2021).

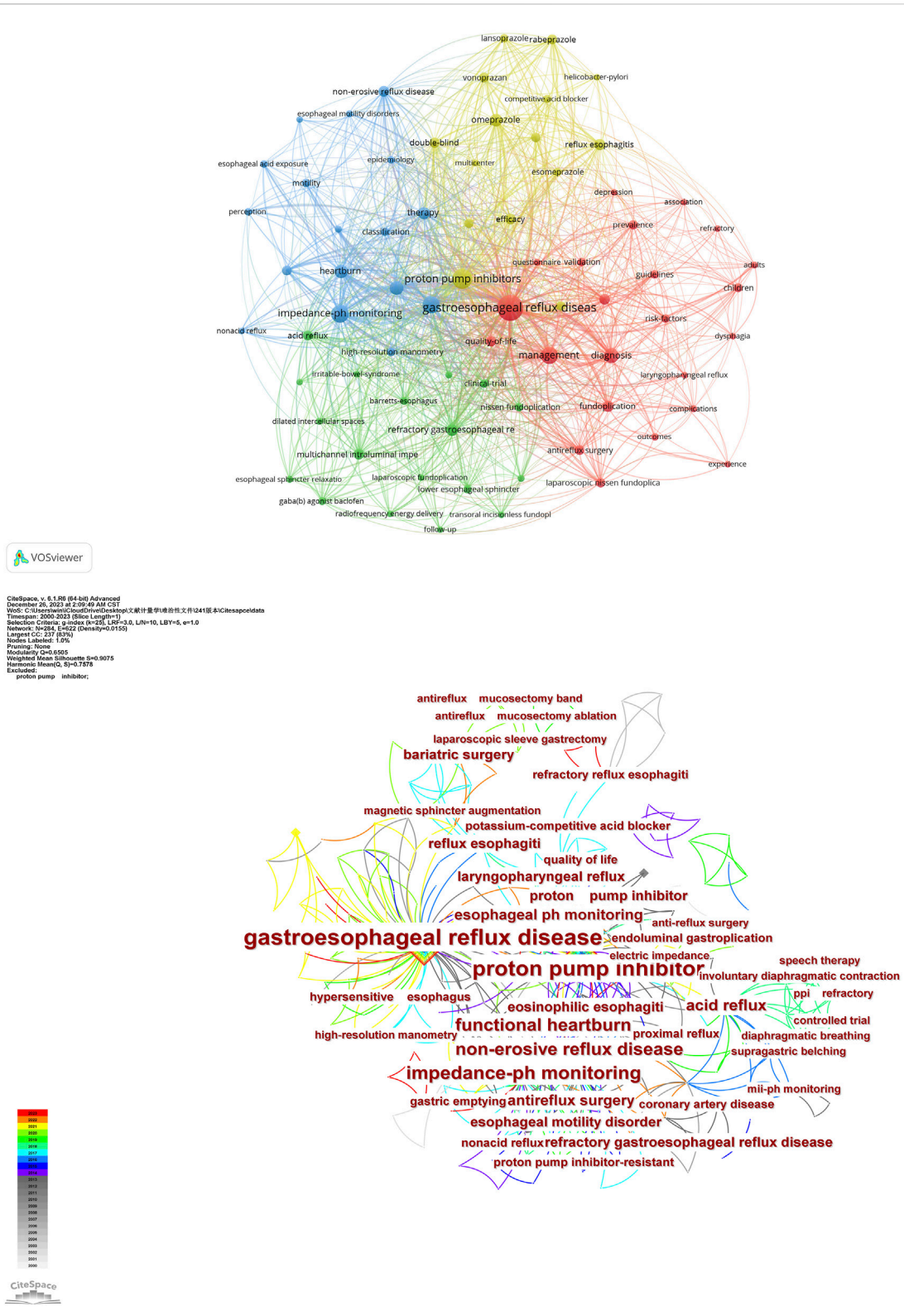

FIGURE 6
Map of keyword clustering in refractory GERD.

Although Japan ranks second in total publications, it accounts for 7 out of the top 10 institutions, indicating the significant prominence of refractory GERD within Jap anese academic circles, possibly related to the country's advanced endoscopic technology and public health awareness regarding early esophageal cancer screening (Pathirana and Poston, 2001; Dobashi et al., 2022; Fujishiro and Matsumoto, 2022). The United States and the Netherlands exhibit closer collaborations with other countries,

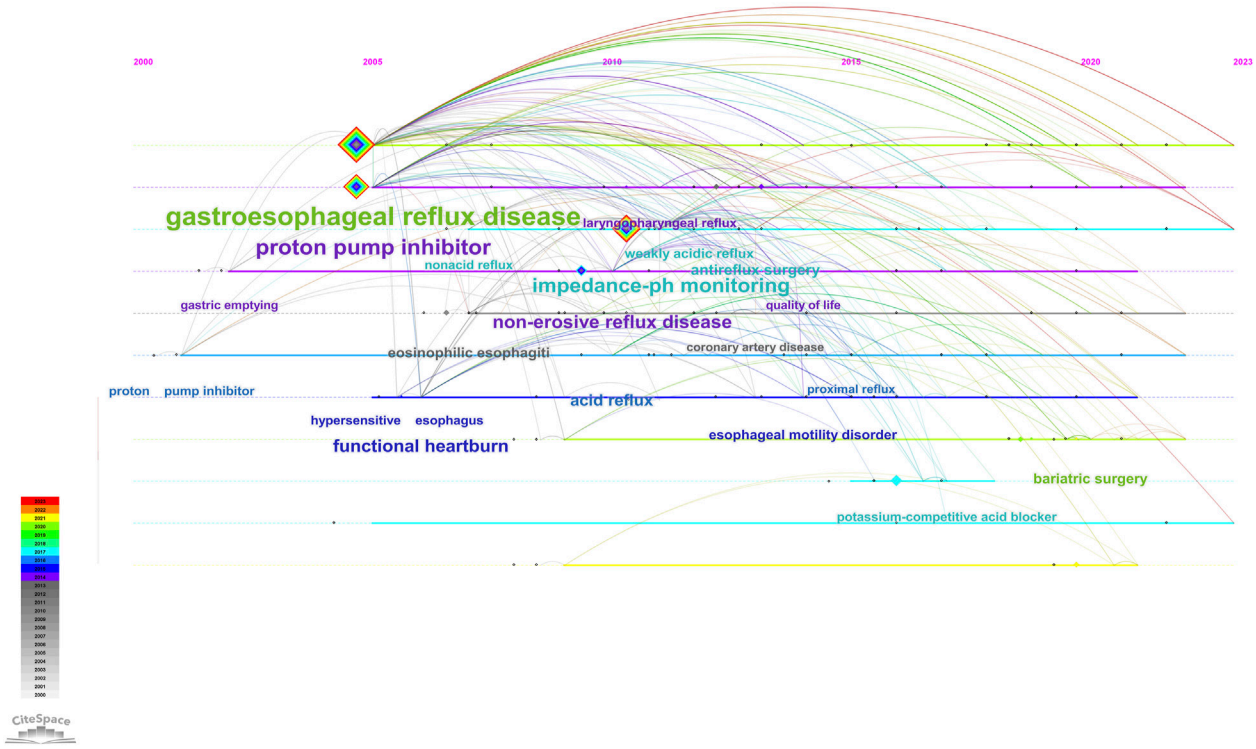

FIGURE 7
Timeline view and evolutionary path in refractory GERD.

serving as a valuable example for other countries to enhance cooperation and exchange to broaden research ideas.

Potential reasons for the leading position of these countries and institutions include the following. The first reason is related to social and demographic factors. A study showed that refractory GERD patients in the United States have the highest proportion among all the countries and regions surveyed, which accounts for 54.1% of the patients with GERD (Delshad et al., 2020). This has a serious adverse impact on the quality of life and work and increases the economic burden, but also triggers the attention and thinking of clinicians and researchers. Secondly, the United States has made significant contributions to the advancement of detection technology through the invention and development of impedance-pH monitoring and high-resolution manometry. Meanwhile, Japan's contribution lies in pioneering gastric fiberscope prototypes, which have greatly facilitated the progress of electronic endoscopy. These remarkable achievements have served as catalysts for extensive research conducted by various institutions and scholars.

In the 1950s, Charlie at Mayo Clinic pioneered the systematic study of esophageal motility, and led to the landmark discovery of the Lower Esophageal Sphincter (LES) (Code et al., 1956). Later, many American gastroenterologists focused on esophagology and became prominent esophagologists, including Donald Castell, who was called the "Pope of Esophagology." He took the initiative to establish a multidisciplinary collaboration with Radiology and cardiothoracic surgery, forming a cohesive team. He initiated multidisciplinary collaborations including esophageal, radiology, and cardiothoracic surgery, as well as establishing close contact with researchers from Japan and Australia. Additionally he innovatively integrated radiologic imaging with manometry techniques to clarify esophageal muscle function and summarized various concepts pertaining to esophageal motility disorders that hold significant value for further investigation. Such as ineffective esophageal motility (IEM) (Leite et al., 1997), achalasia (Olsen et al., 1957), TLESRs (Dent et al., 1980). He also collaborated closely with Tom DeMeester, a cardiothoracic surgeon, who proposed the DeMeester score for assessing reflux events and acid exposure. This contribution played a significant role in the subsequent advancement of surgical techniques for refractory GERD (Grubic and Crookes, 2021). We can gain valuable insights from their successful experience through the aforementioned development process. Multi-disciplinary treatment (MDT) and multi-regional and institutional collaboration serve as the driving forces for advancing progress in the research field. This model provides experts with the opportunity to discuss the issues they face and express different views, as well as the possibility of innovation. In addition, the rapid development of modern science and technology is also crucial to the impact of this disease. Endoscopic technology,

pressure transducer technology, and the pressure topography plots mentioned above are the technical support and guarantee for progress.

In terms of authors, Professor Iwakiri Katsuhiko has the highest number of publications and collaborates most closely with other authors. The author with the highest H-index is Professor Ronnie Fass. The most highly cited author is Professor Daniel Sifrim, who has expertise in neurogastroenterology and motility and pathophysiology of GERD. These three distinguished authors hold prominent positions within this domain and are poised to contribute significantly towards groundbreaking discoveries in refractory GERD. The most cited journals are *GUT* and *GASTROENTEROLOGY*, while *DIGESTION* has emerged as a popular choice among authors for publication purposes. This information can serve as a valuable reference for assessing journal quality and selecting appropriate outlets within this specialized field.

Although the number of article citations is influenced by publication length, it is undeniable that highly cited articles serve as the foundation for subsequent advancements in the field. These articles encompass valuable knowledge worth learning and the key issues that subsequent researchers are concerned about. From the distribution of the top 15 cited literature types in this study, clinical trials accounted for 80%, and the remaining 20% were consensus and reviews.

Clinical trials primarily focused on the efficacy evaluation and long-term follow-up results of drug interventions and surgical treatments. And the accurate identification of refractory symptoms, the clinical value of new detection methods, and their parameters. However, there remains a scarcity of highly cited basic research in this field.

From the evolution of keywords over time, we can see that researchers' thoughts and strategies are interestingly divided into two different directions. A portion of the group spent a lot of effort upfront (probably before 2010) in the study of the efficacy of PPIs in refractory GERD, and the exploration of the mechanisms of acid reflux. Consequently, they continue to advocate for acid inhibition therapy as the preferred strategy. Their focus lies in enhancing the efficiency of acid inhibition, developing novel and more potent acid inhibition agents, accurately identifying refractory individuals who would benefit from strong acid inhibition therapy, and improving the duration during which stomach pH levels are maintained above 4.

With the researchers' further investigation into the pathogenesis of refractory GERD and the persistence of acid inhibition therapy failure, another part of people are gradually recognizing the intricate multifactorial mechanisms involved. Consequently, their interest and patience in acid inhibition therapy for refractory patients who exhibit resistance to acid suppression medications have waned. They have then shifted their focus to intervening in various alternative mechanisms, such as physiological acid or weakly acidic reflux events, persistent mucosal microscopic damage (dilated intercellular spaces), chemical clearance ability, ineffective esophageal motility, TLESRs, visceral hypersensitivity, neuroimmune-mediated responses, psychological factors (stress/anxiety and hypervigilance). The objective is to utilize these emerging mechanisms for the development of innovative pharmacological and non-pharmacological therapies encompassing behavioral interventions, endoscopy, and surgery.

4.2 Standardized diagnosis and management

The definition of refractory GERD has been a subject of controversy, primarily concerning the treatment regimen and daily dosage of PPIs, as well as the criteria for recognizing ineffectiveness or poor response. In 2006 (Richter, 2006), refractory GERD was defined in a study as the absence of significant symptom improvement after 4–8 weeks of twice-daily PPIs treatment. The 2009 guideline (Fass and Sifrim, 2009b) published by Ronnie Fass and Daniel Sifrim defines refractory GERD as the lack of response to once-daily treatment with PPIs. According to the 2012 guidelines (Sifrim and Zerbib, 2012b) for managing refractory GERD, persistent symptoms of regurgitation or heartburn occurring at least three times per week are observed after more than 12 weeks of treatment with double-dose PPIs.

Since then, researchers have gradually realized that the determination of "refractory" status is largely influenced by patients' subjective perceptions and treatment expectations rather than objective assessments. In 2021, the European Society for Neurogastroenterology and Motility (ESNM) and the American Neurogastroenterology and Motility Society (ANMS) (Zerbib et al., 2021b) have made significant advancements in distinguishing subjective symptoms from objective evidence by proposing three definitions: refractory GERD, refractory reflux-like symptoms, and refractory GERD symptoms. Among them, refractory GERD is defined as objective evidence of GERD that has not disappeared after standardized drug treatment, including (erosive esophagitis, abnormal esophageal acid exposure and/or elevated numbers of reflux episodes on reflux monitoring performed on therapy). The advantage of this classification is that it can change the basis for judging whether the disease is "refractory" or not from the patient's subjective feeling to the objective indicators derived from the clinician's specialized examination, which can help to strictly differentiate the patients with truly refractory GERD, and make more accurate judgments of the patient's condition. This way of defining may be the trend in the future.

4.3 Evolution and characteristics of different drugs

Based on the specificity of the definition of refractory GERD, we need to take into account the changes in physiopathology caused by clinical treatment before the diagnosis of this disease in this population. It has been shown that erosive oesophagitis healing rates can be as high as 90% after 8 weeks of PPIs or vonoprazan treatment (Xiao et al., 2020). The fact that the patient was already receiving regular acid-suppressive therapy prior to the discovery of refractoriness may have led to the healing of the eroded esophageal mucosa, which may explain the following two findings. The first notable observation is the high prevalence of non-erosive reflux disease (NERD) among patients with refractory GERD (Kim et al.,

2015). A second finding reveals that individuals with NERD exhibit lower rates of symptomatic response to PPIs compared to those with RE (Martinez et al., 2003). In addition, the strong acid-inhibiting effect of acid-suppressive drugs may affect the acidic reflux of pH monitoring in patients. Marcelo (Vela et al., 2001) compared esophageal impedance-pH monitoring results in 12 patients with GERD before and after omeprazole treatment and found that the proportion of acid reflux decreased from 45% to 3% before treatment, while the proportion of non-acidic reflux (pH ≥ 4) reflux increased from 55% to 97%. Furthermore, numerous studies have documented the presence of weakly acidic reflux outside of the normal range in patients with refractory GERD (Mainie et al., 2006; Sharma et al., 2008; Frazzoni et al., 2011b; Kawami et al., 2018). Radu (Tutuian et al., 2008) conducted a study in patients with persistent symptoms despite acid suppressive therapy and observed 3,547 reflux events, of which 84.3% were non-acidic. However, it is unclear whether the high percentage of non-acidic reflux is attributable to the treatment or represents unique pathological manifestations associated with the condition and its possible role in the development and progression of refractory GERD. The causality between the high rate of non-acidic reflux and acid-suppressive treatment remains uncertain, as well as its potential role in the pathogenesis and progression of this disease.

Insufficient acid inhibition is one of the underlying mechanisms contributing to this disease, and researchers have turned to replacing potent acid inhibition agents as a potential solution. Since its initial global approval in Japan (Garnock-Jones, 2015) on 26 December 2014, Vonoprazan has emerged as a recommended treatment for refractory GERD due to its superior efficacy in suppressing acid production and promoting esophageal mucosal healing compared to PPIs (Miwa et al., 2019; Tanabe et al., 2019; Ochiai et al., 2021; Xu et al., 2023). Meanwhile, it exhibits no susceptibility to variations in CYP2C19 genotype (Kagami et al., 2016). However, a retrospective analysis of a small sample revealed that even with double doses of P-CAB, some NERD patients still exhibited poor responses. In comparison to the effective group, the ineffective group showed an increased proportion of 4 ≤ pH ≤ 5, along with a decrease in acid exposure time (AET). And researchers propose that a pH of 5 may be the threshold for influencing symptom onset (Abe et al., 2021). In Noriyuki Kawami's study (Kawami et al., 2018), all 42 double-dose P-CAB-resistant NERD patients without esophageal motility disorders had no detectable abnormal acid exposure, and 41.9% of these patients were SI-positive, all with weakly acidic reflux. The possible mechanism of refractory GERD is now more clear. Weakly acidic reflux events and the amplificated sensitivity of the esophagus to refluxants play a crucial role in symptom occurrence, making further acid inhibition an unwise choice for symptom relief.

In response, researchers have attempted a therapeutic shift from antiacid to antireflux therapy, focusing on the transient lower oesophageal sphincter relaxations (TLESRs), a key pathogenesis of refractory GERD (Hershcovici et al., 2011). Baclofen, a gamma-aminobutyric acid (GABA)-B receptor agonist, has been found to effectively reduce TLESRs (Koek et al., 2003). A recent randomized, double-blind, placebo-controlled study (Pauwels et al., 2022; Raymenants et al., 2022) found that Baclofen could significantly reduce SAP positivity, that is, symptoms associated with reflux episodes. However, its clinical application is hindered by the side effects resulting from its ability to penetrate the blood-brain barrier (Kent et al., 2020). Another placebo-controlled cross-over study (Sawada et al., 2020) reported that ONO-8539, an E-type prostanoid 1 receptor antagonist, inhibits TLESRs. These findings suggest that EP1 receptor may be a potential target for the treatment of refractory GERD.

The efficacy and benefit of gastrointestinal prokinetics in this condition remain uncertain. One perspective (Ishimura et al., 2015) suggests that Acotiamide has minimal impact on esophageal body contractions or EGJ compliance in both patients with GERD and healthy individuals. However, other studies (Yamashita et al., 2015; Yamashita et al., 2019) have indicated that Acotiamide can reduce TLESRs, improve esophageal bolus clearance in healthy individuals, alleviate persistent symptoms of refractory NERD, and decrease total reflux episodes. These episodes include acid reflux, proximal reflux, and liquid reflux. A recent meta-analysis (Jung et al., 2021) suggested that combining prokinetics with PPIs is more effective than using PPIs alone.

4.4 Advantages and disadvantages of surgery

For reflux events, surgeons tend to focus on the anatomical structure and physiological motor function of the esophagus to remodel the antireflux barrier. In the 1850s, Allison (Allison, 1951) first reported hiatal hernia repair, while Rudolf Nissen (Nissen, 1956) invented fundoplication, which initiated surgical intervention for GERD. In 1991, antireflux surgery (Dallemagne et al., 1991) entered the laparoscopic era. The development trend now leans towards more minimally invasive and readily accepted endoscopic antireflux surgery. Since the emergence of antireflux surgery, addressing symptoms that are unresponsive to medical treatment or severe esophagitis has become a primary concern. Anti-reflux mucosectomy (ARMS) and radiofrequency energy delivery (STRETTA), as emerging endoscopic antireflux procedures, have demonstrated comparable clinical efficacy. The former inhibits reflux by inducing scar contractures in the damaged cardiac mucosa, making it a minimally invasive and effective treatment for refractory GERD. The 270 ARMS is recommended for reducing the incidence of postoperative dysphagia (Sumi et al., 2021; Yang et al., 2022; Zhang et al., 2022).

The latter is proven to induce LES muscle remodeling through stimulation, thereby reducing the occurrence of TLESRs and esophageal acid exposure. During a follow-up period of 10 years after surgery, 72% of patients with refractory GERD returned to a normal quality of life, and 64% reduced their use of PPIs by at least half (Noar et al., 2014). It is recommended as the primary choice for endoscopic treatment following unsuccessful fundoplication (Katz et al., 2013c; Wang Y. et al., 2023).

Additionally, a range of surgical options, such as transoral incisionless fundoplication (TIF) and magnetic sphincter augmentation (MSA), are available to alleviate refractory symptoms (McCarty et al., 2018; Roark et al., 2020; Kalapala et al., 2022; Patel et al., 2022). However, most of the current exploration of surgical interventions for this condition is based on retrospective studies and case reports. Randomized controlled trials and long-term follow-up, as well as the development of

personalized surgical recommendations for refractory GERD, are the focus of future research.

It is important to acknowledge that a high level of confidence in the diagnosis of refractory GERD is crucial before considering any invasive surgical intervention. The ICARUS guidelines highlight the necessity of preoperative esophageal manometry and impedance-pH monitoring if endoscopy is negative. The results of high-quality randomized, controlled trials (Spechler et al., 2019) in 2019 suggested that surgery is superior to medical therapy for patients with truly established refractory GERD. In September 2023, the Lyon consensus 2.0 recommended a switch to surgery in patients with refractory GERD who had both AET > 4% and more than 80 reflux episodes (Spechler et al., 2019; Gyawali et al., 2023).

Visceral hypersensitivity is a complex mechanism mediated by multiple factors that have been suggested to be potentially involved in the development of refractory GERD, including the expression of acid-sensing ion channels, localization of sensory nerve, as well as interactions between inflammatory mediators and neurotransmitters (Guarino et al., 2010; Ustaoglu et al., 2021; Ustaoglu and Woodland, 2023). A study by Rohof (Rohof et al., 2014) found that patients with refractory GERD had more proximal reflux than patients who responded to PPIs. Philip (Woodland et al., 2015) compared the mucosal integrity of the proximal and distal esophagus in healthy subjects and localized calcitonin gene-related peptide (CGRP)-immunoreactive nerve fibers and protein gene product (PGP) 9.5 immunoreactivity in nerve fibers and found that mucosal integrity was essentially the same at both ends, but that the proximal mucosa had more superficial afferent nerves. This unique feature provides anatomical evidence for proximal esophageal hypersensitivity. This finding is supported by a previous study conducted by Radu (Tutuian et al., 2008), which demonstrated a significant correlation between proximal reflux and the onset of symptoms in patients with refractory GERD, irrespective of whether the refluxants were acidic or non-acidic. Furthermore, esophageal hypervigilance may independently contribute to symptom perception in individuals with GERD (El-Serag et al., 2010b; Guadagnoli et al., 2021).

4.5 Novel mechanisms such as neuroimmune interaction

In recent years, with the advancement of research in neurology and immunology, the interaction between neuroimmunity has attracted the attention of researchers, particularly in peripheral organs such as the gastrointestinal tract. It has been reported that immune activation interacts with various gastrointestinal disorders, and visceral hypersensitivity may be associated with the stimulation of sensitive neurons by other algogenic mediators secreted by immune cells (Vanuytsel et al., 2023; Argüero and Sifrim, 2024; Leech and Peiris, 2024). The mast cells, which are part of the immune system, exhibit sensitivity to the endogenous microenvironment of immune cells. They induce sensitization of peripheral nerve function by releasing neuropeptides, such as histamine and other nociceptive mediators that act as pain inducers (Rosa and Fantozzi, 2013; Gupta and Harvima, 2018; Leech and Peiris, 2024). Ustaoglu's study revealed an upregulation of nerve growth factor (NGF) expression in mast cells infiltrating the esophageal mucosa of patients suffering from reflux esophagitis (Ustaoglu et al., 2023). NGF plays a pivotal role in the development of chronic pain by binding to tyrosine kinase receptor A (NTRK1) on nerve fibers, thereby augmenting their numbers and eliciting hypersensitive pain sensations (Dothel et al., 2015; Eskander et al., 2015; Gupta and Harvima, 2018). Additionally, they assessed the association between mast cells and deep afferent nerve endings and discovered that these two are closely juxtaposed within the papillary structure of the esophageal mucosa, potentially contributing to hypersensitivity responses. They suggested that topical NGF antagonists could be a prospective therapeutic option for refractory GERD (Ustaoglu et al., 2023). The abundance of neuroimmune-related receptors offers diverse possibilities for selecting therapeutic targets.

Spechler's team hypothesized (Souza et al., 2009) as early as 2009, following esophagoduodenostomy in rats and *in vitro* studies, that the tissue damage observed in reflux esophagitis may not be attributed to chemical corrosion caused by stimulation from refluxed gastric juice. Instead, it is more likely induced by acidified bile salts stimulating esophageal epithelial cells to secrete chemokines (IL-8 and IL-1β) and promoting inflammation. To further substantiate this hypothesis (Dunbar et al., 2016), a recent clinical study involving GERD patients with recurrent esophageal mucosal erosion after PPIs withdrawal found that the predominant inflammatory cells infiltrating the esophagus were T lymphocytes rather than neutrophils. Surface cell loss did not occur immediately but was observed subsequent to basal cell and papillary hyperplasia, providing evidence in support of a cytokine-mediated pathogenesis. Subsequent experiments have demonstrated (Huo et al., 2017; Souza et al., 2017) the crucial involvement of HIF-2α in RE pathogenesis, as it is activated by acidified bile salts and subsequently amplifies NF-kB/p65 activity, thus facilitating proinflammatory cytokine synthesis.

4.6 Considerations for various detection techniques

The Lyon Consensus, published in 2018, is based on the 2004 Porto (Sifrim et al., 2004) Consensus and, for the first time, puts forward the key recommendation based on a large number of clinical studies: impedance-pH monitoring is the "gold standard" for diagnosing GERD. The consensus also focuses on new parameters such as post-reflux swallow-induced peristaltic wave (PSPW), mean nocturnal baseline impedance (MNBI), and enriching objective detection indicators for refractory GERD. PSPW and MNBI are parameters that reflect the chemical clearance ability of the esophagus and the integrity and permeability of the esophageal mucosa, respectively. These two parameters have been shown to better identify pathological reflux (Wu et al., 2022) in patients with refractory reflux symptoms and can be used as characteristic indicators of refractory GERD (Frazzoni et al., 2023).

Clinicians are faced with two choices before recommending patients for endoscopy and impedance-pH monitoring, on or off PPIs? Lyon consensus 2.0 provides the answer; for patients with refractory GERD who have a previous diagnosis based on objective evidence, it is recommended that impedance-pH monitoring be performed during PPIs therapy, which can help to predict the

outcome of surgery based on the regurgitation results (Gyawali et al., 2021). Conversely, if there is no objective diagnostic basis, impedance-pH monitoring after discontinuation of PPIs is recommended (Hemmink et al., 2008a; Katz et al., 2022). In addition, simple pH monitoring without impedance function is also recommended after withdrawal to rule out the effect of PPIs on acid reflux (Sifrim and Zerbib, 2012b). Considering the high rate of esophageal mucosal healing with antiacid drugs, the American College of Gastroenterology (ACG) suggests (Katz et al., 2022) that diagnostic endoscopy should be conducted 2–4 weeks after withdrawal PPIs for maximizing the accuracy of diagnosis of refractory GERD.

According to previous researches (Gawron et al., 2012), up to 42% of patients with refractory GERD continue taking PPIs despite negative findings on endoscopy and impedance-pH monitoring. This has prompted researchers to consider whether patients with refractory GERD can be terminated from long-term use of PPIs. A recent clinical trial (Yadlapati et al., 2021) has suggested that the number of days of AET ≥4% can be used to determine the necessity of continuing PPIs in patients with endoscopy-negative refractory GERD through prolonged wireless reflux monitoring. However, with the limitations of the paucity of relevant data and the fact that prolonged wireless reflux monitoring is not widely available in clinical practice, this issue remains to be resolved.

While impedance-pH monitoring is widely recommended as a reliable diagnostic tool, it has been regarded by certain scholars as a delicate examination susceptible to temporal variations. Roberto (Penagini et al., 2015b) performed prolonged wireless pH monitoring in 50 AET-negative patients with refractory heartburn, and found that half of them exhibited positive acid exposure after the second or third day. In a study by Yadlapati (Yadlapati et al., 2019), patients who did not respond to PPIs showed three distinct acid exposure trajectories during prolonged pH monitoring. Stephen (Hasak et al., 2020) found that the results of AET varied from day to day in patients with refractory GERD, with dominant AET patterns from long-term wireless pH monitoring being poorly correlated with AET measured on the first day. The above studies suggest that longer monitoring time may make AET and other indicators closer to real events and improve the accuracy of diagnosis.

4.7 Inspiration from research hotspots and emerging trends

The aforementioned research focal points and emerging trends will serve as valuable references for future research orientations in the subsequent areas. The first aspect involves the introduction of a new definition and diagnosis, where it is clinically essential to differentiate refractory GERD from refractory reflux-like symptoms and refractory GERD symptoms. Objective indicators (including emerging ones such as PSPW and MNBI) should be prioritized over subjective feelings as the key diagnostic criteria. This is beneficial in precisely identifying refractory GERD and facilitating a more objective and accurate assessment of the patient's condition. Furthermore, an extended application of prolonged wireless pH monitoring would effectively reflect the most realistic reflux events and contribute to clinical diagnosis. This kind of precise diagnosis will undoubtedly become the prevailing trend in the future.

The second aspect involves the prognostication of therapeutic approaches grounded in innovative mechanisms. The release of pain mediators by immune cells can induce sensitization of peripheral nerves. Current research has demonstrated that there are numerous receptors upstream and downstream, which are associated with neuroimmunity, including neuropeptides, histamine, nociceptive mediators, NGF, and HIF-2α, may serve as crucial targets for the treatment of this disease and hold significant therapeutic potential. Therefore, the development of inhibitors such as NGF antagonists and HIF-2α inhibitors represents a promising direction for novel drug discovery. This is particularly advantageous for patients experiencing physiological acid regurgitation or non-acid regurgitation, with heartburn and retrosternal pain being the primary clinical manifestations.

The findings of various studies have demonstrated that 17-phenyl PGE2, acting as an EP1 agonist, exhibits a biphasic impact on esophageal mucosal inflammation. Specifically, it exerts a protective effect on the esophageal mucosa at lower doses (0.1 and 0.3 mg/kg), while displaying damaging effects at higher doses (1 mg/kg). Both of these effects are mediated through the activation of the EP1 receptor (Yamato et al., 2005; Takeuchi and Amagase, 2018). In addition, a randomized, single-blind, placebo-controlled, cross-over trial in recent years found that EP1 receptor may also be involved in the occurrence of TLESRs. An EP1 receptor antagonist, ONO-8539, has been shown to significantly reduce the frequency of TLESRs (Sawada et al., 2020).These findings suggest that targeting the EP1 receptor could be a promising therapeutic approach for patients with refractory GERD and further researches on drugs acting on EP1 receptor should be designed. The development of these novel drug types remains a highly active research field, playing a pivotal role in addressing the concerns of patients who are apprehensive about undergoing endoscopic or surgical interventions.

The third is that joint behavioral interventions may be beneficial. We hypothesized that developments in the field of psychogastroenterology may provide a new treatment for this disease until new breakthrough drugs are invented. Presently, an increasing number of studies are uncovering the significant role played by psychosocial factors in refractory GERD (Riehl and Chen, 2018; He et al., 2022) such as depression (Kimura et al., 2016), anxiety (Ribolsi et al., 2023) and sleep disorders (Kawara et al., 2017). The repeated and refractory discomfort experienced by patients often leads to feelings of disappointment and helplessness, which not only amplifies the perception of symptoms but also impacts medication adherence and responsiveness to medications such as PPIs.

For these patients, neuromodulators such as selective serotonin reuptake inhibitors (SSRIs) can serve as crucial adjuncts to stabilize mood and ameliorate symptoms. Fluoxetine demonstrated superiority over omeprazole in improving heartburn symptoms, particularly among patients without esophageal mucosa erosion and normal acid exposure (Ostovaneh et al., 2014). One study have shown that taking 20 mg of citalopram daily can improve symptoms in patients with hypersensitive esophagus by 61.5% compared to 33.3% with placebo (Viazis et al., 2012). Therefore, gastroenterologists should consider collaborating with

psychological experts to conduct a thorough evaluation of patients with anxiety, depression, and other psychological disorders. In cases where these conditions coexist, combination therapy should be considered to enhance the efficacy of refractory GERD treatment. In addition, there is an increasing utilization of behavioral interventions targeting these underlying mechanisms, such as cognitive behavioral therapy (CBT), which advises clinicians to guide patients away from solely seeking all the symptoms relief. Encouraging the acceptance and tolerance of residual symptoms may help alleviate the negative emotions experienced by the patient (Riehl et al., 2015; Sawada et al., 2019). The intervention of diaphragmatic breathing training (DBT) has the potential to enhance therapeutic outcomes for GERD, while also contributing to improved sleep quality and overall life satisfaction. This improvement may be attributed to the mechanism of increasing the disparity between LES and stomach pressure to reduce postprandial reflux events (Halland et al., 2021; Zdrhova et al., 2023; Mosa et al., 2024). Moreover, Esophageal directed hypnotherapy has been shown to be an effective mode of treatment for regulating discomfort by adjusting the patient to a state of deep relaxation and concentration (Riehl et al., 2016).

4.8 Potential barriers and challenges

In fact, despite decades of extensive research, there are only two drugs, namely PPIs developed early on and P-CAB approved in 2016, which have profound impact and are widely recommended by consensus for the research field of GERD (whether RE, NERD or refractory GERD) (Talley and Zand, 2021). Currently, there are ongoing studies aimed at optimizing acid-suppressive therapy to enhance the management of refractory conditions. For example, from the perspective of pharmacokinetics and pharmacodynamics, they recommend refractory GERD patients to take acid inhibitors before meals as an important drug administration strategy because there is indirect evidence that this is more effective in alleviating symptoms (Zerbib et al., 2021a). Moreover, refractory GERD patients with persistent esophagitis or continuous esophageal acid exposure would derive greater benefits from stronger PPIs medications (Kinoshita et al., 2018). However, both drugs are essentially acid suppressants that act on the acid reflux mechanism by acting on the H+/K+-ATPase of gastric parietal cells. Given the intricate pathogenesis, an increasing number of researches have directed their attention towards factors other than acid reflux. For instance, the therapeutic effects of alginates are achieved by displacing the postprandial gastric acid pocket to alleviate distress in patients experiencing breakthrough symptoms (De et al., 2014; Leiman et al., 2017). Patients with persistent nocturnal acid breakthrough (impedance-pH monitoring suggested intragastric pH < 4 for more than one continuous hour overnight) can try to add Histamine type-2 receptor antagonists (H2RAs) at bedtime, but the supporting evidence is limited (Fackler et al., 2002; Mainie et al., 2008; Wang et al., 2009). The recent phase IIb study has confirmed that IW-3718, a gastric-retentive and extended-release formulation containing the bile acid sequestrant, effectively binds and sequesters bile acids enroute to the esophagus, providing significant relief for refractory heartburn and acid reflux symptoms (Vaezi et al., 2020). There are also Baclofen, a gamma-aminobutyric acid (GABA)-B receptor agonist and Prokinetics mentioned above. All of these medications have the characteristic of being only adjunctive or experimental to acid suppressant to attempt to alleviate the distress of patients with different clinical characteristics.

This poses the first significant challenge in the management of refractory GERD. Despite the development of various drugs targeting mechanisms beyond acid inhibition, their clinical application and evidence support remain limited, failing to provide definitive critical efficacy. Currently, there is an unmet need for new first-line drug options for refractory patients, as large-scale clinical studies lack evidence in this regard. Ongoing research focusing on novel mechanisms such as neuroimmunology and psychosomatic factors holds great promise.

The second challenge is that the pathogenesis of refractory GERD is not simple and homogenous, it is influenced by dozens of factors as mentioned above, which means that we cannot be sure which factor plays the most important role in this disease. In response, multi-targeted therapies and multidisciplinary collaborative treatment programs may be the way and the hope to overcome these barriers.

The third challenge lies in accurately identifying patients who truly have refractory GERD from a clinical perspective. A rigorous procedure should involve allowing patients to comply with an 8-week pre-treatment of PPIs, completing a questionnaire, and undergoing screening by specialists. Finally, objective indicators are utilized to confirm the suitability of conditions. It may even be necessary to employ prolonged wireless pH monitoring and conduct multiple high-resolution manometry tests. However, the current trend in clinical studies is to overlook this step and place greater emphasis on patients subjective outcomes. It is imperative for clinicians and researchers to allocate more attention to this aspect.

5 Conclusion

According to our bibliometrics insights, the following directions are expected to be developed in the future. Firstly, high-quality clinical studies should not overly rely on patients subjective perceptions but instead adhere strictly to diagnostic criteria and include only those patients who genuinely meet the requirements. This is crucial for minimizing misclassification bias in statistical findings. Secondly, the research in the field of neuroimmunology and psychopsychology is poised to become a pioneering area, with potential for significant advancements. The collaboration among diverse disciplines, including joint expertise from psychological professionals, can greatly facilitate the progress in this domain. Moreover, behavioral intervention is currently a prominent area of research and is anticipated to function as an alternative therapeutic approach until novel, stable medications targeting multiple mechanisms are developed. Finally, close cooperation between different disciplines and regions and keeping up with the frontiers of modern science and technology should be an effective way to promote the innovative development of research on refractory GERD.

It is worth emphasizing that, our study is the first bibliometric analysis of the global literature on refractory GERD, elucidating the research process and evolutionary trends in this field from 2000 to 2023. This work visually demonstrates the development status, cooperative

relationship, research focus, and possible hotspots of this disease. Currently, research in this area focuses on standardized diagnosis and management, and in-depth exploration of novel mechanisms such as neuroimmune interaction. Meanwhile, efforts should also be made to design simpler, more accurate, and more stable monitoring methods and seek new treatment options based on different mechanisms, including the development of innovative drugs and procedures. In conclusion, it is hoped that this research will provide valuable insights for researchers, enabling them to quickly and comprehensively understand the historical background, current status, and future directions of refractory GERD.

5.1 Limitations

This study has limitations. Firstly, due to the constraints of bibliometric tools, we exclusively relied on the more authoritative and comprehensive database-Web of Science for data retrieval. Secondly, although our retrieval method was designed to be relatively comprehensive, it inevitably failed to capture certain articles. Additionally, subjective and professional experience limitations may have influenced the data-cleaning process. These above issues might have impacted the accuracy of our retrieved data.

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

NZ: Conceptualization, Data curation, Methodology, Software, Visualization, Writing–original draft, Writing–review and editing. MH: Conceptualization, Data curation, Methodology, Visualization, Writing–original draft, Writing–review and editing. Q-WZ: Conceptualization, Writing–review and editing. M-YZ: Data curation, Writing–review and editing. W-LZ: Conceptualization, Writing–review and editing. J-JL: Conceptualization, Writing–review and editing. L-XC: Data curation, Writing–review and editing. J-LT: Data curation, Writing–review and editing. YW: Data curation, Writing–review and editing. S-QF: Funding acquisition, Project administration, Resources, Supervision, Validation, Writing–original draft, Writing–review and editing.

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Conflict of interest

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

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EDITED BY
Daniele Maria-Ferreira,
Instituto de Pesquisa Pelé Pequeno Príncipe, Brazil

REVIEWED BY
Shanu Jain,
Indian Institute of Technology Kanpur, India
Fuguo Shi,
Zunyi Medical University, China

*CORRESPONDENCE
Qun Liu,
liuquncpu@126.com
Lian-Wen Qi,
qilw@cpu.edu.cn

†These authors have contributed equally to this work and share first authorship

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CPS1 augments hepatic glucagon response through CaMKII/FOXO1 pathway

Xiao-Meng Sun[1,2†], Xin Wu[1†], Meng-Guang Wei[1,2], Li-Zeng Zhu[1,2], Wen-hui Wu[1,2], Xin-Yue Zhou[1,2], Lian-Wen Qi[2*] and Qun Liu[1,2*]

[1]State Key Laboratory of Natural Medicines, School of Traditional Chinese Pharmacy, China Pharmaceutical University, Nanjing, China, [2]Clinical Metabolomics Center, China Pharmaceutical University, Nanjing, China

Introduction: Elevated glucagon levels are a characteristic feature of type 2 diabetes. This abnormal increase in glucagon can lead to an accelerated rate of gluconeogenesis. Glucagon also stimulates hepatic metabolism of amino acids, particularly promoting the formation of urea. The specific role of carbamoyl phosphate synthetase 1 (CPS1), a rate-limiting enzyme in the urea cycle, in the development versus the persistence of glucagon-induced hyperglycemia has not been previously established.

Methods: The study employed both *in vivo* and *in vitro* approaches to assess the impact of CPS1 modulation on glucagon response. CPS1 was knockdown or overexpression to evaluate its influence on hepatic gluconeogenesis. In addition, an *in-silico* strategy was employed to identify a potential CPS1 inhibitor.

Results: Knockdown of CPS1 significantly reduced the glucagon response both *in vivo* and *in vitro*. Conversely, overexpression of CPS1 resulted in an overactive hepatic gluconeogenic response. Mechanistically, CPS1 induced the release of calcium ions from the endoplasmic reticulum, which in turn triggered the phosphorylation of CaMKII. The activation of CaMKII then facilitated the dephosphorylation and nuclear translocation of FOXO1, culminating in the enhancement of hepatic gluconeogenesis. Furthermore, cynarin, a natural CPS1 inhibitor derived from the artichoke plant, had the capacity to attenuate the hepatic glucagon response in a CPS1-dependent manner.

Discussion: CPS1 played a pivotal role in mediating glucagon-induced hepatic gluconeogenesis. The discovery of cynarin as a natural inhibitor of CPS1 suggested its potential as a therapeutic agent for diabetes treatment.

KEYWORDS

type 2 diabetes, gluconeogenesis, carbamoyl phosphate synthetase 1, CaMKII/FOXO1 pathway, cynarin

1 Introduction

Type 2 diabetes mellitus (T2DM) is intricately linked with a spectrum of metabolic disorders, including hyperlipidemia, non-alcoholic fatty liver disease, and obesity (Calcutt et al., 2009; Chen et al., 2019; Yu et al., 2019). Hyperglycemia, characterized by elevated blood glucose levels, is a defining clinical feature of T2DM. The maintenance of glucose homeostasis is a complex physiological process that is dependent on both hepatic gluconeogenesis and glycogenolysis. During fasting periods, gluconeogenesis,

a process regulated by glucagon, becomes particularly crucial (Consoli, 1992; Magnusson et al., 1992; Wajngot et al., 2001; Boden, 2004). In the context of T2DM, dysregulated glucagon secretion can lead to an overactivation of gluconeogenesis, and consequently, an increase in fasting plasma glucose concentrations. Therefore, therapeutic approaches that target the reduction of glucagon levels or inhibiting its downstream effects are of paramount importance in the management of hyperglycemia in individuals with T2DM.

Hepatic gluconeogenesis is intricately regulated by a variety of factors, including substrate levels, metabolites, and hormonal influences (Zhang X. et al., 2019; Sharabi et al., 2019). Amino acids and free fatty acids, derived from extrahepatic sources, can modulate this process both directly and indirectly (Mc and Gi, 2018; Pan et al., 2022). During periods of starvation, amino acids released from protein breakdown serve as key substrates for hepatic gluconeogenesis. The hepatic response to glucagon is predominantly mediated through the cyclic AMP (cAMP)/protein kinase A (PKA) signaling pathway (Gonzalez and Montminy, 1989). Glucagon plays a pivotal role in the interplay between amino acid metabolism and the urea cycle within the liver. It facilitates the regulation of amino acid metabolism through its influence on urea production (Hu et al., 2023). In a reciprocal manner, circulating amino acids can also prompt the secretion of glucagon from pancreatic α-cells, establishing a feedback loop (Holst et al., 2017; Wewer Albrechtsen et al., 2019). This interdependence underscores the significant role of amino acid metabolism in maintaining hepatic glucose homeostasis. The dynamic interaction between glucagon and amino acid levels is crucial for the liver's ability to balance glucose production and utilization, ensuring a stable internal glucose environment.

The urea cycle, alternatively referred to as the ornithine cycle, is a critical metabolic pathway responsible for the detoxification and elimination of ammonia generated from the metabolism of amino acids within the body (Schimke, 1962; Gropman et al., 2007). This cycle plays a vital role in the removal of excess nitrogen and ammonia that result from the breakdown of proteins or the synthesis of nitrogenous compounds in humans. Carbamoyl phosphate synthetase 1 (CPS1) is the first and a rate-limiting enzyme in the urea cycle, which catalyzes the condensation of ammonia with bicarbonate to produce carbamyl phosphate, a precursor to urea. CPS1 is indispensable in human metabolism, not only marking the entry point of ammonia into the urea cycle, but also having specific contribution to hepatic gluconeogenesis, however, is not well understood and remains an area of ongoing research. Understanding the interplay between CPS1 and gluconeogenesis could provide insights into the broader metabolic processes within the liver and their implications for health and disease.

Currently available therapeutic agents for T2DM have demonstrated remarkable efficacy. Unfortunately, these medications may also be associated with undesired side effects (Upadhyay et al., 2018; Shetty et al., 2022). By utilizing Traditional Chinese Medicine (TCM) syndrome differentiation and selecting appropriate drugs based on specific symptoms, Chinese medicine can effectively maintain lipid and glucose homeostasis while minimizing side effects, thereby preventing further deterioration of diabetes (Zhang et al., 2019b; 2019a; Lou et al., 2019; Wu G. D. et al., 2022). The treatment of diabetes using TCM holds great potential in achieving broad therapeutic outcomes.

This study was designed to elucidate the involvement of CPS1 in metabolic disorders, with a particular focus on its role in hepatic gluconeogenesis. Our findings indicated that CPS1 exerts its regulatory effect on hepatic gluconeogenesis predominantly through the Ca^{2+}/CaMKII/FOXO1 signaling cascade. Furthermore, the research revealed that cynarin, a compound derived from the artichoke plant, had the capacity to attenuate the hepatic glucagon response in a CPS1-dependent manner. This discovery suggested that CPS1 may serve as a novel target for therapeutic interventions aimed at modulating gluconeogenesis and addressing metabolic dysregulations associated with conditions such as diabetes.

2 Materials and methods

2.1 Animals

For long-term model, C57BL/6J male mice were fed rodent chow diet (10% kcal from fat; Xietong Organism, China) or switched to a high-fat diet (HFD, 60% kcal from fat; D12492; Research diet, America) at 8 weeks of age and fed for 12 weeks. Body weights and food intake were recorded twice a week. For glucagon model, C57BL/6J male mice were injected with 2 mg/kg glucagon intraperitoneally. Mice were housed with a 12-h/12-h light-dark cycle with free access to food and water. All animal treatments were approved by the Animal Ethics Committee of China Pharmaceutical University (protocol no. 2020-12-009). Metformin (200 mg/kg) or cynarin (50, 100 mg/kg) were given by gavage.

2.2 AAV8-mediated gene expression and knockdown

AAV8-CPS1 shRNA were injected to generate hepatocyte-specific CPS1 knockdown mice or AAV8- NC for control mice. 6 to 8-week-old mice were intraventricularly injected with AAV8 using a 29-gauge insulin syringe (BD). All tests were performed 4–5 weeks after AAV injection. The shRNA oligo used in our experiments are as follows:

NC shRNA: TTCTCCGAACGTGTCACGT; CPS1 shRNA: GCUCUUGCACAGCCACUAAUU.

2.3 Glucagon, pyruvate and oral glucose tolerance tests

For glucagon tolerance tests (GTT), mice were fasted for 12 h before i.p. injections of 2 mg/kg glucagon in saline solution. For oral glucose tolerance tests (OGTT), mice were fasted for 12 h before administered glucose intragastrically (i.g.) at a dosage of 2.5 g/kg body weight. For pyruvate tolerance tests (PTT), mice were fasted for 12 h before i.p. injections of 2 mg/kg glucagon in saline solution. Blood glucose levels were measured at 0, 15, 30, 60, 90, and 120 min after the injection.

TABLE 1 Primer pairs for qPCR.

Genes	Sense (5′-3′)	Anti-sense (5′-3′)
G6pc (mice)	CGACTCGCTATCTCCAAGTGA	GTTGAACCAGTCTCCGACCA
Pck1 (mice)	AAGCATTCAACGCCAGGTTC	GGGCGAGTCTGTCAGTTCAAT
Pgc1a (mice)	TATGGAGTGACATAGAGTGTGCT	GTCGCTACACCACTTCAATCC
Actb (mice)	AGTGTGACGTTGACATCCGTA	GCCAGAGCAGTAATCTCCTTCT
Cps1 (mice)	ACATGGTGACCAAGATTCCTCG	TTCCTCAAAGGTGCGACCAAT

TABLE 2 Primary antibodies for Western blotting.

Antibody	Catalog	Company	Application	Dilution
CPS1	Ab129076	Abcam	WB	1:1,000
β-actin	bs-00612R	Bioss	WB	1:1,000
phospho-PKA substrates	9621S	CST	WB	1:1,000
FOXO1	2880S	CST	WB/IF	1:1,000/1:200
p-CaMKII	AF3493	Affinity	WB	1:1,000
CaMKII	WL03453	Wanlei	WB	1:1,000
Mouse anti-rabbit IgG	ZB-2301	Zhongshanjinqiao	WB	1:1,000
Goat anti-mouse IgG	ZB-2305	Zhongshanjinqiao	WB	1:1,000

2.4 Hepatocyte culture and transfection

Isolated primary hepatocytes were cultured in the Dulbecco's modified eagle medium (DMEM) with 10% (v/v) fetal bovine serum (FBS). Cells were transfected with CPS1 siRNA using Lipofectamine® 2000 transfection reagent (ThermoFisher, America) at 70% confluence. Negative control siRNA was used as a control. CPS1 plasmid were also used to promote the expression of CPS1 while pcDNA3.1 were used as a control.

NC siRNA: UUCUCCGAACGUGUCACGUTT
ACGUGACACGUUCGGAGAATT;
CPS1 siRNA: GCUCUUGCACAGCCACUAAUU
AAUUAGUGGCUGUGCAAGAGC.

2.5 Quantitative PCR

Total RNA was extracted from cells and tissue samples using High Pure RNA Isolation Kit (Vazyme, China). Equal amounts of RNA samples were used for cDNA synthesis with a HIFair® III 1st Strand cDNA Synthesis SuperMix for qPCR (gDNA digester plus) (Yeasen, China). Quantitative PCR analysis was carried out using a Hieff® qPCR SYBR Green Master Mix (Low Rox Plus) (Yeasen, China). The primers used for quantitative PCR are listed in Table 1.

2.6 Western blotting

The total protein concentration was quantified with a BCA assay kit (Beyotime, China) for the normalization of assayed samples. The samples were applied to 8%–12% SDS-PAGE gels and probed with antibodies. Antibodies are listed in Table 2. Band intensity was measured using ImageJ software.

2.7 Hepatic glucose production

After fast the cells for 2 h in Krebs-Ringer HEPES (KRH) buffer, primary hepatocytes were washed by PBS and incubated in KRH buffer supplemented with 10 mM pyruvate, 100 nM glucagon for 6 h. The cell supernatant was collected for glucose analysis using the Glucose Assay Kit (Jiancheng bioengineering, China), and normalized to total cellular protein content.

2.8 Immunofluorescence

For fluorescence visualization of antibody reactions, primary antibodies were detected using secondary antibodies labeled with the fluorochromes FITC (Beyotime, China). To detect cell nuclei, primary hepatocytes were fluoresced with DAPI (Beyotime, China). Confocal images were acquired using a confocal scanning microscope (Olympus, Japan). All images were acquired with an 63× oil (NA 1.42) immersion objective lenses.

2.9 Molecular docking

We apply Surflex-Dock, a submodule of SYBYL software, for virtual screening. Download the three-dimensional structure of CPS1 protein from the protein database PDB.org and hydrogenate CPS1 protein. Select the active natural products

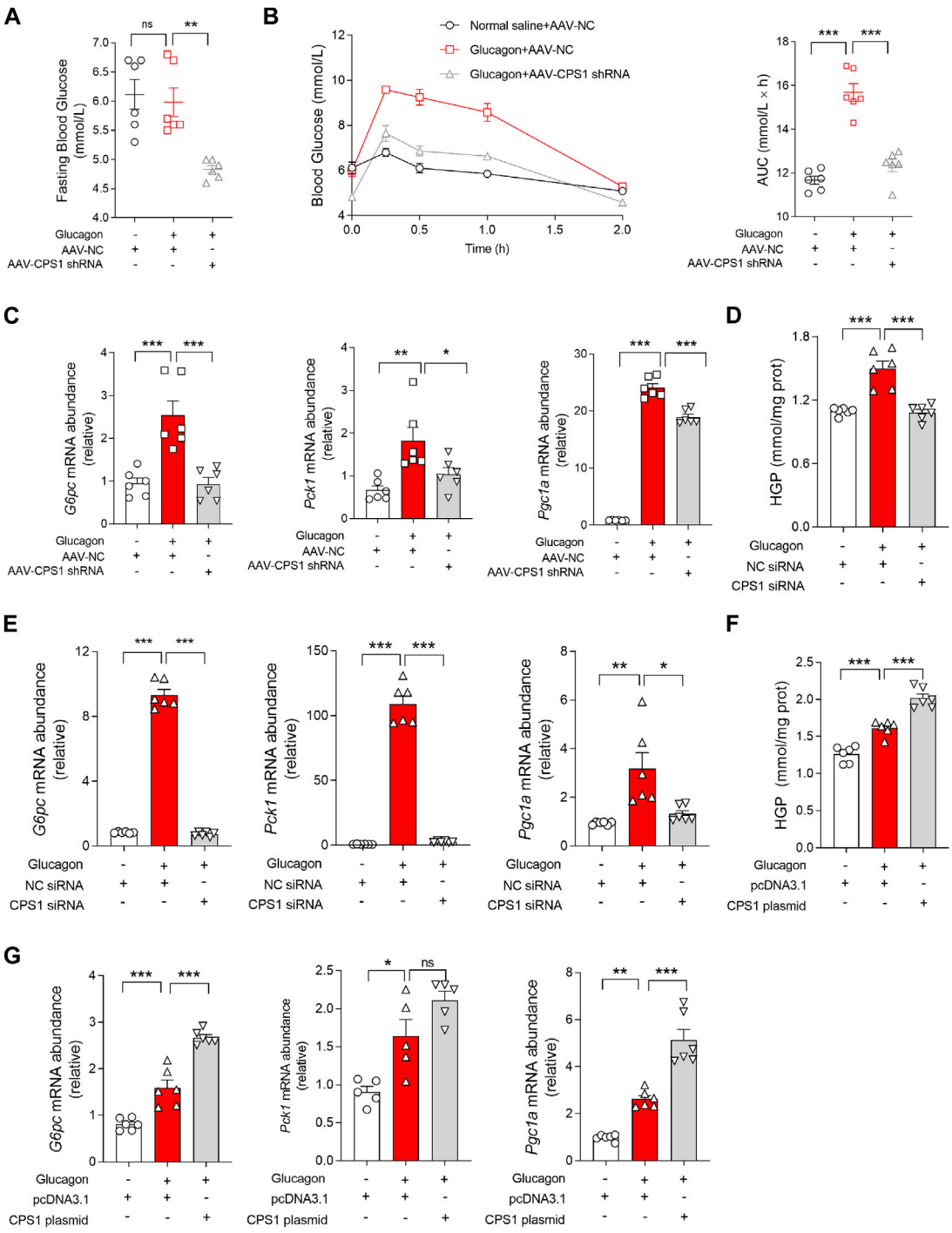

FIGURE 1
CPS1 activated glucagon-induced gluconeogenesis. **(A)** Fasting blood glucose of CPS1 liver specific knockdown mice (n = 6). **(B)** Blood glucose levels and AUC for mice treated with AAV8-CPS1 shRNA or AAV8-NC with glucagon challenge (2 mg/kg, n = 6). **(C)** qPCR analysis of the expression of *G6pc*, *Pck1*, and *Pgc1a* in the liver from the mice in panel **(B)** (n = 6). **(D)** Hepatic glucose production treated with CPS1 siRNA or NC siRNA stimulated by glucagon (100 nM, 1 h) in hepatocytes (n = 6). **(E)** qPCR analysis of the expression of *G6pc*, *Pck1*, and *Pgc1a* in panel **(D)** (n = 6). **(F)** Hepatic glucose production treated with CPS1 plasmid or pcDNA3.1 stimulated by glucagon (100 nM, 1 h) in hepatocytes (n = 6). **(G)** qPCR analysis of the expression of *G6pc*, *Pck1*, and *Pgc1a* in panel **(F)** (n = 6). AUC, area under the curve; HGP, hepatic glucose production; AAV, adeno-associated virus. Data were analyzed by one-way ANOVA. All values are represented as mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

library containing 724 compounds from www.MedChenExpress.com, download the three-dimensional structure of natural product molecules, and hydrotreat them. The structure optimization of the compounds should be made in advance. The docking process: 1) Generate ligand fragments to reduce the image construction space; 2) Superimpose the ligand fragment onto the prototype probe; 3) The ligand fragment for docking the remaining part. The docking results will be analyzed.

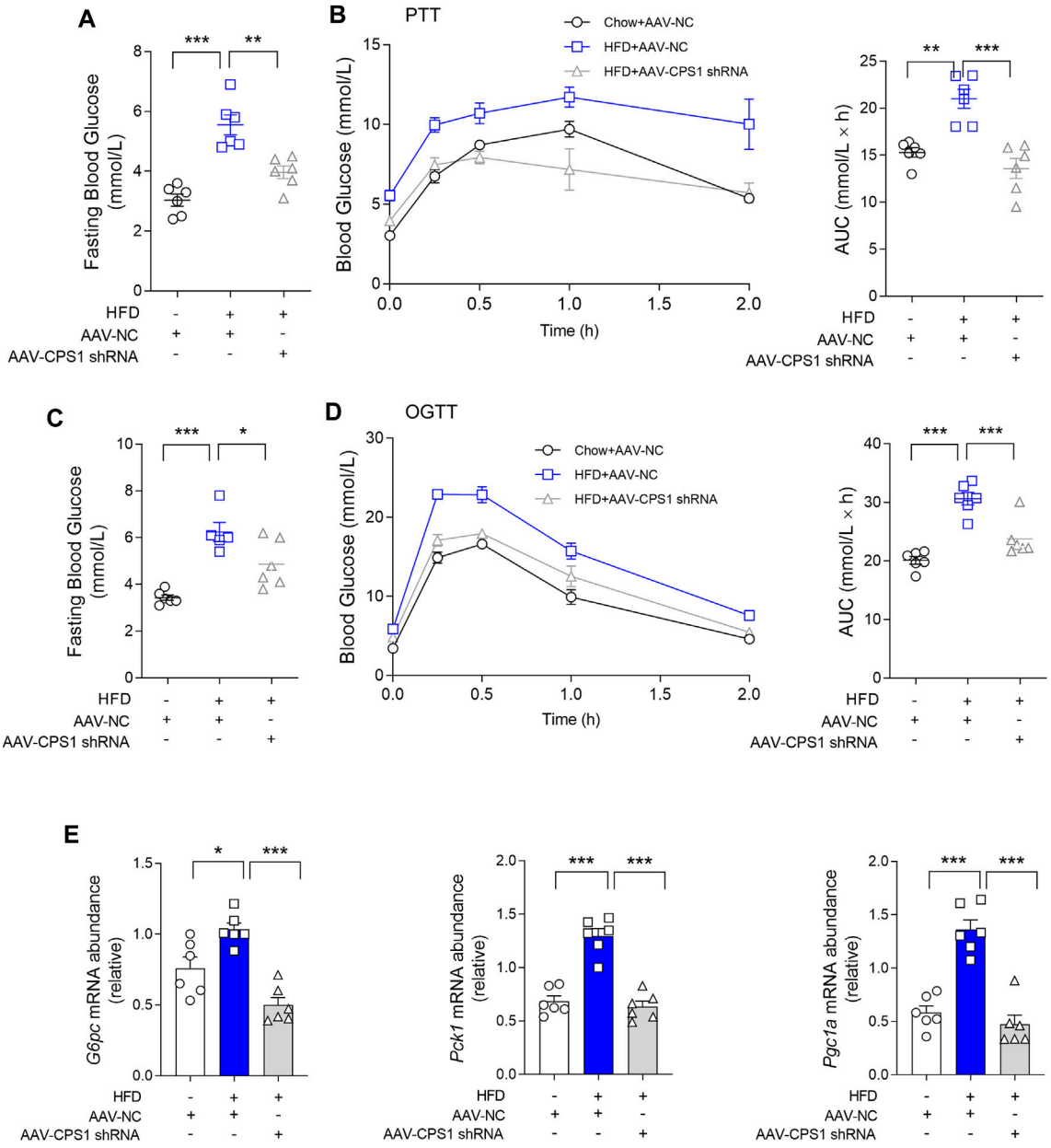

FIGURE 2
Liver specific knockdown of CPS1 attenuated hepatic gluconeogenesis in HFD-fed mice. **(A)** Fasting blood glucose of CPS1 liver specific knockdown mice after 11 weeks HFD feeding (n = 6). **(B)** Pyruvate tolerance test (2 g/kg body weight) after 11 weeks HFD feeding in mice treated with AAV8-CPS1 shRNA or AAV8-NC (n = 6). **(C)** Fasting blood glucose of CPS1 knockdown mice after 12 weeks HFD feeding (n = 6). **(D)** Oral glucose tolerance test (2.5 g/kg) after 12 weeks HFD feeding in mice treated with AAV8-CPS1 shRNA or AAV8-NC (n = 6). **(E)** qPCR analysis of the expression of *G6pc*, *Pck1*, and *Pgc1a* in the liver from the mice in panel **(D)** (n = 6). HFD, high-fat diet; PTT, pyruvate tolerance tests; OGTT, oral glucose tolerance tests; AUC, area under the curve; AAV, adeno-associated virus. Data were analyzed by one-way ANOVA. All values are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

2.10 Statistical analysis

Data analysis was carried out by software GraphPad Prism 9. Data were expressed as mean ± standard error of the mean (SEM). Statistical analysis of difference between two groups was performed using a two-tailed Student's t-test, and comparison for multiple groups was performed by one-way ANOVA. p values of less than 0.05 and 0.01 were to be considered significant and very significant, respectively, and they were expressed as *p < 0.05, **p < 0.01, ***p < 0.001.

3 Results

3.1 CPS1 activates glucagon-induced hepatic gluconeogenesis

To explore the role of CPS1 in hepatic gluconeogenesis, we utilized adeno-associated virus serotype 8 (AAV8) carrying small hairpin RNA (shRNA) to generate mice with liver-specific knockdown of CPS1 (Supplementary Figure S1). In

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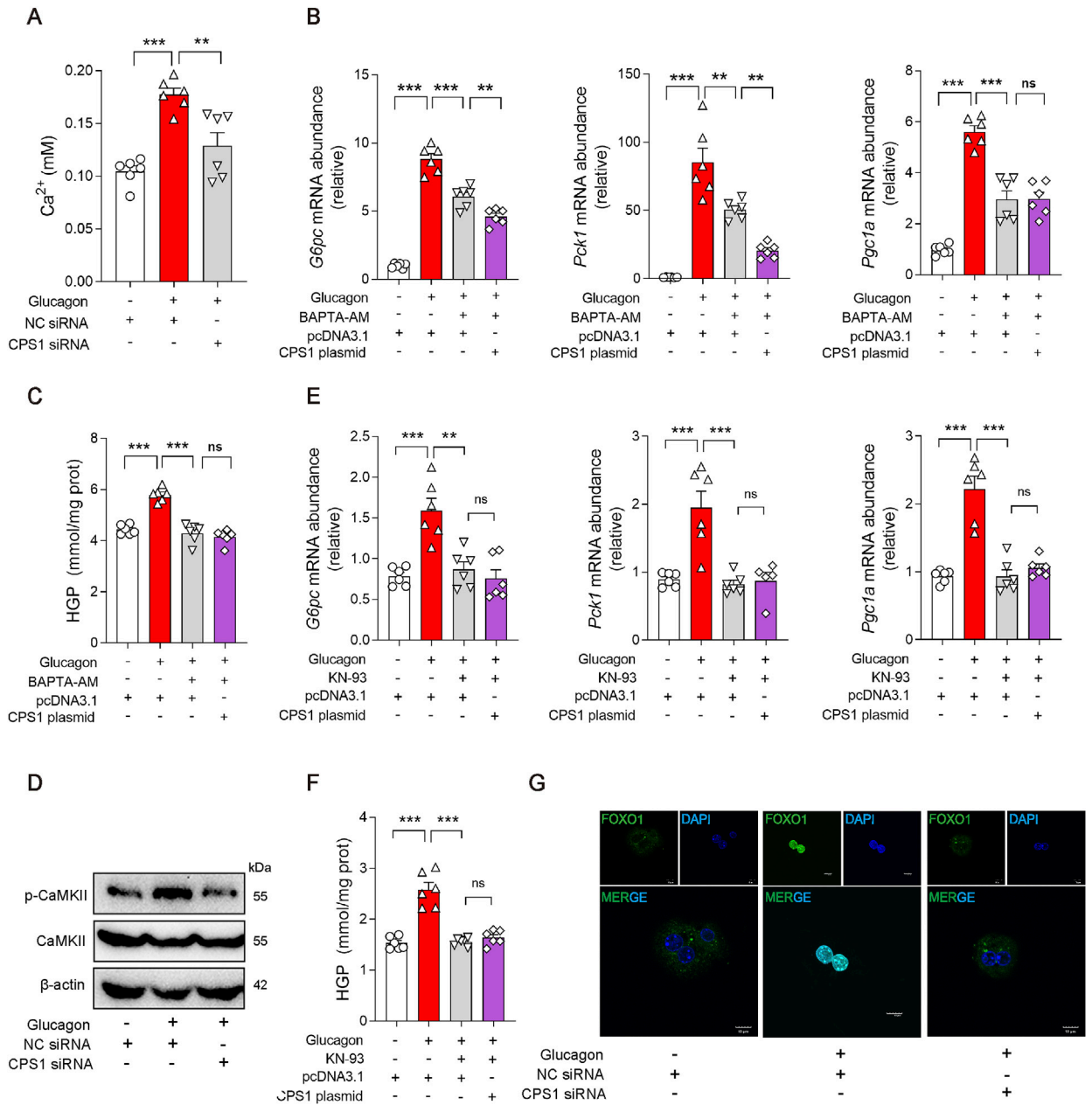

FIGURE 3
CPS1 activated CaMKII/FOXO1 pathway promoted by Ca^{2+} release in glucagon-stimulated hepatocytes. **(A)** Ca^{2+} level in hepatocytes treated with CPS1 siRNA or NC siRNA stimulated by glucagon (100 nM, 1 h) (n = 6). **(B)** qPCR analysis of the expression of *G6pc*, *Pck1*, and *Pgc1a* transfected with CPS1 plasmid stimulated by glucagon (100 nM, 1 h) after pretreated with BAPTA-AM (5 μM) for 1 h (n = 6). **(C)** Hepatic glucose production in panel **(B)** (n = 6). **(D)** Protein levels of CaMKII and p-CaMKII treated with CPS1 siRNA or NC siRNA stimulated by glucagon (100 nM, 1 h) (n = 3). **(E)** qPCR analysis of the expression of *G6pc*, *Pck1*, and *Pgc1a* transfected with CPS1 plasmid stimulated by glucagon (100 nM, 1 h) with KN-93 (10 μM, 1 h) (n = 6). **(F)** Hepatic glucose production in panel **(E)** (n = 6). **(G)** Confocal images of FOXO1 nuclear translocation in panel **(D)**. Scale bars, 10 μm. HGP, hepatic glucose production. Data were analyzed by one-way ANOVA. All values are represented as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$.

the glucagon tolerance test, we observed that the hepatic knockdown of CPS1 notably reduced fasting blood glucose levels (Figure 1A) and subsequently weaked the glucagon-induced hyperglycemia in mice (Figure 1B). Furthermore, the expression levels of key hepatic gluconeogenic genes, including *Pck1*, *G6pc*, and *Pgc1a*, were markedly decreased in CPS1 knockdown mice when subjected to a glucagon challenge (Figure 1C).

Similarly, *in vitro* studies using primary hepatocytes mirrored the *in vivo* findings. CPS1 knockdown (Supplementary Figure S2) effectively counteracted the glucagon-induced expression of hepatic glucose production (Figure 1D) and hepatic gluconeogenic genes, including *G6pc* and *Pck1* (Figure 1E). In contrast, overexpression of CPS1 (Supplementary Figure S3) significantly enhanced glucagon-stimulated hepatic glucose production (Figure 1F) and gluconeogenic genes such as *G6pc* and *Pgc1a* (Figure 1G). These

TABLE 3 Ranking of natural products binding with CPS1.

Catalog_No.	Name	Docking score	Glide gscore	Glide emodel
HY-13680	Meisoindigo	−11.479	−11.479	−75.095
HY-N0359	Cynarin	−10.783	−10.783	−83.779
HY-N0114	Evodiamine	−10.712	−10.712	−67.671
HY-N0058	4,5-Dicaffeoylquinic acid	−10.685	−10.685	−89.305
HY-N0261	Aurantio-obtusin	−10.650	−10.691	−84.624
HY-N0134	Tanshinone I	−10.644	−10.644	−73.372
HY-13065	Isobavachalcone	−10.576	−10.623	−79.175
HY-N0120A	Polydatin	−10.562	−10.562	−87.920
HY-B1756	Rotenone	−10.454	−10.454	−75.489

results underscored the pivotal role of CPS1 in the regulation of hepatic gluconeogenesis and its responsiveness to glucagon signaling.

3.2 CPS1 knockdown attenuates hepatic gluconeogenesis disorders in HFD-fed mice

To further investigate the role of CPS1 in hyperglycemia *in vivo*, hepatic CPS1 was knocked down by tail vein injection of AAV8-shRNA in HFD-fed mice (Supplementary Figure S4A). HFD feeding resulted in fasting hyperglycemia at weeks 10 and 12 (Figures 2A, C) and impaired pyruvate and oral glucose tolerance in mice (Figures 2B, D). Our findings revealed that the knockdown of CPS1 effectively reduced fasting blood glucose levels (Figures 2A, C) and ameliorated both pyruvate tolerance (Figure 2B) and oral glucose tolerance (Figure 2D). In line with these metabolic improvements, the hepatic expression of gluconeogenic genes *Pck1*, *G6pc*, and *Pgc1a* were significantly downregulated in HFD-fed mice following CPS1 knockdown (Figure 2E).

Of note, CPS1 knockdown also correlated with a decrease in body weight gain and reduced fat mass. Additionally, we observed a notable reduction in lipid accumulation within the liver of mice maintained on HFD feeding (Supplementary Figures S4B, D–E). The experimental outcomes demonstrated that the knockdown of CPS1 in liver resulted in a reduction of abdominal adipose tissue accumulation (Supplementary Figure S4B). Concurrently, this intervention was associated with a notable reduction in overall body weight (Supplementary Figure S4D). Oil Red O staining indicated a significant reduction in lipid deposition within the hepatic tissue (Supplementary Figure S4E). It is important to note that these beneficial effects were achieved without any significant alteration in food intake (Supplementary Figure S4C). These results highlighted the potential therapeutic implications of CPS1 modulation in the context of diet-induced metabolic disorders.

3.3 CPS1 promotes Ca^{2+} release to modulate hepatic gluconeogenesis

The hepatic response to glucagon is predominantly orchestrated through the activation of the cAMP/PKA signaling pathway. However, knockdown of CPS1 in hepatocytes did not modulate the expression levels of PKA (Supplementary Figure S5A). Correspondingly, the PKA inhibitor H-89 did not participate in the gluconeogenesis regulatory process governed by CPS1 (Supplementary Figures S5B–C). These findings suggested that the regulatory influence of CPS1 on gluconeogenesis bypasses the canonical pathway.

Glucagon also promotes gluconeogenesis by activating specific calcium ion channels or releasing calcium ions from the endoplasmic reticulum. Interestingly, the release of Ca^{2+} in glucagon-stimulated hepatocyte was abrogated by CPS1 knockdown (Figure 3A), suggesting CPS1 as a Ca^{2+} regulator in glucagon response. Calcium chelating agent BAPTA-AM was subsequently used to detect the effect of Ca^{2+} in CPS1 initiated gluconeogenesis. Obviously, the effect of CPS1 on initiating gluconeogenesis was abrogated by BAPTA-AM (Figures 3B, C), indicating that CPS1 could promote gluconeogenesis by upregulating calcium. Inositol 1,4,5-trisphosphate receptor (IP3R) is a Ca^{2+}-release channel and plays a crucial role in intracellular calcium signaling. In response to glucagon, IP3 binds to IP3R, leading to the release of Ca^{2+} from the endoplasmic reticulum into the cytosol. IP3R inhibitor XesC was subsequently used to determine the action of IP3R in CPS1-mediated gluconeogenesis. Notedly, the augment of gluconeogenesis by CPS1 overexpression was not affected by XesC (Supplementary Figures S6A, B). Thus, CPS1 regulated hepatic gluconeogenesis is not mediated by IP3R.

3.4 CPS1 augment glucagon response by CaMKII/FOXO1 pathway

Calcium-mediated CaMKII/FOXO1 axis plays a significant role in the regulation of gluconeogenesis. In response to glucagon, CaMKII is activated and delivered FOXO1 into the nucleus, where it transcriptionally promotes gene induction of *Pck1* and *G6pc* in cooperation with the co-activator *Pgc1a*. Based on this, we further investigated whether CPS1 mediated hepatic gluconeogenesis through CaMKII/FOXO1 pathway. Of note, knockdown of CPS1 significantly diminished the phosphorylation of CaMKII in response to glucagon stimulation (Figure 3D). KN-93, a specific inhibitor of CaMKII, significantly attenuates the phosphorylation of CaMKII.

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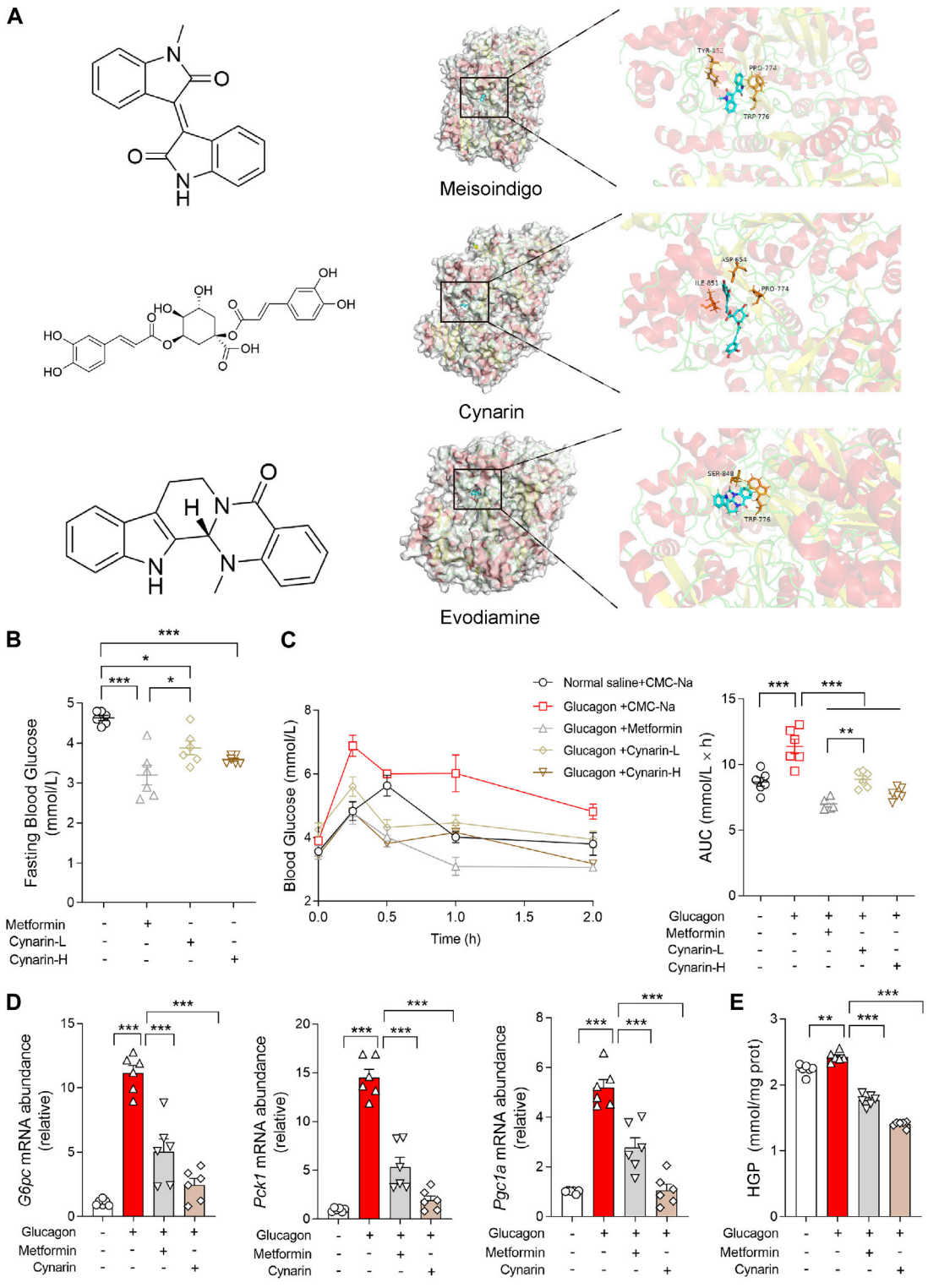

FIGURE 4
Gluconeogenesis inhibition effects of compounds screened binding to CPS1 by molecular docking. **(A)** Chemical structure formula of meisoindigo, cynarin, evodiamine and prediction of binding sites with CPS1 crystal structure. **(B)** Fasting blood glucose of mice pretreated with cynarin (L: 50 mg/kg; H: 100 mg/kg, 1 h) or metformin (200 mg/kg, 1 h) in mice (n = 6). **(C)** Blood glucose levels and AUC for mice pretreated with cynarin (L: 50 mg/kg; H: 100 mg/kg, 1 h) or metformin (200 mg/kg, 1 h) with glucagon challenge (2 mg/kg, n = 6). **(D)** qPCR analysis of the expression of *G6pc*, *Pck1*, and *Pgc1a* pretreated with metformin (1 mM, 4 h) or cynarin (20 μM, 4 h), 100 nM glucagon stimulation for 1 h in hepatocytes (n = 6). **(E)** Hepatic glucose production in panel **(D)** (n = 6). AUC, area under the curve; L, Low concentration; H, High concentration. Data were analyzed by one-way ANOVA. All values are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

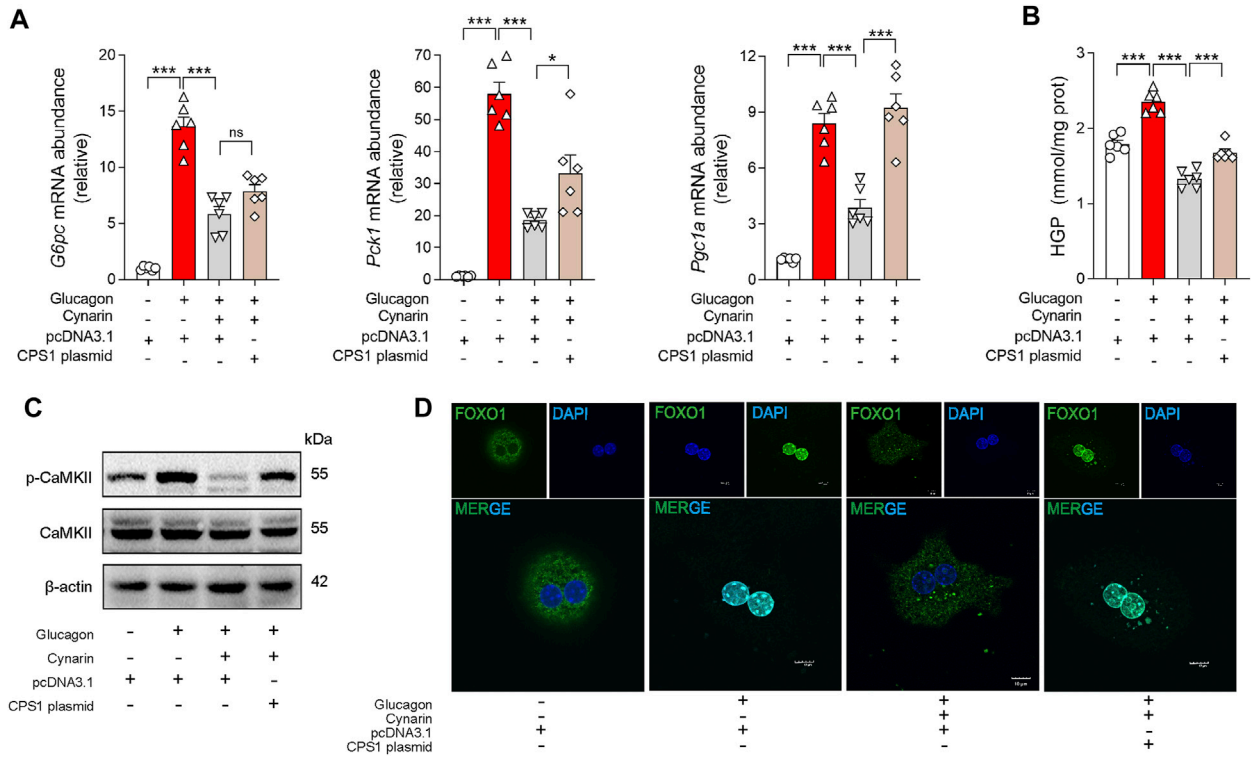

FIGURE 5
Cynarin suppressed CPS1/CaMKII/FOXO1 pathway to diminish hepatic glucagon response in hepatocytes. **(A)** qPCR analysis of the expression of *G6pc*, *Pck1*, and *Pgc1a* transfected with CPS1 plasmid or pcDNA3.1 with cynarin (20 μM, 4 h) stimulated by glucagon (100 nM, 1 h) (n = 6). **(B)** Hepatic glucose production in panel **(A)** (n = 6). **(C)** Protein levels of CAMKII and p-CaMKII in panel **(A)** (n = 3). **(D)** Confocal images of FOXO1 nuclear translocation in panel **(A)**. Scale bars, 10 μm. HGP, hepatic glucose production. Data were analyzed by one-way ANOVA. All values are represented as mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$.

Overexpression of CPS1 did not affect the inhibitory effect of KN-93 on gluconeogenesis (Figures 3E, F). This suggested that the regulatory role of CPS1 in hepatic gluconeogenesis is dependent on CaMKII. The phosphorylation status of FOXO1 is a critical determinant of its nuclear exclusion. CPS1 knockdown elevated the phosphorylation of FOXO1. Furthermore, immunofluorescence imaging provided evidence that CPS1 knockdown favors the nuclear exclusion of FOXO1 (Figure 3G). Summarily, we proposed that CPS1 modulated hepatic gluconeogenesis by impinging upon the CaMKII/FOXO1 signaling cascade through the regulation of calcium ion release.

3.5 *In silico* screening reveals cynarin as a natural CPS1 inhibitor

Given the pivotal role of CPS1 in the regulation of hepatic gluconeogenesis, pharmacological CPS1 inhibition is a potential therapeutic approach for modulating hepatic gluconeogenesis. To this end, an *in silico* molecular docking analysis was conducted utilizing a comprehensive library of natural compounds. Out of 724 natural compounds assessed, we have identified and highlighted the top 10 natural products with the highest binding affinity scores for CPS1 (Table 3). The predicted binding interactions between the top three natural products and the crystal structure of CPS1 were visually represented in Figure 4A, with their corresponding chemical structure formulas displayed in Figure 4A.

Of particular interest, cynarin, a bioactive compound derived from artichoke, was found to effectively reduce the fasting blood glucose that was upregulated by glucagon (Figures 4B, C). Specifically, we have documented the inhibitory of cynarin on gluconeogenesis, findings were further substantiated through *in vitro* experimentation. The data evinced that both cynarin and metformin exert ameliorative effects on gluconeogenesis, with cynarin demonstrating a superior efficacy in comparison to metformin (Figure 4D). This observation was congruent with the outcomes of our hepatic glucose production assays (Figure 4E). In light of these findings, we posited that cynarin may serve as a hypoglycemic agent by specifically targeting and modulating CPS1 activity.

3.6 Cynarin suppressed hepatic glucagon response via CPS1/CaMKII/FOXO1 pathway

To elucidate the mechanisms of cynarin in preventing gluconeogenesis, primary hepatocytes were transfected with CPS1 plasmid. The hypoglycemic influence of cynarin was significantly attenuated in hepatocytes with CPS1 overexpression (Figures 5A, B). This observation underscored the crucial role of CPS1 in mediating the hepatic gluconeogenesis regulatory effects of cynarin.

In addition, we examined the role of cynarin in modulating the CaMKII/FOXO1 pathway. Cynarin markedly elevated the

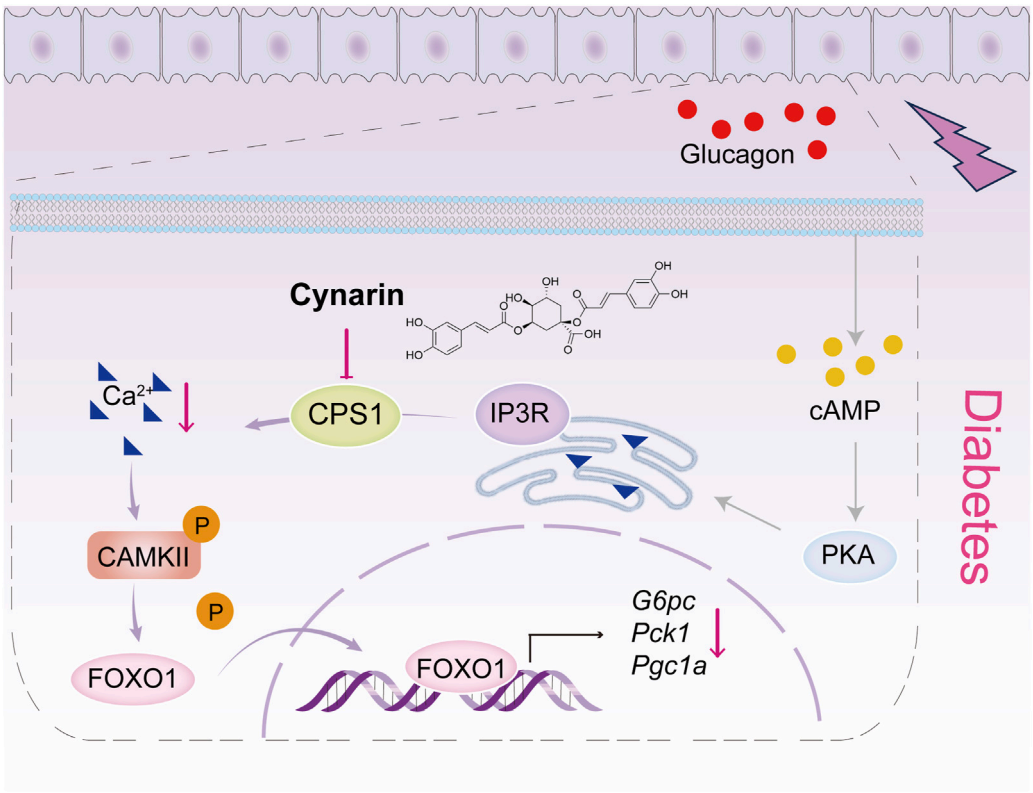

FIGURE 6
Scheme of the role for CPS1 in activating Ca^{2+}/CaMKII/FOXO1 signaling cascade to enhance hepatic gluconeogenesis. The CaMKII pathway is positioned downstream of the cAMP/PKA signaling cascade. Under normal conditions, Ca^{2+} are maintained in a steady state, and CaMKII remains inactive. This inactivation prevents the nuclear translocation of the FOXO1 transcription factor by modifying its phosphorylation state, which is crucial for the regulation of gluconeogenic genes and hepatic glucose production. Upon glucagon stimulation, CPS1 triggers the release of Ca^{2+} from the endoplasmic reticulum into the cytosol. This release activates CaMKII, which in turn enhances the transcriptional activity of FOXO1, thereby promoting hepatic gluconeogenesis. The natural compound cynarin targets CPS1 to inhibit this gluconeogenic process.

phosphorylation of FOXO1 in the presence of glucagon and concurrently reduced the phosphorylation levels of CaMKII. Intriguingly, this effect was abrogated by CPS1 overexpression in primary hepatocytes (Figure 5C). Furthermore, overexpression of CPS1 hindered the regulatory influence of cynarin on the nuclear translocation of FOXO1 (Figure 5D). These findings suggested that cynarin's suppressive effect on the hepatic glucagon response is linked to its regulatory actions on the CaMKII/FOXO1 pathway, with CPS1 playing an indispensable role.

4 Discussion

In this work, we delineated the pivotal role of CPS1 in hepatic gluconeogenesis. Our findings revealed that CPS1, a rate-limiting enzyme in the urea cycle, significantly exacerbates glucagon-induced hyperglycemia. Conversely, the knockdown of CPS1 attenuated the glucagon response. Notably, CPS1 modulated hepatic gluconeogenesis predominantly through the CaMKII/FOXO1 signaling pathway, diverging from the conventional activation of the cAMP/PKA pathway. Additionally, the hypoglycemic effects of cynarin, a compound derived from the artichoke plant, were partially mediated by CPS1 (Figure 6). Collectively, our data suggested a promising therapeutic approach targeting CPS1 as a potential intervention for type 2 diabetes.

Typically, glucagon activates PKA to directly phosphorylate CREB, which in turn transcriptionally induces the PGC-1α, a key coactivator of FOXO1 transcription factor (Herzig et al., 2001). However, in livers overexpressed CPS1, no significant difference in p-CREB levels were observed, suggesting alternative pathways may be at play. Beyond the cAMP/PKA signaling axis, glucagon also engages the calcium/CaMKII pathway, which operates downstream of the cAMP/PKA cascade. CaMKII, a serine/threonine kinase, is activated by cAMP and glucagon in a calcium- and IP3R-dependent manner (Ozcan et al., 2012). This activation is pivotal for mediating the effects of Ca^{2+} (Couchonnal and Anderson, 2008; Singer, 2012). For instance, glucagon and cAMP are known to elevate intracellular Ca^{2+} levels, and the chelation of Ca^{2+} has been demonstrated to attenuate glucagon-induced hepatic gene expression and glucose production (Staddon and Hansford, 1989; Bygrave and Benedetti, 1993; Mine et al., 1993). Previous studies have also correlated intracellular Ca^{2+} with the regulation of gluconeogenesis (Friedmann and Rasmussen, 1970; Kraus-Friedmann and Feng, 1996; Marques-da-Silva et al., 1997).

The inhibition of CaMKII has been shown to impede the nuclear translocation of FOXO1 by altering its phosphorylation state, thereby disrupting fasting- or glucagon-induced gluconeogenesis (Ozcan et al., 2012). Similarly, our findings suggested that CPS1 may modulate hepatic gluconeogenesis by influencing the CaMKII/FOXO1 signaling cascade, potentially through the regulation of calcium ion release. While glucagon operates in the liver alongside other pathways to ensure the nuclear localization of an array of transcription factors that mediate hepatic glucose production, the precise mechanism by which CPS1 regulates calcium ions remains to be elucidated and warrants further investigation.

Glucose, fatty acids, and amino acids serve as the primary sources of cellular energy. The breakdown of amino acids can result in the production of toxic ammonia. Detoxification of ammonia through the urea cycle is essential for preventing hyperammonemia and hepatic encephalopathy (Gropman et al., 2007). Glucagon, a hormone secreted by the pancreas, stimulates hepatic amino acid metabolism, particularly ureagenesis. Conversely, glucagon secretion is also sensitive to amino acids, which can stimulate glucagon secretion by increasing the number of alpha cells, thus establishing a reciprocal feedback loop (Holst et al., 2017; Wewer Albrechtsen et al., 2019). It has been documented that the activities of the five key enzymes in the urea cycle are elevated during fasting and in response to glucagon (Schimke, 1962; Husson et al., 1987; Li et al., 2016), predominantly through transcriptional, posttranscriptional, and posttranslational mechanisms (Ulbright and Snodgrass, 1993; Estall et al., 2009). Under the influence of CPS1, ammonia is converted to carbamoyl phosphate, which then reacts with ornithine to form citrulline through the catalytic action of ornithine transcarbamylase. Citrulline subsequently channels ammonia into the urea cycle. CPS1, as the rate-limiting enzyme, controls the influx of substrates under physiological conditions. Overexpression of CPS1 has been shown to exacerbate HFD-induced excessive activation of gluconeogenesis, as well as glucagon-induced gluconeogenesis both *in vivo* and *in vitro*. However, our study did not include the assessment of CPS1 enzyme activity, which could be a critical factor in hepatic gluconeogenesis. Further investigation is necessary, including the use of liver-specific CPS1 knockout mice for *in vivo* experiments to confirm the role of CPS1 in hepatic gluconeogenesis.

Artichoke, scientifically known for its compounds such as cynarin and chlorogenic acid, is a traditional Chinese medicinal herb with a diverse range of pharmacological properties. Studies have suggested that cynarin can be utilized in the treatment of liver damage (Tong et al., 2017), gouty arthritis (Wu C. et al., 2022), endothelial inflammation (Kim et al., 2022) and intervertebral disc degeneration (Zhang et al., 2023). These effects are mediated through the inhibition of inflammatory or pyroptosis. E. Heidarian and colleagues reported that artichoke extract, rich in cynarin, can effectively reduce blood glucose levels (Heidarian and Rafieian-Kopaei, 2013). However, the precise hypoglycemic mechanism of cynarin remains to be fully elucidated. Our research indicated that overexpression of CPS1 negates the inhibitory effect of cynarin on hepatic gluconeogenesis *in vitro*. We discovered that cynarin, acting as an inhibitor of CPS1, mitigates the excessive activation of hepatic gluconeogenesis via the CaMKII/FOXO1 signaling pathway. These findings offer novel insights into the hypoglycemic effects of cynarin.

In conclusion, this study has delineated a previously unappreciated mechanism by which CPS1 modulates glucagon-induced hepatic gluconeogenesis through the CaMKII/FOXO1 signaling cascade and offered a potential natural inhibitor of CPS1, which may show therapeutic benefits in the management of glucose metabolism disorders.

Data availability statement

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

Ethics statement

The animal study was approved by Animal Ethics Committee of China Pharmaceutical University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

X-MS: Formal Analysis, Investigation, Methodology, Visualization, Writing–original draft, Writing–review and editing. XW: Investigation, Writing–original draft, Writing–review and editing. M-GW: Visualization, Writing–original draft, Writing–review and editing. L-ZZ: Investigation, Writing–original draft, Writing–review and editing. W-hW: Formal Analysis, Writing–original draft, Writing–review and editing. X-YZ: Formal Analysis, Writing–original draft, Writing–review and editing. L-WQ: Project administration, Supervision, Validation, Writing–original draft, Writing–review and editing. QL: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing–original draft, Writing–review and editing.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2024.1437738/full#supplementary-material

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EDITED BY
Marcelo Biondaro Gois,
Federal University of Rondonópolis, Brazil

REVIEWED BY
Felix Kwame Amevor,
Sichuan Agricultural University, China
Yun Ji,
China Agricultural University, China

*CORRESPONDENCE
Yan Cui,
dryancui@163.com
Hao Li,
lihbys@163.com

†These authors have contributed equally to this work

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New insights into the intestinal barrier through "gut-organ" axes and a glimpse of the microgravity's effects on intestinal barrier

Hong-Yun Nie[1†], Jun Ge[2†], Guo-Xing Huang[3†], Kai-Ge Liu[1], Yuan Yue[4], Hao Li[1*], Hai-Guan Lin[1], Tao Zhang[1], Hong-Feng Yan[1], Bing-Xin Xu[5], Hong-Wei Sun[1], Jian-Wu Yang[1], Shao-Yan Si[5], Jin-Lian Zhou[6] and Yan Cui[1*]

[1]Department of General Surgery, The Ninth Medical Center of PLA General Hospital, Beijing, China, [2]Clinical laboratory, The Ninth Medical Center of the PLA General Hospital, Beijing, China, [3]306th Clinical College of PLA, The Fifth Clinical College, Anhui Medical University, Beijing, China, [4]Department of Disease Control and Prevention, The Ninth Medical Center of PLA General Hospital, Beijing, China, [5]Special Medical Laboratory Center, The Ninth Medical Center of PLA General Hospital, Beijing, China, [6]Department of Pathology, The Ninth Medical Center of PLA General Hospital, Beijing, China

Gut serves as the largest interface between humans and the environment, playing a crucial role in nutrient absorption and protection against harmful substances. The intestinal barrier acts as the initial defense mechanism against non-specific infections, with its integrity directly impacting the homeostasis and health of the human body. The primary factor attributed to the impairment of the intestinal barrier in previous studies has always centered on the gastrointestinal tract itself. In recent years, the concept of the "gut-organ" axis has gained significant popularity, revealing a profound interconnection between the gut and other organs. It speculates that disruption of these axes plays a crucial role in the pathogenesis and progression of intestinal barrier damage. The evaluation of intestinal barrier function and detection of enterogenic endotoxins can serve as "detecting agents" for identifying early functional alterations in the heart, kidney, and liver, thereby facilitating timely intervention in the disorders. Simultaneously, consolidating intestinal barrier integrity may also present a potential therapeutic approach to attenuate damage in other organs. Studies have demonstrated that diverse signaling pathways and their corresponding key molecules are extensively involved in the pathophysiological regulation of the intestinal barrier. Aberrant activation of these signaling pathways and dysregulated expression of key molecules play a pivotal role in the process of intestinal barrier impairment. Microgravity, being the predominant characteristic of space, can potentially exert a significant influence on diverse intestinal barriers. We will discuss the interaction between the "gut-organ" axes and intestinal barrier damage, further elucidate the signaling pathways underlying intestinal barrier damage, and summarize alterations in various components of the intestinal barrier under microgravity.

This review aims to offer a novel perspective for comprehending the etiology and molecular mechanisms of intestinal barrier injury as well as the prevention and management of intestinal barrier injury under microgravity environment.

KEYWORDS

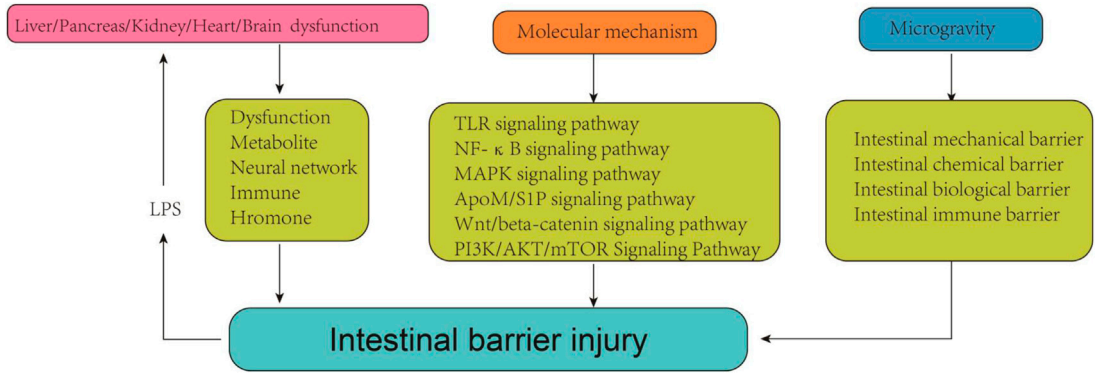
intestinal barrier damage, gut-organ axis, extraintestinal organs, signaling pathway, microgravity

1 Introduction

Gut is the largest digestive and immune organ, as well as the principal interface between humans and the environment (Yang and Yu, 2021). In addition to its role in nutrient digestion and absorption, the gut also establishes a distinctive mucosal barrier that protect the host from harmful substances and pathogens (Hao et al., 2022). In general, intestinal barrier can be categorized into four components: the mechanical, immune, chemical, and biological barriers. Each component is supported by its corresponding structural foundation. An intact intestinal barrier plays a crucial role in upholding gastrointestinal function and overall human health (Di Sabatino et al., 2023). Once the intestinal barrier is damaged, it may further trigger strong inflammatory responses, autoimmune disorders, metabolic dysregulation, and deterioration of organic lesions (Albillos et al., 2020). Upon the critical importance of intestinal barrier integrity, extensive research has been conducted to investigate the causes and underlying mechanisms of intestinal barrier damage.

Previous studies have shown that intestinal barrier damage is often associated with intestinal diseases such as intestinal infection, irritable bowel syndrome, ischemia-reperfusion (I/R) injury, inflammatory diseases and intestinal malignancies (Gao et al., 2023; Zhang et al., 2020). In recent years, the interconnection between the gastrointestinal tract and extraintestinal organs has gradually been elucidated. The concepts of "gut-liver axis," "gut-kidney axis," and "gut-heart axis" have been proposed, unequivocally indicating the intricate communication network between the intestines and other organs (Lombardi et al., 2024; Xiao et al., 2023). Studies have demonstrated that functional impairment of certain extraintestinal organs can induce oxidative stress, provoke an inflammatory response, and disrupt metabolic homeostasis in the intestine, thereby impacting intestinal barrier function. When the integrity of the intestinal barrier is compromised, it results in increased intestinal permeability and subsequent release of gut-derived endotoxin (Nie et al., 2023). Subsequently, these endotoxins can exert their effects on the target organs via the circulatory system, leading to further deterioration of the extraintestinal organs and eventually forming a vicious cycle of "gut-organ" axis disorder, which brings greater challenges to the management of intestinal barrier injury. It can be speculated that the disturbance of these axes plays a crucial role in the occurrence and progression of intestinal barrier damage (Figure 1). It is noteworthy that during the initial phase of functional impairment in certain organs, such as liver injury, kidney injury, and cardiovascular disease, increased intestinal permeability and endotoxin release can be detected, which helps to identify some high-risk adverse events early (Lee et al., 2018). At the same time, restoring intestinal barrier function could potentially serve as a therapeutic approach to mitigate extraintestinal organs injuries.

Signal transduction pathway, serving as the foundation for various cellular functions such as proliferation, differentiation, apoptosis, metabolism, oxidative stress, and immune response, facilitates the transmission of extracellular signaling molecules to the cell via cell membrane or intracellular receptors. Its proper functioning is indispensable for maintaining cellular and organ homeostasis (Chou et al., 2022). Dysregulation of cell signaling pathways has been demonstrated to be closely associated with the disruption and restoration of the intestinal barrier in previous studies (He et al., 2022). The elucidation of the molecular mechanism underlying intestinal barrier injuries holds significant importance in preventing such injuries and enhancing intestinal barrier homeostasis. Simultaneously, a comprehensive understanding of relevant mechanisms can aid in the development of targeted drugs to reverse ectopic signaling



GRAPHICAL ABSTRACT

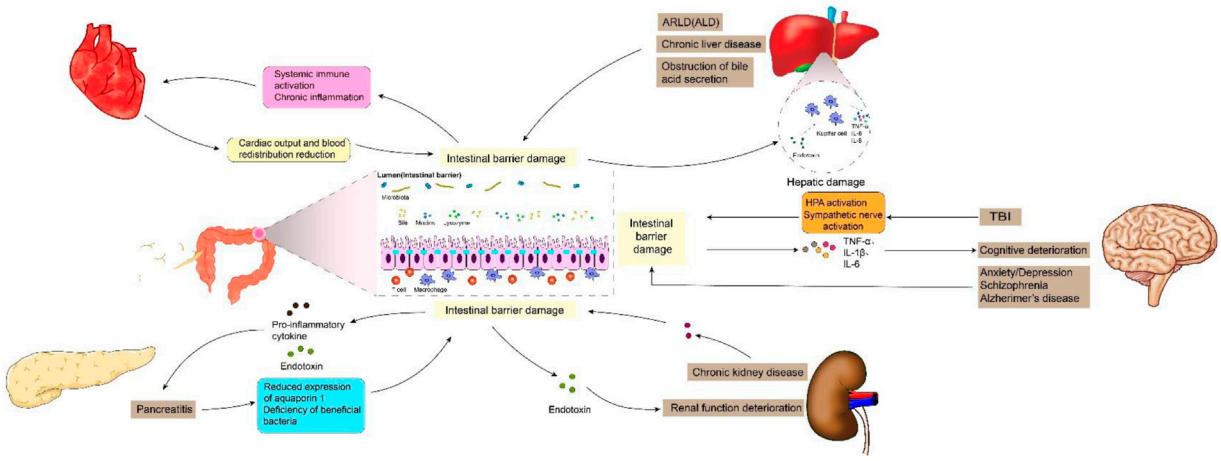


FIGURE 1
Intestinal barrier damage in the "gut-organ" axes.

pathways, thereby offering potential therapeutic approaches for treating intestinal barrier injury.

The exploration of space by humanity is consistently accompanied by fervent enthusiasm. Aerospace medicine, as a prominent domain in contemporary medical research, also garners significant attention. Weightlessness, being an essential and inevitable factor within the space environment, exerts a profound and intricate influence on organisms (Bharindwal et al., 2023). Studies have demonstrated that microgravity or simulated microgravity (SMG) can induce alterations in the physiopathological state of the digestive system and exert diverse effects on various intestinal barriers, encompassing mechanical, immune, chemical, and biological barriers (Yang et al., 2020). Consequently, it is imperative to summarize the modifications occurring in the intestinal barrier under microgravity conditions and to uphold gastrointestinal homeostasis among astronauts in space.

2 The key roles of intestinal barrier damage in "gut-organ" axes cross talk

2.1 Gut–liver axis

The gut and liver are anatomically and physiologically closely interconnected, with this intimate association originating from a shared derivation from the ventral foregut endoderm during embryogenesis (Zorn and Wells, 2009). The gut-liver axis (GLA) is characterized by bidirectional interaction between the intestine and the liver. Bile acids and bilirubin synthesized by hepatocytes are released into the duodenum via the biliary tract, while nutrients from the intestinal lumen are transported to the liver through the portal circulation. The optimal functioning of GLA is crucial for efficient nutrient absorption and waste elimination. The liver damage, however, can lead to a decline in intestinal barrier function and the production of enterogenic endotoxins, thereby breaking the balance of GLA and exacerbating further deterioration of both the liver and intestine. The reported findings indicate a significant increase in the sensitivity of intestinal epithelial cell apoptosis and the nitration of intestinal tight junction (TJ) and adhesive junction (AJ) proteins in mice with alcohol-related liver disease (ARLD) (Rungratanawanich et al., 2023). Xiao et al. (2020) reported a decrease in the expression of ZO-1, Claudin-1, Claudin-4, and Reg3g in the intestines of mice with liver injury, leading to an increase in intestinal permeability. Furthermore, there was a negative correlation observed between the expression levels of these proteins and serum endotoxin levels (Assimakopoulos et al., 2012). Hypoxia-inducible factor 1a (HIF-1a) plays a crucial role in the transcriptional regulation of intestinal barrier integrity and inflammation, governing the expression of various genes involved in barrier protection, including intestinal trefoil factor (ITF), CD73, p-glycoprotein (P-gp), cathelicidin, claudin-1, and MUC3 (Fan et al., 2015; Saeedi et al., 2015). Shao et al. (2018) reported a significant reduction of hif-1a in the intestines of mice afflicted with alcoholic liver disease (ALD), leading to compromised intestinal barrier function and gut leakiness. Subsequent investigations demonstrated that upregulating hif-1a could serve as a potential therapeutic approach for ALD (Shao et al., 2018). In addition, the intestinal biological barrier is also affected by liver disease. Jiang et al. (2020) reported that liver injury can result in an increase of the relative abundance of potentially pathogenic *Escherichia* and *Staphylococcus*, as well as a reduction in the presence of SCFA-producing bacteria, such as *Prevotella, Faecalibacterium*, and *Clostridium*. Additionally, the presence of bacterial overgrowth in the small intestine and impaired intestinal motility have been observed in patients with chronic liver disease, leading to enterogenic endotoxemia (Voulgaris et al., 2021). The obstruction of bile acid secretion in patients with liver disease also plays a crucial role in the development of enterogenic endotoxemia. Bile acids exert direct antibacterial effects by inducing farnesol X receptor-mediated antimicrobial peptides such as angiopoietin 1, which effectively prevent bacterial overgrowth and enhance the integrity of intestinal epithelium. The bile acids and their salts, on the other hand, can also degrade endotoxin molecules into non-toxic subunits or polymerize them into colloidal molecules, thereby exhibiting antibacterial properties and regulating intestinal pH. In

patients with liver disease, the impaired bile acid secretion and excretion compromise its ability to inhibit bacteria and regulate pH levels, as well as reduce the clearance of intestinal endotoxins. Moreover, elevated bile acid levels hinder the uptake and clearance of endotoxins by Kupffer cells (de Faria Ghetti et al., 2018).

The impairment of the intestinal barrier can also lead to hepatic damage via endotoxemia. The endotoxin, upon reaching the liver, binds to TLR4 and co-receptors CD14 and MD-2 in Kupffer cells, thereby initiating downstream signaling pathways that result in excessive production of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-8 (Cho et al., 2018). The mice were initially engineered by Shao et al. (2018a) to develop intestinal barrier injury through targeted knockout of intestinal epithelial HIF-1a. Subsequently, they observed a significant increase in serum alanine aminotransferase and lipopolysaccharide levels, along with the presence of liver steatosis. The biologically active lysophospholipid sphingosine 1-phosphate (S1P) plays a crucial role in various biological processes, including cell adhesion, regulation of barrier function, proliferation, differentiation, migration, and protection of the intestinal barrier (Li et al., 2023a). Chen et al. (2023) discovered that elevating the level of S1P can effectively restore intestinal barrier function, reduce lipopolysaccharide (LPS) levels in plasma and liver, mitigate inflammation and apoptosis, as well as enhance liver function. Similarly, the findings of Wu et al. (2023) also demonstrated that upregulation of rat intestinal epithelial tight junction protein expression and modulation of rat intestinal microbiota imbalance can significantly attenuate levels of endotoxin and inflammatory cytokines in rats, while inhibiting the TLR4/NF-κB signaling pathway, thereby ameliorating hepatic pathological damage and oxidative stress. In conclusion, the normalization of GLA has garnered increasing attention through repairing intestinal barrier damage and reducing enterogenic endotoxin production. The levels of serum endotoxin in the blood have also been demonstrated to exhibit a positive correlation with the severity of advanced liver diseases, such as fibrosis and cirrhosis (Rungratanawanich et al., 2023).

2.2 Gut-pancreas axis

The gut and pancreas are intricately interconnected both anatomically and functionally (Floyel et al., 2023; Svegliati-Baroni et al., 2020). Previous studies have consistently demonstrated a strong association between intestinal barrier damage and pancreatic diseases. Epithelial cells (ECs) play a crucial role in the mechanical barrier of the intestine and are indispensable for maintaining its integrity (Benede-Ubieto et al., 2024). However, pancreatitis or local pancreatic injury can induce the release of a plethora of inflammatory factors such as TNF-α and IL-1β, thereby triggering activation of the caspase-3 pathway and subsequent apoptosis of intestinal mucosal epithelial cells (Chen et al., 2018). In addition, the expression of intestinal tight junction proteins, such as occludin and ZO-1, significantly decreases in acute necrotizing pancreatitis (ANP), leading to a significant increase in intestinal permeability. This phenomenon is closely associated with high-mobility group box-1 (HMGB1) (Huang et al., 2019). Lu et al.'s study demonstrated that pancreatic tissue damage exacerbates small intestinal capillary endothelial barrier dysfunction, which may be attributed to reduced expression of aquaporin 1 (AQP1) in the small intestine (Lu et al., 2017). The integrity of the intestinal biological barrier, which is established through the close adhesion of symbiotic bacteria to the intestinal epithelial mucosa, is compromised during acute pancreatitis (Li et al., 2020). Li et al. (2023) discovered alterations in the diversity of intestinal microbiota and a deficiency of beneficial bacteria in patients with pancreatitis. Specifically, a reduction in *Bacteroides* uniformis abundance among these patients resulted in decreased taurine levels and increased release of IL-17 within the intestine. This subsequently triggered neutrophil extracellular trap (NET) formation, exacerbating pancreatic injury. The abundance of probiotics, such as Blautia, exhibited a inverse association with the severity of acute pancreatitis (Zhu et al., 2019). Conversely, the presence of detrimental bacteria, including *Escherichia coli* and *Shigella*, amplified the NF-κB-mediated inflammatory response while concurrently suppressing protein expression (e.g., MUC2), thereby compromising intestinal barrier integrity (Zheng et al., 2019).

Following an increase in intestinal permeability, enterogenic endotoxins and inflammatory mediators can be transported to the pancreas *via* systemic circulation and mesenteric lymphatic pathways, ultimately exacerbating acute pancreatic inflammation. The results of pancreatic studies have demonstrated that promoting apoptosis in intestinal inflammatory cells to inhibit the activation of the NF-κB signaling pathway, reducing endotoxin secretion, decreasing phosphorylated-p65 (p-p65) expression, and increasing IκBα expression can effectively enhance the pathological outcome of both pancreatic and intestinal tissues (Pan et al., 2024). Similarly, Mei et al. (2021) discovered that the mitigation of pro-inflammatory cytokine production (TNF-α, IL-1β, CXCL2 and MCP1) and endotoxin in the ileum, along with activation of the Nrf2/HO-1 pathway, could effectively restore ileum injury and barrier dysfunction associated with severe pancreatitis. Furthermore, there was a significant reduction observed in serum amylase levels, lipase levels, and pancreatic pulp peroxidase activity. The team led by Li et al. demonstrated enhanced intestinal barrier function and reduced intestinal inflammation in mice through microbiota transplantation (MT) and NLRP3 knockout. Subsequently, they observed a decrease in pancreatic neutrophil infiltration and necrosis (Li et al., 2020). In fact, there are limited therapeutic interventions for intestinal damage associated with acute pancreatitis, and the majority of treatments focus on hormone-mediated inhibition of pancreatic enzyme secretion, which does not effectively address intestinal injury. However, during the early stages of acute pancreatitis, enterogenic endotoxins and inflammation can propagate to the pancreas and other organs through the gut-pancreas axis, leading to severe consequences (Pasari et al., 2019). Therefore, it is imperative to address intestinal injury in association with AP by conducting comprehensive research on the gut-pancreas axis.

2.3 Gut-kidney axis

The correlation between the gut and renal is gradually being elucidated. Simultaneously, multiple studies have demonstrated the

potential involvement of kidneys in the progression of intestinal barrier impairment. Clinical trials have demonstrated that patients with stage IIIb-IV chronic kidney disease (CKD) exhibit the accumulation of enterogenous uremic toxins (UT), such as indoxyl sulfate (IS) and p-toluene sulfate (PCS), along with increased intestinal permeability and constipation (Cosola et al., 2021). The presence of chronic kidney disease (CKD) has been associated with intestinal villi shortening, elongation of crypts, and infiltration of the lamina propria (Georgopoulou et al., 2024). In addition, serum levels of endotoxin, IL-6, IL-8, and IL-10 were significantly elevated in patients with stage I-IV CKD, while intestinal occludin and claudin-1 exhibited significant reduction in expression. Furthermore, their expression showed a negative correlation with systemic endotoxemia (Georgopoulou et al., 2024). The impairment of the intestinal barrier is partially facilitated by urea (Lau et al., 2015). With an increase in intestinal barrier permeability, endotoxins, bacteria, and toxins are able to enter the circulatory system, leading to further deterioration of renal function. Additionally, kidney dysfunction can also impact the gut microbiome by causing a reduction in the population of proteolytic bacteria, altering the ratio of aerobic and anaerobic bacteria, and compromising intestinal epithelial barrier integrity. Simultaneously, changes in the microbiome can lead to the generation of potentially toxic compounds that are typically eliminated by renal excretion (Mertowska et al., 2021; Jaglin et al., 2018; Alexeev et al., 2018). Microorganisms such as *Enterobacterium*, *Enterococcus*, *Bifidobacterium*, and *Bacteroides*, which are responsible for the production of short-chain fatty acids, were found to exhibit reduced abundance in blood and fecal samples from patients with CKD (50). Furthermore, there was a negative correlation observed between the concentration of short-chain fatty acids in the bloodstream and the severity of renal insufficiency (Wang et al., 2019).

The discovery of the gut-kidney axis has also established that disruption of intestinal homeostasis can promote the onset and progression of renal disease. Several recent studies have initiated the development of therapies for renal dysfunction by focusing on restoring intestinal barrier function and microbial balance. The study conducted by Yang et al. (2024) demonstrated that the renal toxicity induced by zinc could be mitigated through rectifying colon injury, restoring ZO-1 protein expression, and reestablishing the structure of intestinal flora. Wang et al. (2024) demonstrated that oral administration of Lactococcus cremoris D2022 can enhance the production of short-chain fatty acids (SCFA) in cecum samples, ameliorate intestinal barrier function, and ultimately mitigate kidney inflammation. Additionally, in the study conducted by Yang et al. (2024), it was observed that oral administration of the probiotic *Lactobacillus* reuteri exhibited significant enhancement in intestinal barrier function impairment associated with AKI, while also regulating the composition of intestinal microbiota and its related metabolites. Consequently, there was a reduction in serum creatinine and urea nitrogen concentrations, along with protection against renal cell necrosis and apoptosis (Yang et al., 2024). The findings suggest that the preservation of gut barrier integrity and regulation of gut microbiota and associated metabolites should not be overlooked in the treatment of kidney disease. The significance of the kidney in maintaining proper physiological functioning of the human body goes without saying (Li et al., 2024); however, it remains one of the most overlooked organs. This can be attributed not only to insufficient knowledge and action in the field of prevention but also to the fact that most kidney diseases are asymptomatic during their initial stages. Early detection and diagnosis of kidney disease are severely limited due to a lack of sensitive and specific molecular markers indicating the progression towards a particular disease entity. Therefore, it is crucial to explore novel disease markers for predicting and tailoring personalized treatments for kidney diseases. Evaluation of gastrointestinal function through comprehensive analysis of gut microbiota composition holds potential for enhancing diagnostic capabilities, necessitating further investigation into the enterorenal axis.

2.4 Gut-heart axis

The concept of the gut-heart axis elucidates the intricate connection between intestinal pathology, the intestinal microbiome, and cardiovascular disease. Disorders of the gut-heart axis are characterized by alterations in intestinal permeability and disruption of the microbiome. Then, the gastrointestinal microbiota or its derivatives traverse the intestinal epithelial barrier in a non-physiological manner, leading to systemic immune activation and chronic inflammation, which subsequently impact cardiovascular function (Witkowski et al., 2020). Additionally, the contribution of cardiovascular disease to intestinal barrier damage is significant. In patients with heart failure, reduced cardiac output and blood redistribution result in decreased intestinal perfusion, leading to ischemia and hypoxia in the mucous membrane of the villous structure of the intestinal wall. This alteration in the intestines increases permeability of the intestinal wall, causing disturbances in fluid metabolism, disruptions in intestinal microbial balance, translocation of bacteria from the intestines into the circulatory system, and a increase in endotoxins, thereby promoting a characteristic inflammatory state (Xu et al., 2024). The lactulose/mannitol test revealed a 35% elevation in small intestine permeability, while the sucralose test demonstrated a significant increase of 210% in large intestine permeability among patients with chronic heart failure (CHF) (Sandek et al., 2007). The disruption of intestinal barrier integrity and the downregulation of tight junction protein expression have been reported to be associated with neutrophil extracellular trap (NET) (Wang et al., 2018). Hoel et al. (2020) discovered that COVID-19 patients with cardiac involvement exhibited elevated levels of intestinal leakage markers (LPS-binding protein, LBP) and intestinal epithelial cell damage markers (intestinal fatty acid-binding protein, IFABP), indicating the potential existence of a Gut-heart axis in COVID-19. Recently, Blöbaum et al. (2023) conducted a groundbreaking study where they successfully diagnosed intestinal barrier dysfunction in patients with atrial fibrillation (AF). Their research findings revealed an elevation in circulating biomarkers associated with intestinal mucosal inflammation, such as mucosal adhesion molecule MAdCAM-1, and indicators of intestinal epithelial damage like intestinal fatty acid binding protein (IFABP) present in plasma among individuals experiencing early stages of atrial fibrillation (AF). Additionally, surrogate plasma

markers indicating increased intestinal permeability were also detected, including LPS, CD14, and LPS-binding proteins. Moreover, Du et al. (2020) observed alterations in the composition of intestinal microbiota (particularly *firmicutes* and *Bacteroidetes*) as well as significant elevations in metabolites associated with the microbiome, including short/medium chain fatty acids, arginine, and tryptophan derivatives, in rats exhibiting cardiac hypertrophy. These changes are implicated in the impairment of intestinal barrier integrity induced by heart failure.

In recent years, the restoration of intestinal barrier integrity has emerged as a novel approach for the treatment of heart dysfunction by researchers. The correlation between intestinal permeability and markers of heart injury was found to be positive (Wu et al., 2024). Cui et al. (2023) demonstrated that enhancing the expression of tight junction proteins (ZO-1, occludin) and reducing intestinal permeability and inflammation can lead to improved cardiac function, as well as decreased serum CK-MB and LDH expression. The microbiota, as a crucial component of the intestinal biological barrier, is increasingly being targeted for heart failure treatment (Violi et al., 2023; Bianchi et al., 2022). Regulating gut microbiota through dietary interventions, probiotics administration, antibiotic therapy, fecal transplants, and microbial enzyme inhibitors can effectively enhance cardiac function and reduce mortality associated with heart failure. These interventions involve strategies such as optimizing the firmicutes/Bacteroidetes ratios and promoting the growth of beneficial microbiota (bacteroidetes and heterobacteroidetes) (Jia et al., 2019). The recognition of intestinal barrier damage as a risk factor for cardiovascular disease is increasingly growing. In certain cases of early heart disease, gut-derived low-grade endotoxemia may be present (Violi et al., 2023). Therefore, it is imperative to conduct early assessment of intestinal permeability and detection of endotoxins in high-risk groups for cardiovascular disease to facilitate timely intervention.

2.5 Gut-brain axis

The bidirectional communication between the brain and gut occurs *via* systemic immune pathways, neural networks, endocrine hormones, and microbiota axes (Morais et al., 2021). Studies have demonstrated that traumatic brain injury (TBI) exerts detrimental effects on the gastrointestinal tract through hormonal regulation. TBI triggers activation of the hypothalamic-pituitary-adrenal (HPA) axis, leading to an elevation in cortisol levels (Li et al., 2023). This surge in cortisol enhances intestinal barrier permeability, ultimately resulting in intestinal leakage (Morais et al., 2021). Consequently, enteric pathogens infiltrate the bloodstream and induce systemic inflammatory response syndrome, thereby releasing a plethora of cytokines and chemical mediators into the systemic circulation. The sharp escalation of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 accelerates cognitive function deterioration (Milleville et al., 2021). In addition, gut can be influenced by brain injury through neuroregulation. TBI can induce sympathetic overactivity, leading to an elevation in circulating catecholamine levels, subsequently resulting in reduced blood flow within the gastrointestinal tract and causing gut dysmotility as well as damage to the intestinal barrier (Montgomery et al., 2016). There is also a correlation between anxiety and intestinal disorders. A clinical study investigation revealed that patients with depression/anxiety disorder exhibited dysregulation of the gut microbiota and significantly elevated levels of LPS, zonulin, and FABP2 in comparison to healthy subjects. Assessing markers of intestinal permeability can aid in interventions for depression and anxiety (Stevens et al., 2018). Furthermore, studies have demonstrated increased intestinal permeability in individuals with schizophrenia, characterized by tight junctions disruption, adhesive junctions dysfunction, and heightened bacterial translocations (Maes et al., 2019). The assessment of the lactulose to mannitol ratio revealed a significantly higher index in patients with schizophrenia compared to the control group (Ishida et al., 2022). There is a strong correlation between gut flora and the central nervous system. The microbiota has the ability to regulate the synthesis of neurotransmitters that impact the gut-brain axis, such as tryptophan. Tryptophan serves as a crucial precursor for aminergic neurotransmitters and cannot be synthesized by the human body; however, it is produced by gut microbes through the shikimate pathway. Notably, Alzheimer's disease onset is associated with tryptophan secretion in intestinal flora. Although the gut and brain are physiologically and anatomically distant, neuroimmune links between the two are gradually being discovered, which could help in the development of more intestinal biomarkers to make the prevention and diagnosis of neurological diseases more effective.

2.6 Gut-liver-brain axis

Gut-liver-brain axis serves as a tripartite communication pathway, garnering increasing attention due to its paramount significance (Muhammad et al., 2022). Maintenance of homeostasis within the gut-hepato-brain axis is imperative for optimal brain functionality, contingent upon the intestinal barrier's integrity and hepatic filtration efficacy. Intestinal tract is the primary reservoir of bacteria within the human body, and its barrier function effectively prevents the translocation of intestinal toxins into capillaries. The liver functions as a prominent "detoxification factory," efficiently metabolizing and filtering exogenous and endogenous metabolites, bacterial products, and toxins (Wiest et al., 2017). Impairment of either the liver or intestinal barrier can lead to an exacerbation of abnormal gut-liver-brain axis states. Specifically, the impairment of the intestinal barrier facilitates the translocation of bacteria or their metabolites into the liver, thereby precipitating various hepatic disorders (Bauer et al., 2022). In addition, the gut can also affect nerve signals between the gut and the brain by influencing the production of various peptides or hormones. Subsequently, the brain innervates liver and intestinal activity through neuroimmunomodulation, exacerbating the disorder of the enteroliver-brain axis (Socala et al., 2021). Studies have demonstrated that alterations in the composition of intestinal microbiota and its metabolites, along with increased intestinal permeability, play a pivotal role in the pathogenesis of hepatic encephalopathy (Aguirre Valadez et al., 2016). In individuals with liver dysfunction, compromised integrity of the intestinal

barrier can further exacerbate hepatic failure. In patients with cirrhosis, impaired liver function hampers ammonia metabolism, leading to elevated blood ammonia levels. Subsequently, ammonia traverses the blood-brain barrier and accumulates within the brain parenchyma, thereby triggering hepatic encephalopathy and cognitive impairment (Milosevic et al., 2019). Hyperammonemia can induce microglial activation, indicating the generation of inflammation in the central nervous system (Aitbaev et al., 2017). Furthermore, the gut microbiota also plays a pivotal role in hepatic encephalopathy development. Research has demonstrated that alterations in intestinal motility and decreased bile acid levels among cirrhosis patients contribute to an overgrowth of intestinal bacteria, resulting in dysbiosis of the gut flora. Bajaj et al. demonstrated that, in comparison to the control group, patients with hepatic encephalopathy exhibited an elevated proportion of enterobacteriaceae, Fusobacteriaceae, and Veillonaceae in their intestinal mucosal flora. Furthermore, these microbial groups were positively correlated with intestinal inflammation. Additionally, enterobacteriaceae was found to be associated with astrocyte changes linked to hyperammonemia (Ahluwalia et al., 2016). These findings suggest a potential association between specific gut microbiota and cirrhosis-related brain dysfunction.

3 Causes of intestinal barrier damage

The occurrence of intestinal inflammation is a pathological process triggered by external stimuli or pathogen invasion in the intestinal tissues (Medzhitov, 2010). In the early stages of inflammatory diseases, such as acute intestinal infections, pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) are released in large quantities and cause an inflammatory cascade. Conversely, anti-inflammatory cytokines (IL-4, IL-10, and IL-13) can suppress the inflammatory response and mitigate damage caused by excessive inflammation. The dysregulation of inflammatory/anti-inflammatory factors results in the infiltration of immune cells, concurrently activating signaling targets such as NF-κB and MAPK, thereby influencing the expression of downstream proteins (intestinal barrier proteins, immune receptors, and related enzymes) (Dong et al., 2019), ultimately leading to an elevation in intestinal permeability (Wells et al., 2017). In addition, intestinal infection can induce apoptosis of intestinal epithelial cells, resulting in the subsequent release of damage-associated molecular patterns. This leads to a persistent state of heightened inflammation within the intestinal mucosa and impairs the functionality of goblet cells and mucosal epithelial regeneration, thereby compromising the repair process of the damaged intestinal barrier (Jiang et al., 2010). However, the presence of ulcers, bleeding, and an imbalance in the mucosal environment can further compromise the integrity of the intestinal mucosal barrier, ultimately resulting in a detrimental cycle of ongoing mucosal damage and destruction, impaired reconstruction capacity, and exacerbated homeostatic imbalance (Chang et al., 2013).

Studies have demonstrated that cigarette smoking exerts both direct and indirect effects on the gastrointestinal tract, encompassing oxidative damage, compromised immune cell functionality, alterations in epigenetic patterns, as well as changes in microbial composition that collectively contribute to intestinal barrier dysfunction (Papoutsopoulou et al., 2020). The study conducted by Berkowitz et al. (2019) demonstrated that smoking adversely affects the integrity of the intestinal barrier in the small intestine through its impact on Pan's cells, specialized epithelial cells located in this region responsible for secreting antimicrobial peptides crucial for regulating microbial growth. When mice were exposed to cigarette smoke condensate (CSC), specific alterations in Paneth cell granules in the ileum were observed, leading to a decrease in antimicrobial peptide production and bactericidal capacity. Furthermore, CSC induced an imbalance in the gut bacterial community and heightened susceptibility to bacterial infection-induced ileal damage in mice. The potential risk of intestinal injury due to chronic smoke exposure may be attributed to modifications in mucin distribution within the intestinal epithelium and alterations in flora composition (Allais et al., 2016).

In addition, other irritants including alcohol, radiation, and drug abuse are important triggers for intestinal barrier damage. The activation of pro-oxidases/genes and the inhibition of antioxidant levels, including glutathione, have been reported as key factors contributing to alcohol-induced intestinal damage, leading to heightened oxidative and nitrification (nitrosation) stress. Similarly, factors such as physical stimulation (radiation) and bacterial infection can also impact intestinal barrier damage by inducing imbalances in intestinal oxidative stress. When there is an excessive oxidation of intestinal tissues, it can hinder the regeneration of intestinal epithelial cells (IECs), increase the disruption of tight junction integrity, and reduce the secretion of antioxidants and other physiological processes that affect intestinal barrier function (Gao et al., 2023). Oxidative stress has the ability to directly impair IECs and activate various pathways related to oxidative and anti-oxidative stress, thereby regulating the extent of intestinal damage.

The occurrence of intestinal barrier damage is often not limited to a single cause. In addition to complex infection, inflammation, oxidative stress, apoptosis, and other pathological processes, intestinal ischemia/hypoxia and genetic factors are also closely associated with intestinal barrier damage, involving intricate mechanisms of interaction among them. To achieve a more accurate understanding of the etiology of intestinal injury, comprehensive and in-depth research on the molecular mechanism and injury mechanism needs to be conducted.

4 Molecular mechanism of intestinal barrier damage

4.1 TLR signaling pathway

Toll-like receptor (TLR) is an innate immune pattern recognition receptor, promptly triggering intracellular signaling cascades upon detection of pathogen-associated molecular patterns (PAMPs), including proteins, nucleic acids, and lipids derived from invading pathogenic microorganisms. Ultimately, this activation leads to the initiation of both non-specific and specific immune responses aimed at eliminating pathogens (Liu et al., 2024). LPS/TLR4 signaling pathway can be classified into

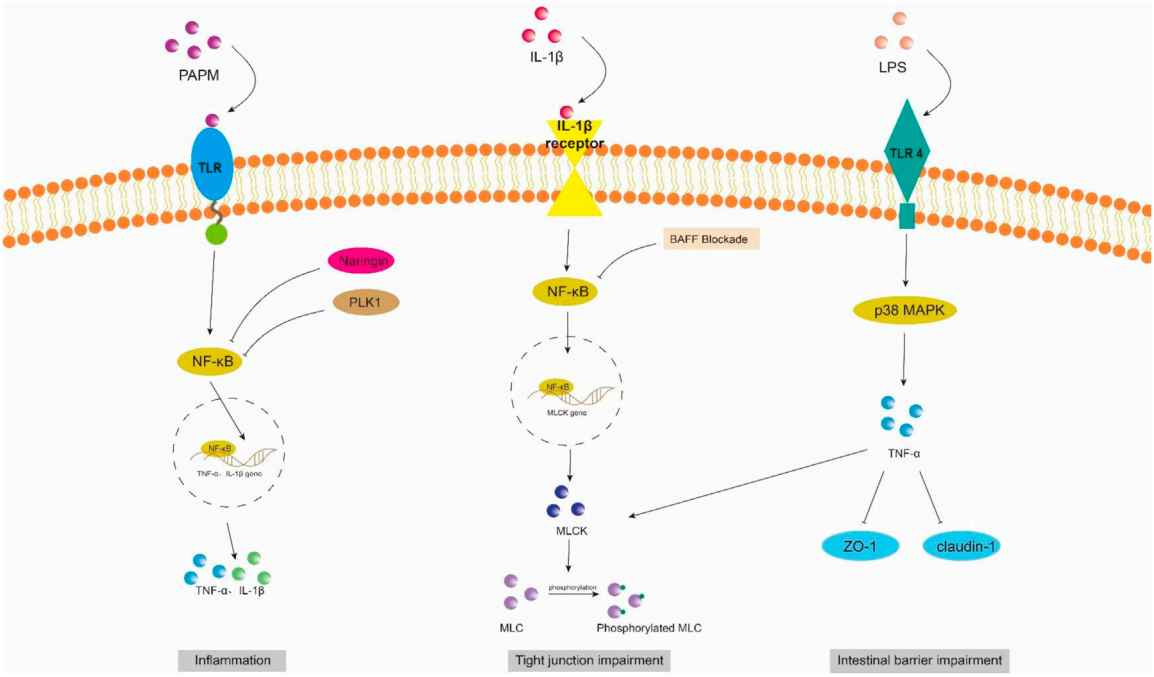


FIGURE 2
NF-κB and MAPK signaling pathway in intestinal barrier damage. PAPM, pathogen-associated molecular pattern; LPS, Lipopolysaccharides; TLR, Toll-like receptors; PLK1, Polo-like Kinase 1; ZO-1, zonula occluden-1.

MyD88-dependent and MyD88-independent pathways, both of which activate the NF-κB and MAPK pathways that regulate inflammation (Izadparast et al., 2022). In intestinal inflammatory diseases, the activation of the LPS/TLR signaling pathway induces a robust inflammatory response, resulting in increased apoptosis of intestinal epithelial cells (IECs) and downregulation of tight junction protein expression, ultimately leading to impaired intestinal barrier function (Figure 2). Recent studies have demonstrated that neutrophil extracellular traps (NETs) induce the activation of Toll-like receptor 9 (TLR9) signaling pathway in septic patients, thereby triggering endoplasmic reticulum (ER) stress in intestinal epithelial cells (IECs), leading to increased intestinal inflammation, apoptosis of IECs, tight junction injury, and ultimately compromising the mechanical barrier of the intestinal mucosal epithelium. Inhibition of TLR9-ER stress signaling can significantly ameliorate NETs-induced apoptosis of IECs and improve intestinal function (Sun et al., 2021). As custodians of small intestine crypts, stem cell-derived Panes cells not only enhance the function of stem cells and promote epithelial regeneration but also secrete highly potent antimicrobial peptides such as alpha-defensin and lysin.

However, in sepsis mice, it has been observed that the activation of the TLR4/ATF/CHOP signaling pathway by ER stress can induce apoptosis or dysfunction in Panzer cells, leading to exhaustion and subsequently impairing intestinal stem cell mobility, reducing secretion of antimicrobial peptides, exacerbating intestinal injury, and ultimately increasing mortality (Wang et al., 2022). Scholars have also discovered that the overexpression of pentosan-3 (PTX3) can inhibit the TLR signaling pathway, thereby reducing levels of inflammatory factors such as TNF-α, IL-1β, and interferon (INF)-γ. This inhibition helps alleviate apoptosis in intestinal epithelial cells (IECs) and promotes the expression of tight junction proteins ZO-1 and occludin between these cells, ultimately leading to a reduction in damage to the intestinal epithelium (Li et al., 2022).

4.1.1 NF-κB signaling pathway

NF-κB is a crucial nuclear transcription factor involved in inflammation and immune response, while also regulating apoptosis and stress response. In sepsis, the activation of Transforming growth factor kinase 1 (TAK1) can be mediated by inflammatory factors such as TNF-α and IL-6. Upon activation, TAK1 phosphorylates the downstream inhibitory protein IκBα, leading to dissociation of NF-κBp65 from IκBα and subsequent translocation into the nucleus for transcriptional regulation of related genes. It has been observed that Polo-like kinase 1 (PLK 1) is suppressed in septic rats, leading to reduced expression of IκB-α and enhanced nuclear translocation of NF-κB p65. The combined downregulation of PLK1 and activation of NF-κB result in apoptosis of intestinal epithelial cells, thereby compromising the integrity of the intestinal mechanical mucosal barrier (Cao et al., 2020; Cao et al., 2018). At the same time, the expression levels of pro-caspase-3 and IκB-a were significantly upregulated upon pretreatment of human colon cells with NF-κB activity-inhibiting drugs, suggesting that inhibition of NF-κB can reduce the apoptosis of intestinal epithelial cells (Cao et al., 2020). Additionally, NF-κB signaling pathway can impair the integrity of the intestinal barrier by influencing the junctions between intestinal epithelial cells. The activated NF-κB p65 interacts with the promoter region of myosin light chain kinase (MLCK) and regulates the transcription of MLCK (Al-Sadi et al., 2008). Consequently, phosphorylation of MLC by MLCK triggers

actin-myosin filament contraction, leading to downregulation of tight junction protein expression and increased intestinal permeability (Gatica-Andrades et al., 2017). Quan et al. (2020) discovered that rats treated with LPS exhibited significant increases in the phosphorylation levels of NF-κB p65 and IκBa, as well as MLCK and MLC, leading to a decrease in ZO-1 and occludin expression which compromised intestinal epithelial cell integrity and increased permeability. However, B-cell activator (BAFF) can inhibit the NF-κB/MLCK/MLC signaling pathway while increasing ZO-1 and occludin expression. Furthermore, a study conducted on animals demonstrated that the administration of gadolinium chloride (GdCl3) to sepsis rats resulted in a reduction of both systemic and intestinal inflammatory responses. This was primarily achieved through inhibition of NF-κB activation, leading to decreased MLCK expression, while promoting the expression of atrexin and ZO-1 (Zhao et al., 2021). Therefore, the restoration of the intestinal TJ barrier in endotoxemia can potentially be achieved by employing chemical compounds that possess inhibitory effects on NFκB and MLCK.

4.1.2 MAPK signaling pathway

Mitogen-activated protein kinase (MAPK) is a cytoplasmic serine/threonine protein kinase that plays a crucial role in various physiological and pathological processes, including cell proliferation, differentiation, apoptosis, and survival. MAPK signaling network comprises three distinct pathways: the p38 mitogen-activated protein kinase (p38MAPK) pathway, the extracellular signal-regulated protein kinase 1/2 (ERK 1/2) pathway, and the c-Jun amino-terminal kinase (JNK/SAPK) pathway (Yang et al., 2013). ERK1/2 regulates cell survival, differentiation, and proliferation. The involvement of JNK and p38MAPK in inflammation is characterized by their ability to impede cell cycle progression and facilitate apoptosis. Moreover, these signaling pathways may exert crucial pro-apoptotic functions in damaged intestinal epithelial cells (He et al., 2015). Luo et al. (2023) demonstrated that LPS treatment markedly upregulated the mRNA expression of p38 MAPK in the ileum of mice. Upon activation, the p38 MAPK signaling pathway induces robust oxidative stress response and triggers the release of a plethora of inflammatory factors, including TNF-α, IL-1β, and IL-6. Consequently, this leads to an increase in crypt depth and a decrease in villus height and the ratio of villus height to crypt depth (V/C) of the intestine. Simultaneously, there was a significant reduction observed in the expression levels of intestinal barrier proteins such as zonula occludens 1 (ZO-1), occludin, claudin, mucin 2 (MUC2), and junctional adhesion molecule 2 (JAM2). The activation of the MAPK pathway has been demonstrated to be closely associated with the impairment of intestinal barrier function, which is attributed to the phosphorylation of myosin light chain (MLC) (Raj et al., 2023). Phosphorylation of MLC by myosin light chain kinase (MLCK) regulates cellular actomyosin contraction, a crucial step in maintaining barrier integrity through the opening of paracellular pathways (Turner et al., 1997). After MAPK activation, the phosphorylation of MLCK is increased by a significant number of inflammatory factors (such as TNF-α), subsequently leading to MLC phosphorylation and ultimately resulting in the disruption of intestinal tight junctions and an increase in intestinal permeability. Moreover, inhibition of MLCK enhances the barrier function of TNF-α-stimulated intestinal epithelial cells (Zolotarevsky et al., 2002). In addition, TNF-α can also exert an inhibitory effect on the promoter activity of occludin, leading to the rearrangement of ZO-1 and claudin-1 (Wang et al., 2005). Wang et al. (2017) demonstrated that IL-1β induces phosphorylation of p38 MAPK, upregulates MLCK expression, and enhances paracellular permeability in the intestinal epithelial cells. Additionally, they discovered that curcumin inhibits IL-1β-induced activation of p38 MAPK, thereby attenuating the increased expression of MLCK and safeguarding the integrity of the intestinal epithelial barrier against translocation of bacterial LPS from the intestine into systemic circulation. It has been reported that the JNK inhibitor SP600125 has been shown to reduce intestinal inflammatory response and prevent intestinal barrier breakdown by increasing ZO-1 and closure protein expression (Samak et al., 2015). Similarly, Sotetsuflavone exhibited protective effects on the intestinal barrier by suppressing the JNK and p38 signaling pathways in inflammatory conditions (Ge et al., 2023). Xu et al. (2024b) demonstrated that through the inhibition of inflammatory macrophages, regulation of extracellular redox homeostasis, and downregulation of the MAPK/ERK signaling pathway, it is possible to suppress over-activated inflammation and restore cell-tight junction proteins. This leads to a reshaping of the intestinal microenvironment and achieves the purpose of treating endotoxemia.

4.2 ApoM/S1P signaling pathway

Gut vascular barrier (GVB), a novel anatomical structure in the mouse and human gut described by Spadoni et al., in 2015, is composed of vascular endothelial cells, pericellular fibroblasts, enteric glial cells, as well as junction complexes including tight junctions (TJs) and adhesion junctions (AJs) (Ge et al., 2020). GVB serves to prevent the entry of microorganisms into the bloodstream and regulate antigen translocation (Sorribas et al., 2019). As an intestinal barrier, GVB has gained increasing attention in recent years. Studies have found that GVB may be impaired in sepsis and its permeability increases (Liu et al., 2020). Sphingosine-1-phosphate (S1P) is a sphingomyelin metabolite that exhibits diverse biological activities, including involvement in cell growth, apoptosis, and regulation of the immune and clotting systems through binding to G protein-coupled receptors. Within the intestinal epithelium, S1P serves as a crucial regulator of epithelial cell barrier function by activating the receptor S1PR1 located in intestinal blood vessels. This activation leads to an increase in trans-monolayer electric resistance of endothelial cells and helps maintain vascular integrity during inflammatory bowel disease (Karuppuchamy et al., 2017). The primary carrier of S1P in plasma is ApoM, with approximately 5% of HDL particles containing ApoM (Frej et al., 2016). In conclusion, the ApoM/S1P axis plays a crucial role in safeguarding the integrity of the intestinal barrier (Li et al., 2023a). Studies have demonstrated that LPS, TNF-α, and IL-1 exert inhibitory effects on hepatic ApoM production (Jiang et al., 2015). Kumaraswamy et al. (2012) observed a significant reduction in plasma ApoM levels among patients with varying degrees of sepsis, septic shock, and SIRS compared to healthy controls, with the extent of reduction reflecting the severity of SIRS/sepsis. Christensen et al. (2016) confirmed an increased intestinal permeability in ApoM (−/−) mice as opposed to ApoM (+/+) mice, suggesting that disruption of the ApoM/S1P axis is responsible for

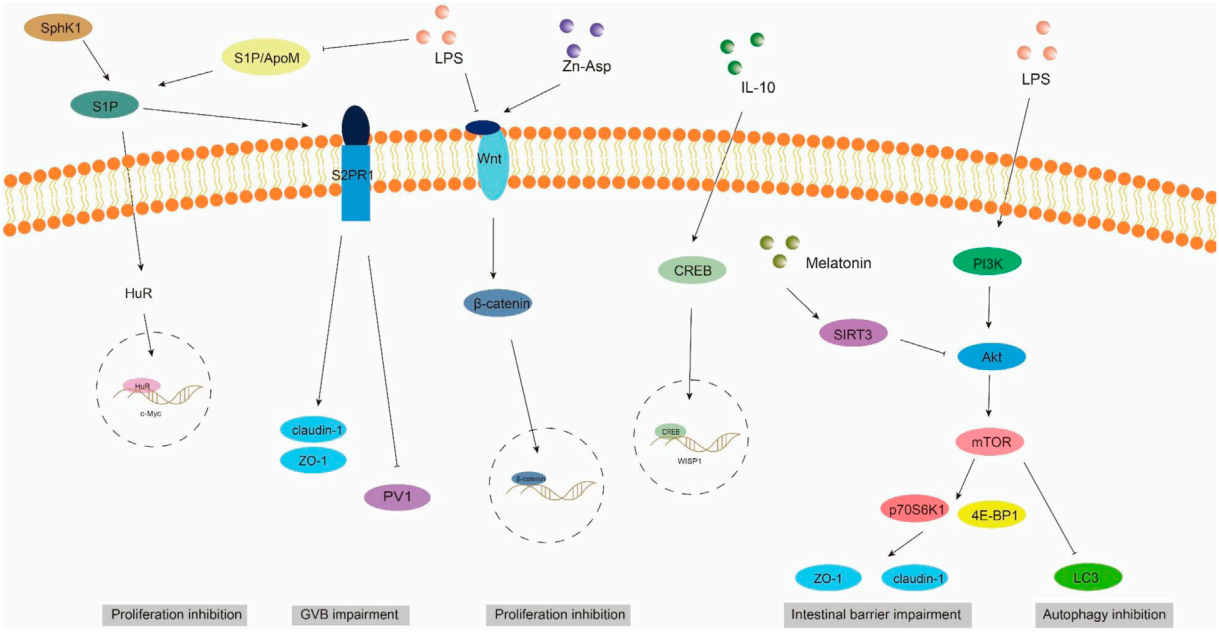


FIGURE 3
ApoM/S1P, Wnt/beta-catenin, and PI3K/AKT/mTOR signaling pathways involved in intestinal barrier damage. S1P, sphingosine 1-phosphate; S2PR1, sphingosine 2-phosphate receptor 1; SPHK1, sphingosine kinase 1; PV1, plasmalemmal vesicle-associated protein-1.

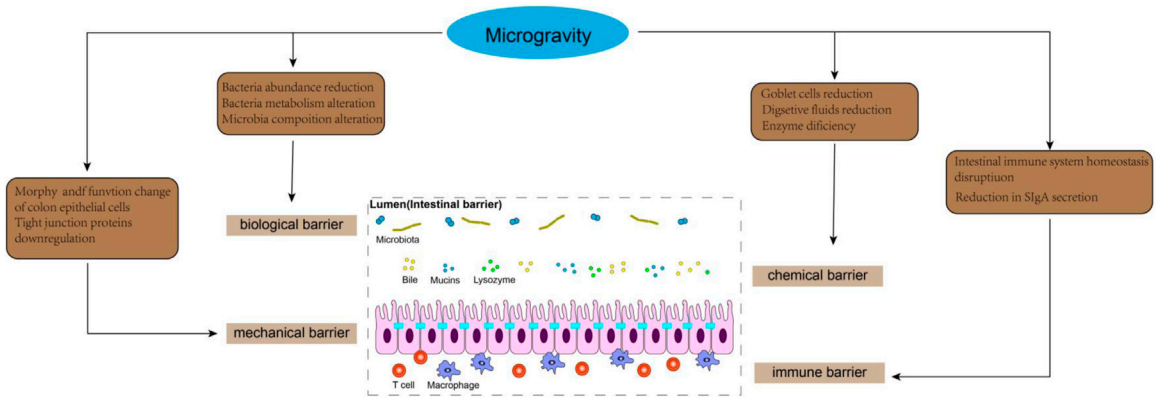


FIGURE 4
Effects of microgravity on intestinal barrier.

heightened intestinal leakage and damage to the gut-vascular barrier. Sphingosine kinase (SPHK) is a rate-limiting enzyme involved in the synthesis of S1P and plays a pivotal role in maintaining the integrity of the intestinal epithelial barrier (Li et al., 2023d). Chen et al. (2023) discovered an elevation in plasma lipopolysaccharide (LPS) levels in mice with alcoholic liver disease, accompanied by a reduction in SPHK2 protein expression and its regulated S1P, ultimately leading to disruption of the intestinal barrier. Their study substantiated that targeting SPHK2 and increasing S1P levels can ameliorate gut microbiota, reduce plasma LPS levels, and restore intestinal barrier function. In addition, Li et al. (2020) discovered that berberine can augment plasma ApoM and APOM-binding S1P levels during sepsis by inhibiting gluconeogenesis, insulin resistance, and secretion of pro-inflammatory molecules. Subsequent investigations revealed that APOM-bound S1P reduced the expression of PV1, an endothelial permeability marker induced by LPS. Furthermore, the levels of occludin and β-catenin suppressed by LPS were elevated (Li et al., 2020; Spadoni et al., 2015). It is postulated that targeting the ApoM/S1P axis could be a novel approach for treating LPS-induced intestinal barrier damage (Figure 3).

4.3 Wnt/beta-catenin signaling pathway

The WNT pathway is a complex signaling network that encompasses the classical WNT/β-catenin pathway and the non-

classical WNT pathway. It plays a pivotal role in various biological processes, including cell proliferation, apoptosis, and migration (Moparthi and Koch, 2019). The translocation of β-catenin to the nucleus occurs following activation by the Wnt ligand, thereby facilitating transcription of downstream gene transcription factors that play a pivotal role in shaping the morphology, growth, and regeneration of intestinal epithelial cells (Jung and Park, 2020). Within the gastrointestinal tract, activation of the Wnt/β-catenin signaling pathway is indispensable for maintaining epithelial homeostasis and holds dominance in recognizing and sustaining epithelial stem cells (Kuhnert et al., 2004) (Figure 2). Moreover, this pathway exhibits close association with inflammatory signaling pathways such as NF-κB and MAPK signaling, exerting influence on epithelial homeostasis and tissue regeneration; inhibition of this pathway results in loss of intestinal crypts and tissue denaturation (Moparthi and Koch, 2019). Xie et al. (2020) reported an over-activation of the Wnt/β-catenin signaling pathway following intestinal infection, resulting in aberrant proliferation of intestinal stem cells and crypts, leading to the disruption of the intestinal mucosal barrier and subsequent onset of diarrhea. Mouries et al. (2019) confirmed that fatty liver disease development is accompanied by impairment of GVB and the intestinal barrier, which is closely associated with perturbations in the WNT/β-catenin signaling pathway. Additionally, driving β-catenin activation in endothelial cells prevents damage to GVB and inhibits NASH progression. Studies have demonstrated that during the onset of weaning stress, piglets experience a range of complications including impairment to the integrity of the intestinal epithelial barrier, transient intestinal inflammation, and diarrhea (Wang et al., 2024), which are closely associated with perturbations in the wnt/β-catenin signaling pathway (Wang et al., 2022). It has been reported that upregulation of β-catenin expression can impede the proliferation of pathogenic bacteria, thereby mitigating weaning stress-induced intestinal inflammation and damage to the intestinal barrier (Tong et al., 2023). In addition, the repair of the intestinal mucosal barrier by the anti-inflammatory factor IL-10 has been demonstrated to partially rely on the activation of the Wnt pathway in epithelial cells (Quiros et al., 2017). Regulating the Wnt/β-catenin pathway can also confer protection on GVB function in sepsis (He et al., 2018). The findings of Zhou et al. (2020) demonstrated that zinc L-aspartate (Zn-Asp) effectively augmented the renewal and regeneration of intestinal stem cells (ISCs) through activation of the Wnt/β-catenin signaling pathway. Additionally, it successfully mitigated inflammation in jejunal epithelial cells and preserved intestinal barrier integrity against deoxynivalenol (DON)-induced damage. The preservation of intestinal stem cells (ISCs) is crucial for the sustained regeneration and repair of the intestinal mucosal epithelium following injury, as ISCs possess the capacity to generate multiple cell lineages within the intestinal epithelium, which relies on the proper functioning of the Wnt/beta-catenin signaling pathway (Ma et al., 2023).

4.4 PI3K/AKT/mTOR signaling pathway

Autophagy exerts beneficial effects on cellular, tissue, and organ homeostasis, while also playing a crucial role in maintaining intestinal barrier function (Wu et al., 2019). The phosphatidylinositol 3-kinase (PI3K)/Protein kinase B (AKT)/Mammalian target of rapamycin (mTOR) signaling pathway serves as a pivotal transduction factor in autophagy and is involved in the regulation of diverse cellular functions such as cell survival, growth, proliferation, and metabolis (Guo et al., 2019). It has been documented that LPS induces a significant upregulation of mRNA and phosphorylation levels within the AKT/PI3K/mTOR signaling pathway in mice, leading to intestinal inflammatory response and impairment of barrier function. Simultaneously, inhibition of the AKT/PI3K/mTOR signaling pathway can effectively safeguard the intestine against LPS-induced damage to its barrier integrity (Cheng et al., 2024). Wang et al. (2024) demonstrated that LPS significantly attenuated autophagosome formation and suppressed the expression of LC3, a key protein involved in autophagy, within the gastrointestinal tract. Consequently, this impaired elimination of damaged cellular components, leading to heightened intestinal oxidative stress and an exaggerated immune response. Subsequently, they discovered that inhibition of the PI3K/Akt/mTOR signaling pathway reversed LPS-induced suppression of autophagy, thereby mitigating damage to the intestinal barrier. The study conducted by Xu et al. (2021) showed that melatonin induces the upregulation of Sirtuins3 (SIRT3), which in turn modulates the AMPK/mTOR pathway and enhances autophagy, thereby mitigating small intestine damage in sepsis. In addition to affecting autophagy, the activation of the PI3K/Akt/mTOR signaling pathway in intestinal epithelial cells is also closely associated with the expression of tight junction proteins (Wang et al., 2015). Akt activation facilitates mTOR phosphorylation, leading to downstream substrate activation including p70S6K1 and 4E-BP1, which subsequently promote protein synthesis (Manning and Cantley, 2007). Yan and Ajuwon (2017) discovered that stimulation with LPS results in a reduction in the levels of phosphorylated Akt and total Akt, subsequently leading to a decrease in the abundance of phosphorylated 4E-BP1. This cascade effect ultimately causes a decline in the expression of tight junction proteins such as occludin, claudin-4, ZO1and 2. Furthermore, they observed that upregulation of Akt signaling counteracts the LPS-induced decrease in tight junction protein synthesis. These findings suggest that disruption of the epithelial barrier induced by LPS may be achieved through inhibition of the Akt/mTOR signaling pathway. It can be speculated that the PI3K/Akt/mTOR signaling pathway may lead to LPS-induced intestinal barrier damage through autophagy and regulatory protein synthesis. Inhibition of PI3K/Akt/mTOR may enhance autophagy while potentially reducing protein synthesis, thus emphasizing the criticality of maintaining a balanced state within the PI3K/Akt/mTOR signaling pathway to safeguard intestinal barrier integrity (Figure 3).

5 Effects of microgravity on intestinal barrier

Microgravity is the predominant characteristic of the space environment, which can induce physiological adaptation and pathophysiological alterations in multiple systems, such as muscle atrophy, bone demineralization, and immune system dysregulation

(Nie et al., 2024). As a vital interface with the external milieu, the intestinal barrier function plays a crucial role in safeguarding the internal homeostasis by effectively impeding the entry of harmful substances. In recent years, increasing attention has been devoted to investigating the impact of microgravity on gastrointestinal physiology and function, particularly pertaining to the intestine (Figure 4). Moreover, despite its close interconnection with other extraintestinal organs, our understanding of gut-organ axis under microgravity conditions remains limited. Future comprehensive exploration into the intricate interactions between gut and extraintestinal organs within a microgravity environment will significantly contribute to unraveling the mechanisms underlying intestinal barrier function in space.

5.1 Intestinal mechanical barrier

The intestinal mechanical barrier is primarily comprised of intestinal epithelial cells (IEC), intercellular junctions, basement membrane, and the submucosal lamina propria, which collectively form the structural foundation of the intestinal mucosal barrier. IEC mainly consists of absorptive enterocytes, goblet cells and intestinal endocrine cells (He et al., 2021). The intercellular junctions in intestinal epithelial cells consist of tight junctions, adherens junctions, gap junctions, and desmosomes. The tight junction complex in the intestinal epithelium, comprising ZO-1, β-catenin, cadherin, claudins, and occludin proteins, plays a crucial role in regulating intestinal permeability. The intact structure of intercellular junctions effectively seals the gap between adjacent epithelial cells, thereby impeding the infiltration of bacteria and toxins into the lamina propria of the intestinal mucosa (Crowley et al., 2024). The occurrence of various adaptive and pathophysiological changes in the structure and physiology of the digestive tract has been reported in a microgravity environment, including the disruption of intestinal microvilli architecture and a significant decrease in microvilli surface area (Li et al., 2015). Spaceflight lasting from 7 to 18 days may compromise the morphology and functionality of colon epithelial cells (Rabot et al., 2000). Furthermore, compared to rats exposed to normal gravity, tail-suspended rats exhibited a significant increase in apoptosis of IECs in the ileum, which was associated with upregulated expression of pro-apoptotic protein Bax and downregulated expression of anti-apoptotic protein Bcl-2 (Jin et al., 2018). As the primary proteins in tight junctions, claudin-1 and claudin-5 play a pivotal role in maintaining the integrity of the epithelial barrier (Abdulqadir et al., 2023). Additionally, E-cadherin serves as a fundamental constituent of adhesion junctions, ensuring mechanical strength and stability to the intestinal lining (The zonula adherens matura redefines the apical junction of intestinal epithelia). Li et al. (2021) discovered that tail suspension led to a decrease in the expression of occludin and zonula occludens-1 (ZO-1), while increasing the concentration of DAO and D-lactic acid in plasma. These findings suggest that simulated weightlessness may impair the intestinal barrier function by disrupting tight junctions and enhancing intestinal permeability. Similarly, Jin et al. (2018) demonstrated a significant reduction in the expression of tight junction proteins such as occludin, claudin-1, claudin-5, and E-cadherin in the ileum of rats after hindlimb unloading for 21 days. The study also demonstrated that under simulated microgravity conditions, the downregulation of tight junction expression was closely associated with the activation of TLR4/MyD88/NF-κB signaling. IECs cultured in simulated microgravity using a rotating wall vessel (RWV) for 18 days prior to seeding on normal gravity condition exhibited reduced junctional ZO-1 localization and FITC-dextran (FD4) permeability, compared with static cells (Alvarez et al., 2019). This study suggests that simulated microgravity can induce a potential and sustained susceptibility to epithelial barrier disruption after being removed from the microgravity environment. The study conducted by Wang et al. (2021) demonstrated that exposure to simulated microgravity for a duration of 3 weeks resulted in impairment of the intestinal mucosal barrier, characterized by reduced goblet cell count, enlarged intercellular space, downregulated adhesion molecules, and increased intestinal permeability in rats. Subsequently, employing proteomics techniques, they discovered that simulated microgravity significantly suppressed the expression of adhesion molecules and disrupted several signaling pathways associated with metabolism, adhesion plaques, and regulation of actin cytoskeleton. Notably, Wang et al.'s findings showed that the downregulation of adhesion molecules and the upregulation of myosin-regulated light chain (MLC) phosphorylation mediated by myosin light chain kinase (MLCK) contributed to intestinal barrier dysfunction during simulated microgravity injury (Wang et al., 2021). It can be speculated that the regulation of epithelial MLCK could potentially offer a novel approach for addressing intestinal barrier injury in microgravity. Additionally, intestinal interstitial connective tissue plays a crucial role in maintaining the mechanical barrier function and osmotic balance of the intestines. Research has demonstrated that specific structural and functional rearrangements of the intestinal connective tissue occur in microgravity conditions (Shishkina et al., 2024). Shishkina et al. (2024) found that the content of fibrous extracellular matrix within the connective tissue in the intestinal wall of C57BL/6N mice after 30 days of spaceflight was significantly reduced, especially the expression of reticular skeleton in the lamina propria and the muscularis externa. This is intricately associated with matrix metalloproteinases (encompassing mast cell proteases) that actively contribute to the gravity-induced adaptations in the intestines (Shishkina et al., 2024; Atiakshin et al., 2023a). In summary, the impact of microgravity on the intestinal epithelium, intercellular connections, and connective tissue of the intestinal wall leads to a profound alteration in the mechanical barrier function of the intestine.

5.2 Intestinal chemical barrier

The chemical barrier primarily consists of mucus secreted by the intestinal epithelium, digestive fluid, and antibacterial substances released by probiotics. Mucin (MUC2), which is predominantly produced by intestinal goblet cells and epithelial cells, serves as the principal constituent of the mucous layer covering the intestinal

epithelium surface. Structurally resembling bacterial adhesion receptors, MUC2 hinders bacterial attachment to intestinal epithelial cells through competitive binding sites, thereby facilitating bacteria retention within the mucosal layer and subsequent expulsion during intestinal peristalsis (Cheong et al., 2024). It has been reported that the expression of mucin and the number of goblet cells in the gut of Sprague-Dawley rats were found to be reduced after 14 days of space flight, when compared to age-matched ground-based controls (Rabot et al., 2000). Similarly, a decrease in MUC-19 expression was observed in the digestive acinar cells of mice flown on the US space shuttle Atlantis (STS-135). The researchers also suggest that identifying changes in salivary mucin may facilitate the development of non-invasive methods for assessing astronauts' digestive physiological state (Dagdeviren et al., 2018). Digestive juices play a crucial role in safeguarding the integrity of the gastrointestinal tract. The presence of digestive fluid within the intestines serves to dilute toxins, while also facilitating the removal of pathogenic bacteria from adhering to the intestinal epithelium (Wang et al., 2019). Previous studies indicated an initial increase in intestinal and bile secretions among volunteers subjected to bed rest for a duration of 2 months. However, it was observed that the secretion of digestive fluids gradually declined after this period, potentially contributing to prolonged simulated microgravity-induced injury to the intestinal barrier (Hargens and Vico, 1985). Digestive enzymes are crucial for maintaining normal intestinal function. Studies have shown that Mongolian gerbils subjected to a 12-day space flight exhibited significantly reduced trypsin levels in the stomach and jejunal walls. This enzymatic deficiency led to inadequate mitosis of smooth myocytes in the intestinal walls, resulting in thinning of the smooth muscle layer and subsequently affecting gastrointestinal motility (Atiakshin et al., 2023b). Although direct examination of the impact of digestive enzymes on gut barrier function was not conducted, the correlation between these two factors was evident (Zheng et al., 2024).

5.3 Intestinal biological barrier

The biological barrier is a microecosystem established by the symbiotic bacteria in the intestinal cavity in a specific proportion. Intestinal microbiota plays a crucial role in regulating the integrity of the intestinal barrier and host wellbeing, and maintaining a healthy gut environment necessitates stability in terms of species composition, abundance, and localization. They not only adhere closely to the surface of intestinal epithelial mucosa to form a bacterial membrane barrier but also enhance tight junction protein proliferation, promote secretion of the intestinal mucus layer and IgA, as well as interact with other components of the intestinal barriers (Ge et al., 2020). It has been reported that simulated microgravity significantly reduces the abundance of bacteria associated with anti-inflammatory effects, such as Subdoligranulum, Faecalibacterium, Fusicatenibacter, Butyricicoccus, and Lachnospiraceae-NK4A136-0 group when compared to normal gravity (Han et al., 2022). Additionally, KEGG pathway analysis unveiled that microgravity exerts significant impacts on the metabolism of gut microbiota, including pyrimidine, fatty acid, glyoxylate and dicarboxylate, peptidoglycan biosynthesis, as well as carbon fixation in photosynthetic organisms (Siddiqui et al., 2022). The microgravity environment may disrupt the human intestinal microbiota and subsequently compromise the integrity of the intestinal biological barrier. Jin et al. (2018) demonstrated that hindlimb unloading (HU) resulted in a reduction in the abundance of *Clostridium*, a butyric acid-producing bacterium. Butyric acid plays a crucial role as a regulatory factor in the proliferation and differentiation of intestinal epithelial cells. The decrease in butyric acid content may compromise the morphology of intestinal epithelial cells, thereby impairing intestinal barrier function and increasing intestinal permeability (Koh et al., 2016). Many digestive disorders such as diarrhea, intestinal stress, and ulcerative colitis have been closely associated with alterations in butyric acid levels (Koh et al., 2016). Interestingly, studies have revealed that 9 days of space flight led to a significant increase in short-chain fatty acids (SCFAs) concentration within rat cecal contents; however, there was a concurrent decrease observed specifically for butyric acid proportion (Rabot et al., 2000). Shi et al. (2017) discovered that, in comparison to the ground control group, HU resulted in a significant alteration of the intestinal microbiota composition characterized by an expansion in Firmicutes and a reduction in Bacteroidetes. This dysbiosis of gut flora led to a decline in intestinal goblet cell count, epithelial cell turnover rate, as well as the expression of genes associated with defense mechanisms and inflammatory responses among HU mice. Subsequent investigations demonstrated that alterations in gut microbiota increased susceptibility to colitis development in HU mice. Notably, transplantation of fecal matter from normal mice into HU mice ameliorated the damage inflicted upon the intestinal barrier. Probiotics can effectively address gastrointestinal issues that arise during spaceflight and enhance the function of the intestinal barrier by competing with pathogens, reinforcing tight junctions between intestinal epithelial cells, producing vital metabolites, and interacting with host cells to promote physiological and immune alterations (Cunningham et al., 2021). Shao et al. (2017) demonstrated that L. acidophilus probiotics exhibit resilience in stressful microgravity conditions and persist for an extended period within the gastrointestinal tract while maintaining their adhesion ability, thereby preserving the integrity of the intestinal epithelial barrier and preventing pathogen infiltration. The stability of the Freeze-dried *Lactobacillus* casei Strain Shirota capsule was assessed during a 1-month period aboard the International Space Station, revealing its potential to enhance innate immunity and restore gut microbiome equilibrium (Sakai et al., 2018). The value of probiotics in the space environment is gradually being unraveled (Bharindwal et al., 2023). It is imperative to further investigate the alterations of probiotics in the microgravity environment and apply them to address the issue of intestinal biological barrier breakdown that occurs during spaceflight.

5.4 Intestinal immune barrier

The intestinal immune barrier consists of gut-associated lymphoid tissue (GALT), diffuse immune cells and secretory immunoglobulin (SIgA). GALT primarily consists of mesenteric lymph nodes (MLN) and lamina propria lymphocytes. GALT plays

a crucial role in maintaining the stability of the intestinal immune environment by timely eliminating danger signals. In response to innocuous stimuli, GALT can activate the mechanism of immune tolerance, thereby ensuring the body remains under low feedback immune surveillance (Cukrowska et al., 2017). SIgA is secreted by intestinal immune tissue and plays a crucial role in humoral immunity. It has ability to specifically bind to bacterial antigens, thereby inhibiting bacterial adhesion. Its deficiency can significantly increase the risk of intestinal fistulas and bacterial translocations (Wang et al., 2019). Additionally, macrophages and natural killer cells present in the lamina propria of the intestinal mucosa serve as vital constituents of the intestinal immune barrier and actively participate in immune responses related to enteric functions (Ge et al., 2020). The study conducted by Li et al. (2015) demonstrated that, in comparison to control mice, the population of Treg cells and IL-10 levels in the gut of HU mice were reduced by more than two-fold, while neutrophils and IL-1β exhibited an approximately two-fold increase. These findings provide confirmation that disrupted intestinal immune system homeostasis in mice exposed to simulated microgravity results in a pro-inflammatory shift within the intestinal microenvironment and heightened susceptibility to colitis. The disturbance of the intestinal immune system during space flight is closely associated with alterations in the gut microbiota. Jin et al. (2018) reported that alterations in the gut microbiota of HU rats, characterized by an expansion of *Bacteroides* and a reduction in firmicutes, resulted in significant production of enterogenic endotoxin. This subsequently activated the TLR4/MyD88/NF-κB signaling pathway, leading to increased levels of pro-inflammatory cytokines and decreased SIgA levels, ultimately disrupting intestinal immune homeostasis. Additionally, research has demonstrated that in simulated microgravity environments, the composition of the intestinal microbiota becomes imbalanced, characterized by an elevated proportion of anaerobic and biofilm-forming bacteria, while the proportion of aerobic and Gram-negative bacteria decreases. Moreover, bile acid metabolism is disrupted under conditions of weightlessness (resulting in decreased levels of glycine ursodeoxycholic acid, glycine chenodeoxycholic acid, glycine deoxycholic acid, and glycine cholic acid). Collectively, these factors contribute to a significant rise in intestinal oxidative stress and inflammatory markers in HU rats leading to a reduction in SIgA secretion (Wang et al., 2024). Currently, there is a limited body of research on the intestinal immune barrier in a microgravity environment. Further investigation into the interplay between the immune barrier and other barriers under weightlessness is crucial for comprehending alterations in intestinal function during microgravity conditions.

6 The intervention strategies to intestinal barrier damage

6.1 Nutritional support

Appropriate nutritional support can promote the repair and regeneration of intestinal mucosa and enhance the defense function of intestinal barrier. Leman Arslan Ariturk et al.'s studies have shown that Docosahexaenoic acid (DHA) can reduce the production of reactive oxygen species, reduce the level of pro-inflammatory cytokines, prevent neutrophil infiltration, etc. Thereby reducing epithelial shedding of the colon and improving glandular structure and mucosal integrity (Ariturk et al., 2024). Yin et al. found that dietary fiber from sweetpotato residues (SRDF) can significantly improve intestinal barrier function by improving intestinal morphology and permeability and inhibiting inflammatory response (Yin et al., 2024). Li et al. (2024) demonstrated that VD/VDR can promote Notch-1 transcription to maintain intestinal tight junction integrity and barrier function. Lu et al. (2024) showed that dietary α-Ketoglutarate (AKG) can prevent mitochondrial dynamic dysfunction, endoplasmic reticulum stress, and mitochondria-associated endoplasmic reticulum membrane disorder, ultimately alleviating LPS-induced intestinal damage. Therefore, through reasonable selection of nutritional support methods, optimization of nutritional formula, attention to the detailed management of nutritional support, avoidance of intestinal damage factors and formulation of personalized nutritional support programs, we can effectively protect the intestinal barrier function and promote the recovery of patients.

6.2 Drug treatment

Antibiotics usually rapidly sterilize most bacteria. More and more evidence shows that antibiotics can effectively intervene in intestinal barrier damage, such as rifaximin can significantly increase the level of serum long chain fatty acids and carbohydrate metabolic intermediates, and then affect serum pro-inflammatory cytokines and secondary bile acids, thereby improving the structure of intestinal microbiota and intestinal immune function (Bajaj, 2016). Due to the limitation that antibiotics cannot specifically change the ecology of intestinal flora, bacterial therapy has gradually emerged because of its unique advantages. Probiotics can regulate blood metabolites related to intestinal microbiota, such as cytokines, amino acids and vitamins, which have an impact on intestinal microbiota and thus intervene in intestinal damage (Liu et al., 2021). Studies have shown that probiotics can colonize the human gut and improve the balance of intestinal microbiota. It can improve the integrity of intestinal barrier and reduce intestinal damage by alleviating oxidative stress, enhancing immune response, and increasing the production of short-chain fatty acids (Li et al., 2024; Toritsuka et al., 2024). In addition, other drug interventions can also effectively deal with intestinal barrier damage. Fan et al. (2024) research has shown that Methane saline (MS) can reduce iron death by regulating Nrf2/HO-1 signaling pathway and reduce intestinal ischemia-reperfusion damage. Liu et al. (2024) found that p-Hydroxybenzaldehyde (HD) can combat oxidative stress through the Keap/Nrf2/HO-1 pathway and NF-κB/AP-1 pathway to prevent intestinal barrier damage. Nowadays, many Chinese herbal decoction have shown good therapeutic effect on intestinal barrier damage. For example, modified Zhenwu Decoction can improve intestinal barrier function of experimental colitis by activating sGC mediated

cGMP/PKG signaling (Xu et al., 2024c); Paeoniae decoction (PD) can be regulated by intestinal flora and ILC3 interaction to repair chronic colitis intestinal mucosa damage (Huang et al., 2024); Sijunzi decoction can reduce intestinal epithelial barrier damage by regulating intestinal flora and improving inflammation (Li et al., 2024). In a word, with the continuous deepening of traditional Chinese medicine research and the continuous development of modern science and technology, traditional Chinese medicine decoction has broad application prospects and important research value in the treatment of intestinal barrier damage. It is believed that traditional Chinese medicine decoction will play a more important role in the treatment of intestinal barrier damage.

6.3 Other interventions

Chen et al. found that moxibustion can improve intestinal barrier function by regulating blood lipids, improving insulin resistance, and alleviating inflammation (Chen et al., 2024). Studies by Liu et al. have shown that Electroacupuncture (EA) can regulate the expression of Corticotropin-Releasing Factor (CRF) and its receptor in the brain-gut interaction pathway through the CRF signaling pathway, thereby reducing inflammatory response and damage to the intestinal mucosal barrier (Liu et al., 2024). Sun et al. used autoinducer-2 to enhance the expression of tight inducer protein to reduce intestinal damage (Sun et al., 2024), maintain water and electrolyte balance, reduce intestinal peristalsis and other measures also help improve intestinal barrier damage. In addition, for severe intestinal barrier damage, surgical treatment may be required, such as removal of dead tissue and reconstruction of the intestine.

In conclusion, a multi-faceted strategy is needed to mitigate intestinal barrier damage. This includes maintaining a balanced diet to support gut health, and the rational use of antibiotics to prevent microbial imbalance. In addition, supplementing with probiotics and prebiotics helps restore the beneficial flora of the gut and enhances the integrity of the gut barrier. Managing stress levels and getting enough rest is also important to prevent damage to the gut barrier. In addition, regular exercise, quitting smoking and limiting alcohol intake are essential for maintaining gut health. Finally, patients who already have intestinal barrier damage need to receive specific medical treatment and to promote recovery and prevent further complications under the close supervision of a medical professional.

7 Conclusion

In this review, we initially discuss the role of "gut-organ" axis disruption in the impairment of the intestinal barrier. The compromise of the intestinal barrier can arise from a multitude of factors. Alongside inflammation, stress, tumors, and other intrinsic factors affecting the gastrointestinal tract itself, alterations in extra-intestinal organs also contribute significantly to damage to the intestinal barrier. In general, alterations in the extra-intestinal organs result in damage to the intestinal barrier through a cascade of effects including an inflammatory response, release of metabolites, and influence on intestinal circulation perfusion. Consequently, there is an increase in intestinal permeability and translocation of intestinal flora, leading to the release of enterogenic endotoxins into the bloodstream which act on the original organ via the circulatory system, further exacerbating deterioration of the intestinal organ. The emergence of this detrimental cycle relies on dysregulation within the "gut-organ" axes. It is worth mentioning that in the early stage of certain extra-intestinal organs injury, increased intestinal permeability and the release of endotoxins can be detected, which is necessary for early intervention of the disease. At the same time, the assessment of intestinal barrier damage can also help us to judge the prognosis of extra-intestinal organs. In short, intestinal barrier damage is not only a disease of the intestine itself, but also a "detection agent" for functional changes in other organs. It is noteworthy that during the early stage of certain extra-intestinal organ injuries, there is an increase in intestinal permeability and subsequent release of endotoxins, which plays a crucial role in the timely intervention of the disease. Simultaneously, evaluating the extent of intestinal barrier damage can aid in prognosticating extra-intestinal organ function. In essence, intestinal barrier damage not only affects the intestine itself but also serves as a "detection agent" for functional alterations in other organs.

Secondly, we have summarized the molecular mechanisms underlying intestinal barrier damage (Table 1). In brief, the TLR4 signaling pathway, in conjunction with NF-κB and MAPK, primarily mediates the inflammatory response within the intestine, while inflammatory factors exert a negative impact on tight junction protein expression. The ApoM/S1P pathway predominantly influences the gut vascular barrier (GVB), leading to a significant increase in intestinal permeability. Intestinal stem cells (ISCs) play an indispensable role in sustained regeneration and repair of the intestinal mucosal epithelium, and aberrant activation of WNT/β-catenin can disrupt normal proliferation of ISCs, thereby affecting multiple cell lineages within the intestinal epithelium. Additionally, it is worth noting that LPS-induced intestinal barrier damage may be mediated by autophagy and regulatory protein synthesis through activation of the PI3K/Akt/mTOR signaling pathway. Further exploration into signal pathways associated with intestinal barrier damage holds immense significance for advancing molecular targeted drug development.

Finally, we have concluded the alterations in the intestinal barrier under microgravity conditions (Table 2). In a microgravity environment, there are notable changes observed in intestinal epithelial cells and their tight intercellular connections, which contribute significantly to the increased permeability of the intestines. Rresearch has been conducted on the impact of microgravity on the biological barrier of the intestines. It has been discovered that both the composition and metabolites of intestinal microbiota undergo disturbances in a microgravity environment. These alterations in gut flora also influence various aspects such as intestinal epithelial cell formation, immune responses within the intestines, and mucus production. Furthermore, microgravity affects chemical and immune barriers within the intestines by reducing secretion levels of digestive fluids, mucin, and SIgA (Atiakshin et al., 2019). However, limited studies

TABLE 1 Intestinal injury molecular mechanism.

Signaling Pathway	Description	Results	References
LPS/TLR4	Activate the NF-κB and MAPK pathways that regulate inflammation	Impaired intestinal barrier function	Izadparast et al. (2022)
TLR9	NETs induce the activation of TLR9 signaling pathway leading to increased intestinal inflammation	Mechanical barrier disruption	Sun et al. (2021)
TLR4/ATF/CHOP	The activation of the TLR4/ATF/CHOP signaling pathway by ER stress can induce apoptosis or dysfunction in Panzer cells	Lowered antimicrobial peptide secretion, exacerbating injury	Wang et al. (2022a)
TLR	Overexpression of PTX3 can inhibit the TLR signaling pathway, thereby reducing levels of inflammatory factors such as TNF-α, IL-1β, and INF-γ	Promotes ZO-1 and occludin expression, reducing intestinal epithelial damage	Li et al. (2022)
NF-κB	TNF-α and IL-6 activation of TAK1 leads to dissociation of NF-κBp65	The integrity of the intestinal mechanical mucosal barrier is compromised	Cao et al. (2020), Cao et al. (2018)
NF-κB	The activated NF-κB p65 interacts with the promoter region of MLCK and regulates the transcription of MLCK74	The expression of tight junction protein was downregulated and intestinal permeability was increased	Gatica-Andrades et al. (2017)
NF-κB	Affect the expression of MLCK, MLC, ZO-1 and occludin	Affects the integrity and permeability of intestinal epithelial cells	Quan et al. (2020), Zhao et al. (2021)
p38MAPK	Inhibit cell cycle progression, promote apoptosis and Affect the expression of MLCK, MLC, ZO-1 and occludin	Induces oxidative stress responses and triggers the release of inflammatory cytokines, including TNF-α, IL-1β, and IL-6, Affects intestinal barrier function	Luo et al. (2023), Turner et al. (1997), Zolotarevsky et al. (2002), Wang et al. (2005), Wang et al. (2017)
ERK 1/2	Regulates cell survival, differentiation and proliferation	Leads to the remodeling of the intestinal microenvironment	Yang et al. (2013), Xu et al. (2024b)
JNK/SAPK	Inhibit cell cycle progression and promote apoptosis, Affect the expression of ZO-1 and closure protein	Affecting the intestinal barrier	Samak et al. (2015), Ge et al. (2023)
ApoM/S1P	LPS, TNF-α and IL-1 inhibit the production of ApoM, Targeting SPHK2 and increasing S1P levels can improve gut microbiota	Affects intestinal vascular barrier and permeability	Jiang et al. (2015), Christensen et al. (2016)
Wnt/β-catenin	Overactivation of the Wnt/β-catenin signaling pathway leads to abnormal proliferation of intestinal stem cells and crypts	The intestinal mucosal barrier is destroyed	Xie et al. (2020)
Wnt/β-catenin	Driving β-catenin activation in endothelial cells	Prevents damage to GVB and inhibits NASH progression	Mouries et al. (2019), He et al. (2018)
Wnt/β-catenin	Upregulation of β-catenin expression can impede the proliferation of pathogenic bacteria	Reduces intestinal inflammation and intestinal barrier damage	Wang et al. (2022b)
Wnt/β-catenin	Zn-Asp effectively augmented the renewal and regeneration of ISCs through activation of the Wnt/β-catenin signaling pathway	Protects the integrity of the intestinal barrier	Zhou et al. (2020)
AKT/PI3K/mTOR	It is a key transduction factor in the process of autophagy and is involved in the regulation of a variety of cell functions, such as cell survival, growth, proliferation and metabolism	Affects intestinal inflammatory response and barrier function	(Guo et al., 2019) (Cheng et al., 2024)
AKT/PI3K/mTOR	Melatonin induces the upregulation of SIRT3, which in turn modulates the AMPK/mTOR pathway and enhances autophagy	Reduce small intestinal damage	Xu et al. (2021)
AKT/PI3K/mTOR	Through autophagy and regulation of protein synthesis, such as tight junction protein expression	Affects intestinal barrier integrity	Wang et al. (2015), Manning and Cantley (2007)

have focused on exploring these effects specifically on chemical and immune barriers under microgravity conditions. Further investigation into how microgravity impacts different aspects of intestinal barriers is crucial for preventing damage to these barriers during space travel while ensuring optimal gastrointestinal health for astronauts. Moreover, this knowledge will provide valuable insights for future space exploration endeavors and potential colonization beyond our planet.

TABLE 2 The effects of microgravity on intestinal barrier.

NO	Intestinal barrier	Changes	Reference
1	Mechanical barrier	The disruption of intestinal microvilli architecture and a significant decrease in microvilli surface area	Li et al. (2015)
2	Mechanical barrier	Compromise the morphology and functionality of colon epithelial cells	Rabot et al. (2000)
3	Mechanical barrier	Significant increase in apoptosis in the ileum	Jin et al. (2018)
4	Mechanical barrier	Impair the intestinal barrier function by disrupting tight junctions and enhancing intestinal permeability	Jin et al. (2018) , Abdulqadir et al. (2023), Li et al. (2021), Alvarez et al. (2019)
5	Mechanical barrier	The downregulation of adhesion molecules and the upregulation of myosin-regulated light chain (MLC) phosphorylation mediated by myosin light chain kinase (MLCK)	Wang et al. (2021)
6	Mechanical barrier	Specific structural and functional rearrangements of the intestinal connective tissue	Shishkina et al. (2024), Atiakshin et al. (2023a)
7	Chemical barrier	The expression of mucin and the number of goblet cells in the gut were found to be reduced	Dagdeviren et al. (2018)
8	Chemical barrier	The secretion of digestive fluids gradually declined	Hargens and Vico (1985)
9	Chemical barrier	Significantly reduced trypsin levels in the stomach and jejunal walls	Atiakshin et al. (2023b)
10	Biological barrier	Significantly reduces the abundance of bacteria associated with anti-inflammatory effects	Han et al. (2022)
11	Biological barrier	Significant impacts on the metabolism of gut microbiota	Siddiqui et al. (2022)
12	Biological barrier	A significant alteration of the intestinal microbiota composition	Shi et al. (2017)
13	Immune barrier	Disrupted intestinal immune system homeostasis results in a pro-inflammatory shift within the intestinal microenvironment	Li et al. (2015), Jin et al. (2018)
14	Immune barrier	A significant rise in intestinal oxidative stress and inflammatory markers leading to a reduction in SIgA secretion	Wang et al. (2024d)

Author contributions

H-YN: Data curation, Writing–original draft. JG: Data curation, Writing–original draft. G-XH: Data curation, Writing–original draft. K-GL: Formal Analysis, Writing–review and editing. YY: Investigation, Writing–review and editing. HL: Methodology, Writing–review and editing. H-GL: Supervision, Writing–review and editing. TZ: Validation, Writing–review and editing. H-FY: Methodology, Writing–review and editing. B-XX: Project administration, Writing–review and editing. H-WS: Supervision, Writing–review and editing. J-WY: Project administration, Writing–review and editing. S-YS: Validation, Writing–review and editing. J-LZ: Methodology, Writing–review and editing. YC: Conceptualization, Writing–review and editing.

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Conflict of interest

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

Publisher's note

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EDITED BY
Daniele Maria-Ferreira,
Instituto de Pesquisa Pelé Pequeno Príncipe, Brazil

REVIEWED BY
Mani Abdul Karim,
XIM University, India
Mallikarjun Bachalapur,
Visvesvaraya Technological University, India

*CORRESPONDENCE
Andy Wai Kan Yeung,
ndyeung@hku.hk

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Tracing the historical foundations of infliximab in Crohn's disease treatment: a cited reference analysis

Andy Wai Kan Yeung*

Oral and Maxillofacial Radiology, Applied Oral Sciences and Community Dental Care, Faculty of Dentistry, The University of Hong Kong, Hong Kong, Hong Kong SAR, China

Introduction: The use of infliximab to treat Crohn's disease patients has been evaluated for decades. The current work aimed to identify the historical roots of this research topic.

Methods: The literature database Web of Science Core Collection was searched to identify relevant papers. Cited reference analysis on the identified literature set was performed using CRExplorer, a dedicated bibliometric software. The disruption index was computed with an automated routine described by Leydesdorff and Bornmann, which is freely available online. Based on data from citation count and reference list, the disruption index can range from −1 to +1, with −1 meaning a continuity from existing research and +1 meaning a disruption.

Results: This analysis successfully identified key references dealing with infliximab use on Crohn's disease patients, such as the original report that introduced the Crohn's Disease Activity Index (CDAI) in 1976, the first case series reporting a favourable outcome of infliximab infusion on 10 patients published in 1995, the first randomized controlled trial published in 1997, the ACCENT I and ACCENT II trials published in 1999 and 2002, and a couple of European consensus guidelines on the diagnosis and management of Crohn's disease.

Conclusion: Cited reference analysis could reveal the historical origins of the use of infliximab in treating Crohn's disease. Highly cited references included CDAI, important early clinical studies, and European consensus guidelines. The important cited references identified by the analysis provided solid foundation to support subsequent research.

KEYWORDS

Crohn's disease, infliximab, cited reference analysis, CDAI, ACCENT I

1 Introduction

Crohn's disease is a type of inflammatory bowel diseases (IBD) and has affected many people around the world. The collective data during the period of 1990–2016 have indicated that the prevalence of Crohn's disease is higher among European and North American countries, such as the United States (96.3 per 100,000), but lower in Asian countries, such as Japan (18.6 per 100,000) (Ng et al., 2017). Crohn's disease is a debilitating disease that is chronic inflammatory in nature, progressively damaging the gastrointestinal tract with

periods of relapses and remission; and developing strictures, fistulas, and abscesses that often require surgical management (Torres et al., 2017). Recent papers from medicine (Caprilli et al., 2002; Sands, 2007; Hindryckx et al., 2014) have acknowledged Targan et al. (1997) as the first randomized controlled trial of infliximab on treating Crohn's disease patients. To the best understanding of the author, infliximab was first described in 1993 as a chimeric human/mouse monoclonal anti-tumor necrosis factor alpha antibody (known as cA2) produced by Centocor Inc. (Malvern, Pennsylvania, United States) and experimentally used to treat rheumatoid arthritis (Elliott et al., 1993; Knight et al., 1993). Within the same year, it was used to experimentally treat a patient with severe Crohn's disease with promising results (Derkx et al., 1993). Apart from consulting academic historians who are familiar with the historical development of infliximab, readers may also rely on review papers and meta-analyses to have an idea on the availability of randomized controlled trials published in the literature. In the current work, the method of cited reference analysis was demonstrated to identify the historical roots of infliximab research with Crohn's disease, its first trial, and important references published prior to the first trial.

The pioneering works may provide insights to guide researchers for future studies. For instance, readers should appreciate Crohn et al. (1932) that reported a case series of patients affected by Crohn's disease, described as regional ileitis at that time (Crohn et al., 1932). In order to classify patients into different groups according to disease severity for management triage, Best et al. (1976) developed the Crohn's Disease Activity Index (CDAI) (Best et al., 1976). Also well-known by the research community are the ACCENT I and ACCENT II trials (Present et al., 1999; Hanauer et al., 2002). By identifying and studying these works in a chronological order from bibliometric data, researchers can better understand the development or evolution of treatment regimens with their corresponding patient groups. Several bibliometric studies concerning IBD have been published, including (but not limited to) analyses of the top 25 cited articles on COVID-19 and IBD (Veisman et al., 2022), immunotherapy and biotherapy for IBD (Xiong et al., 2022), original articles of the Crohn's disease research literature (Karabulut and Kaya, 2023), and the use of ustekinumab for Crohn's disease (Chen et al., 2024). However, none of them focused on infliximab. Besides, there is a lack of a historical overview of infliximab in Crohn's disease management using bibliometric tools, which may offer another perspective apart from traditional review papers based on expert opinion. Hence, the current work aimed to demonstrate the use of cited reference analysis to identify and study the historical roots of research on the use of infliximab to treat Crohn's disease. In addition, a disruption index for the most cited references identified from the analysis would be calculated. In short, a disruption index gives a score to a cited reference from −1 to +1, with −1 meaning a continuity from existing research and +1 meaning a disruption (Wu et al., 2019). Please the Materials and Methods section for more methodological details.

2 Materials and methods

The Web of Science Core Collection (WOSCC) literature database was queried on 14 August 2024 to identify studies on the use of infliximab to treat Crohn's disease. The following search terms were used: (Infliximab OR Remicade OR Ixifi OR Renflexis OR Inflectra OR Remsima) AND (Crohn*). The former group of words were searched in the title, abstract, and author keyword fields of papers indexed in WOSCC, whereas the term Crohn* was searched in the title field only to make the dataset more specific. Papers were limited to those labelled as articles (original articles) by WOSCC. Besides this, no other restrictions were placed to limit the search. Finally, the search yielded 1,793 articles.

The full record and cited references of these articles were exported to CRExplorer (Thor et al., 2016) to undergo a method of cited reference analysis called reference publication year spectroscopy (RPYS). In short, the number of times that the cited references were cited by the 1793 articles were counted and sorted by the publication year of the cited references. The positive and negative peaks shown in an RPYS visualize years when the citation count of the cited references deviated from its 5-year median. Take the year 2002 when the ACCENT I trial was published as an example. From the downloaded dataset of 1793 articles, cited references published in 2000–2004 were cited 1,616, 1,647, 2,537, 2,187, and 2,580 times respectively. The 5-year median citation count was 2,187. It meant that references published in 2002 were cited 350 times more than its 5-year median and thus created a positive peak with a magnitude of 350. Many positive peaks were generated by RPYS. The original plan was to follow the routine of CRExplorer developers to identify "important peaks" with Tukey's fences based on the interquartile range of the median deviations with positive values (Haunschild and Bornmann, 2022; Gruber et al., 2023). However, only one peak could survive the lower fence. Therefore, the final decision was to lower the threshold to the upper quartile. Peaks that had a magnitude exceeding the upper quartile were deemed significant in the current work. From each significant peak, the most cited reference was identified. Using CRExplorer, it was possible to identify reference publication years (RPY) that experienced a sudden increase in total citations received and to pinpoint the corresponding references responsible for the increase. This method enables users to identify important cited references that are frequently cited by a predefined literature set but may not have a very high total citation count to be recognized by traditional citation analysis.

Disruption index (DI) for the most cited references from the identified peaks were calculated. In short, a disruption index gives a score to a cited reference (also called a focal paper) from −1 to +1, with −1 meaning a continuity from existing research and +1 meaning a disruption (Wu et al., 2019). The formula considers the number of papers that cite exclusively the focal paper, exclusively the references of the focal paper, and both the focal paper and its references. There are many variants of disruption index in the literature, and the one used here is called DI_5 proposed by Bornmann et al. (Bornmann et al., 2020). By obtaining data from WOSCC, DI_5 of the most cited reference from the important peaks were computed with an automated routine described by Leydesdorff and Bornmann (Leydesdorff and Bornmann, 2021) and available from https://www.leydesdorff.net/software/di/. The computation of a disruption index could complement the findings from CRExplorer, allowing for further differentiation of the contextual nature of highly cited references.

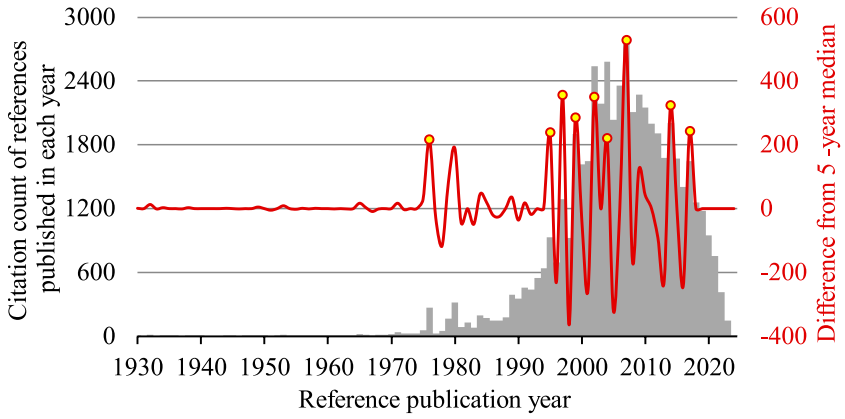

FIGURE 1
Reference publication year spectrogram of research on the use of infliximab to treat Crohn's disease. Peaks with a magnitude that exceeded the upper quartile are identified in 9 years, namely 1976, 1995, 1997, 1999, 2002, 2004, 2007, 2014, 2017 (yellow). A very small peak is visible in 1932, when the seminal paper by Crohn et al. (1932) was published. The bar chart shows the citation count of references published in each reference publication year (grey), whereas the wave form shows the deviation of citation count from the 5-year median (red).

TABLE 1 Details of the most cited reference identified for each significant peak from reference publication year spectrogram shown in Figure 1.

Reference publication year (RPY)	Cited references	No. of citations from the 1,793 articles (overall WOSCC citation count)	% of citations received by all references published in the same RPY	Disruption index (DI_5)
1976	Best et al. (1976)	178 (3,197)	66.2%	0.99
1995	Van Dullemen et al. (1995)	105 (964)	11.3%	0.10
1997	Targan et al. (1997)	426 (2,687)	33.0%	0.09
1999	Present et al. (1999)	412 (2091)	21.7%	0.10
2002	Hanauer et al. (2002)	574 (3,294)	22.6%	0.13
2004	Sands et al. (2004)	315 (1,616)	12.2%	0.01
2007	Colombel et al. (2007)	224 (1,686)	8.0%	−0.002
2014	Ruemmele et al. (2014)	50 (732)	2.5%	−0.03
2017	Gomollón et al. (2017)	72 (1,377)	4.4%	Not computed

The reference list of Gomollón et al. (2017) was placed in its supplementary data, and WOSCC, treated it as having 0 reference. Hence, its DI_5 was not computed.

3 Results

The 1,793 articles entered into the analysis had a total of 21,540 distinctive cited references published since 1900. Overall, there were 9 significant peaks identified between 1976 and 2017 (Figure 1). Table 1 shows a list of the most cited reference identified from each significant peak.

4 Discussion

This cited reference analysis has identified 9 important cited references from studies on the use of infliximab to treat Crohn's disease.

The first important cited reference identified was Best et al. (1976). It reported the development of CDAI, an 8-factor psychometric scale used to evaluate the severity of the disease and developed based on prospective data collected by the National Cooperative Crohn's Disease Study group on 112 patients. Best et al. (1976) also set threshold values of ≤150 for quiescent disease, and >450 for extremely severe disease. It only had three references, none of which was related to the disease activity of Crohn's disease. In this sense, Best et al. (1976) was very innovative to collect patient data and construct a clinical assessment tool to classify Crohn's disease patients according to disease severity. This was reflected by its high DI_5 value of 0.99, almost approaching to 1. The development of CDAI has inspired the subsequent development of a simpler index known

as the Harvey-Bradshaw index (HBI) (Harvey and Bradshaw, 1980), which does not require a patient to complete a diary card 1 week before assessment and does not impose any weighting factors.

The second important cited reference identified was van Dullemen et al. (1995). It was a case series of 10 patients with active Crohn's disease (CDAI value > 200) who were unresponsive to ≥20 mg prednisone for ≥2 weeks. At Academic Medical Center, Amsterdam, Netherlands, an open-label treatment with a single intravenous infusion of cA2 (infliximab) at 10 mg/kg, administered over 2 h, resulted in significant clinical improvement in 8 of the 10 patients that generally lasted for 4 months. This case series explicitly mentioned itself as a follow-up study of a case report, Derkx et al. (1993), that treated one severe Crohn's disease patient in the same hospital with the same treatment protocol. Even though Derkx et al. (1993) could be one of the first, if not exactly the first, to treat Crohn's disease patient with infliximab, their case report did not produce a noticeable peak in the RPYS or have a high DI_5 value (0.15), though its DI_5 value was slightly higher than that of van Dullemen et al. (1995) (0.10).

The third important cited reference identified was Targan et al. (1997). It was recognized as the first-ever randomized controlled trial of infliximab in treating patients with Crohn's disease (Sands, 2007; Hindryckx et al., 2014). A total of 108 patients with moderate-to-severe Crohn's disease (CDAI value at 220–400) who were unresponsive to prior treatment were recruited from 18 centres in North America and Europe. In this double-blinded trial, patients were randomized into receiving a single 2-h intravenous infusion of placebo, or infliximab in the dose of either 5, 10, or 20 mg/kg. Results found that infliximab of 5 mg/kg had the best clinical response rate and remission rate compared to the other two dose regimens, and all of them were significantly better than placebo. Overall, the infliximab treatment was very effective at 4 weeks after the infusion, but patients showed signs of relapse at week 12 in terms of CDAI value and C-reactive protein concentration. The most common adverse effects of a single-dose infliximab infusion reported in this trial included headache, nausea, and upper respiratory tract infection; but the prevalence of these effects were comparable to the placebo group.

The fourth important cited reference identified was Present et al. (1999). Similar to Targan et al. (1997), it was also a double-blinded, placebo-controlled trial. This time, 94 Crohn's disease patients with draining abdominal or perianal fistulas of >3 months were recruited from 12 centres in the United States and Europe. They were randomized into receiving 3 doses of either placebo, infliximab of 5 mg/kg, or infliximab of 10 mg/kg via intravenous infusion at weeks 0, 2 and 6. Results found that infliximab of both dose regimens had significantly better clinical response rate than placebo in terms of healing or complete absence of draining fistulas. Mean CDAI values of infliximab groups were also significantly lower than the placebo group at week 2, but not at week 18. In terms of adverse effects, the prevalence of headache seemed to be comparable across the placebo group and both infliximab groups, but the 10 mg/kg infliximab group tended to have a higher prevalence of having abscess, upper respiratory tract infection, and fatigue. Hence, Present et al. (1999) reaffirmed the initial dose of 5 mg/kg recommended by Targan et al. (1997), and further recommended subsequent identical doses at week 2 and week 6, thus forming the protocol of current intravenous induction regimen of using infliximab to treat Crohn's disease patients.

The fifth important cited reference identified was Hanauer et al. (2002). It was a double-blind, placebo-controlled trial registered as the ACCENT I trial. A total of 573 Crohn's disease patients (CDAI value ≥ 220) were recruited from 55 centres in North America, Europe, and Israel. The patients received an initial dose of 5 mg/kg intravenous infusion of infliximab at week 0, then randomized into receiving repeat infusions at weeks 2 and 6 of either placebo or infliximab in the dose of 5 mg/kg. For the placebo group, patients will then receive repeat infusions of placebo every 8 weeks until week 46. For the infliximab group, patients were further randomized into receiving repeat infusions of either 5 mg/kg or 10 mg/kg of infliximab every 8 weeks during the maintenance period, until week 46. Results found that patients who responded to the initial dose of infliximab were more likely to sustain clinical remission at weeks 30 and 54, discontinue the use of corticosteroid (prednisone, prednisolone, or budesonide), and maintain their response for a more prolonged period. The clinical remission and clinical response rates for the group with repeat infusions of 10 mg/kg of infliximab during the maintenance period were better than the group with 5 mg/kg, but the differences did not reach statistical significance. In fact, Hanauer et al. (2002) commented in its introduction that the maintenance regimen of infliximab infusion every 8 weeks was previously tested by their study group, published as Rutgeerts et al. (1999). In the study by Rutgeerts et al. (1999), survival analysis for time to loss of response showed that the median time to loss of response for the infliximab retreatment group and the placebo group were 48 weeks and 37 weeks, respectively, with a P-value of 0.057. Hanauer et al. (2002) commented that the results of Rutgeerts et al. (1999) were promising but underpowered, and therefore designed a larger study to confirm the efficacy. As such, they established the protocol of current maintenance regimen of repeat infusions every 8 weeks.

The sixth important cited reference identified was Sands et al. (2004). It was a double-blind, placebo-controlled trial registered as the ACCENT II trial. A total of 306 Crohn's disease patients with draining abdominal or perianal fistulas of >3 months were recruited from 45 centres in North America, Europe, and Israel. The patients received the induction regimen of 5 mg/kg intravenous infusion of infliximab at weeks 0, 2, and 6. A total of 282 patients entered randomization at week 14 to receive the maintenance regimen of 5 mg/kg infliximab or placebo every 8 weeks until week 54. Results found that the time to loss of response was significantly longer for patients in the infliximab group than those in the placebo group, and the infliximab group had a significantly higher ratio of patients with a complete absence of draining fistulas than the placebo group at week 54. This trial can be perceived as a follow up study of both Present et al. (1999) and Hanauer et al. (2002) to demonstrate that the recommended maintenance regimen can be equally applicable to patients with draining fistulas.

The seventh important cited reference identified was Colombel et al. (2007). It was a double-blind, placebo-controlled trial registered as the CHARM trial. As stated in its introduction, the CHARM trial was conducted because previous findings showed that repeat infusions of infliximab would lead to the development of antibodies, rendering loss of efficacy, infusion reactions, and delayed

hypersensitivity. Hence, Colombel et al. tried to test adalimumab, another biologics with a similar function. Results found that it was significantly better than placebo in maintaining remission in moderate-to-severe Crohn's disease through 56 weeks. They concluded that adalimumab could be a substitute of infliximab for patients who were intolerant of or failed to respond to the latter.

From van Dullemen et al. (1995) to Colombel et al. (2007), several authors have been involved in 3 or more studies, namely Paul Rutgeerts (n = 5), Stephen B. Hanauer (n = 4), and Sander J. van Deventer (n = 3), as well as many authors who had contributions to 2 studies, such as Jean–Frédéric Colombel, Daniel H. Present, and Bruce E. Sands, to name a few. It showed that many highly cited references identified in this study were authored by a core group of field experts in North America and Europe.

The eighth and ninth important cited references identified were Ruemmele et al. (2014) and Gomollon et al. (2017). These two papers were European consensus guidelines on the management of pediatric Crohn's disease and the diagnosis and management of Crohn's disease, respectively. Both papers were initiatives from the European Crohn's and Colitis Organisation (ECCO).

Meanwhile, it was worthwhile to mention that the first visually noticeable peak, being very small and not reaching the threshold set to identify important peaks, was found in 1932 by RPYS. The most cited reference published in that year was Crohn et al. (1932) that reported a case series of 14 patients with regional ileitis, now known as Crohn's disease (14 citations, or 93.3% of citations received by references published in 1932).

Undoubtedly, there were many clinical trials on the efficacy of biologics (either infliximab or others) in treating Crohn's disease patients. For a comprehensive (but not exhaustive) list of the trials, readers can refer to recent reviews and meta-analyses (Hindryckx et al., 2014; Singh et al., 2021; Barberio et al., 2023; Shehab et al., 2023). Meanwhile, the current study has demonstrated the ability of cited reference analysis to identify seminal papers from a pre-defined literature set, such as papers that reported the invention of CDAI, the very first randomized controlled trial of infliximab on Crohn's disease, and the renowned ACCENT I and II trials. This method of cited reference analysis offers a different perspective than traditional citation analysis. Traditionally, citation analysis focused on the total number of citations received by papers. However, some papers are only important in a small research field. Even if they are frequently cited by papers within the small research field, their overall citation count will still be very low, so that they may not be readily identified by a routine search. Moreover, the historical insights could help inform new research areas or gaps in Crohn's disease treatment. For example, depending on the pharmacological similarity, newer biologics may be tested with similar induction and maintenance regimens. In particular, since repeated infusions of infliximab would result in loss of efficacy, infusion reactions, and delayed hypersensitivity due to the development of antibodies, newer biologics should be tested in these aspects, and have a much more delayed or minimal level of antibody development.

This study had several limitations. First, the accuracy of cited reference and citation count depended on the literature database. It was reasonable to expect that literature databases, such as WOSCC used in this study, may fail to keep a record of the reference list of very old publications, or even fail to index older publications themselves (Yeung, 2023). However, the current study has revealed that even recent publications indexed in WOSCC may suffer from the same issue, as in the example of Gomollón et al. (2017) that placed its reference list in its supplementary data, rendering WOSCC recording it as having 0 reference. Moreover, well-established concepts and treatment regimens may become a common knowledge that the source articles are no longer cited, otherwise known as obliteration by incorporation (Merton, 1965; Yeung, 2021). On the other hand, the calculation of the disruption index makes use of bibliographic data only. Therefore, even though some of the cited references were definitely clinically novel, such as the first attempt to use infliximab to treat Crohn's disease patients or the first randomized controlled trial on the efficacy of infliximab to treat Crohn's disease patients, they were considered not disruptive from the perspective of bibliometric data. These issues might underestimate the scientific impact of the analyzed papers.

In conclusion, this cited reference analysis on the use of infliximab to treat Crohn's disease succeeded in identifying key references of the literature. The first important reference identified within the analyzed dataset was Best et al. (1976), which introduced the CDAI to assess the disease severity of patients. Key case series and randomized controlled trials, as well as consensus guidelines, were also identified. It is anticipated that randomized controlled trials that compare the efficacy between infliximab and other newer biologics in managing Crohn's disease in various stages will become highly cited in the future. Therefore, future bibliometric studies can explore and reveal the research foundation of newer biologics and targeted medicines used to treat IBD, such as interleukin inhibitors, integrin blockers, sphingosine-1-phosphate receptor modulators, and Janus kinase inhibitors.

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

AY: Writing–original draft, Writing–review and editing.

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Conflict of interest

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

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EDITED BY
Daniele Maria-Ferreira,
Instituto de Pesquisa Pelé Pequeno Príncipe, Brazil

REVIEWED BY
Kimberly Crosby,
University of Oklahoma, United States
Marcelo Adrian Estrin,
Interamerican Open University, Argentina
Qiuxia Min,
The First People's Hospital of Yunnan Province, China

*CORRESPONDENCE
Ze Wang,
zew74340@gmail.com

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Association between different GLP-1 receptor agonists and acute pancreatitis: case series and real-world pharmacovigilance analysis

Hui Guo[1], Qian Guo[2], Zhiqiang Li[3] and Ze Wang[1]*

[1]Department of Pharmacy, Shanxi Cardiovascular Disease Hospital, Taiyuan, Shanxi, China, [2]Department of Pharmacy, Second Hospital of Shanxi Medical University, Taiyuan, Shanxi, China, [3]Department of Pharmacy, Shanxi Traditional Chinese Medical Hospital, Taiyuan, Shanxi, China

Objective: Glucagon-like peptide-1 receptor agonists (GLP-1 RAs) have shown notable advancements in managing blood sugar control. Nevertheless, there remains a gap in real-world data regarding the variation in acute pancreatitis (AP) risk among different GLP-1 RAs. Our study aimed to characterize and evaluate AP associated with different GLP-1 RAs (exenatide, lixisenatide, liraglutide, albiglutide, semaglutide, dulaglutide and tirzepatide) in a public adverse events database and to review the relevant case reports.

Methods: We described a case series of patients experiencing AP while on GLP-1 RAs. Additionally, we utilized various algorithms including reporting odds ratio (ROR), proportional reporting ratio (PRR), Bayesian confidence propagation neural network (BCPNN), and multi-item gamma Poisson shrinker (MGPS) to analyze data from the Food and Drug Administration's Adverse Event Reporting System (FAERS) regarding suspected adverse events of AP linked to GLP-1 RAs from January 2005 to September 2023.

Results: Our case series comprised thirty-nine patients who experienced AP events while on GLP-1 RAs. Within the FAERS database, we retrieved a total of 6,751 individual case safety reports (ICSRs) involving various GLP-1 RAs. The median age of the patients included in our study was 57 years (range: 14–99), with 98.3% of cases classified as serious. Signals indicating AP were observed across all GLP-1 RAs, with particular emphasis on exenatide and liraglutide.

Conclusion: There is a notable reporting signal of AP associated with all GLP-1 RAs. Healthcare providers must remain vigilant and closely monitor this potentially life-threatening adverse event.

KEYWORDS

GLP-1 receptor agonists, acute pancreatitis, FAERS, pharmacovigilance, data mining

1 Introduction

Globally, over 95% of diabetes cases are attributed to type 2 diabetes mellitus (T2DM), with subsequent cardiovascular complications emerging as the primary drivers of morbidity and mortality. As a novel antidiabetic agent, glucagon-like peptide-1 receptor agonists (GLP-1 RAs) are seeing an increasing application in the management of patients with

T2DM, given their remarkable efficacy in regulating blood sugar levels without posing an elevated risk of hypoglycemic episodes or weight gain (Drucker and Nauck, 2006; Nauck, 2016). Moreover, promising outcomes from various large-scale cardiovascular outcome trials (CVOTs) have indicated that GLP-1RAs could mitigate the risk of major adverse cardiovascular events (MACE) in T2DM patients with an elevated cardiovascular risk profile (Marso et al., 2016a; Marso et al., 2016b; Hernandez et al., 2018; Pfeffer et al., 2015; Holman et al., 2017; Husain et al., 2019; Gerstein et al., 2019). Due to these favorable attributes, GLP-1RAs have garnered endorsement from authoritative guidelines (Marx et al., 2023; 2024) as a significant therapeutic option for individuals with T2DM, especially those with preexisting atherosclerotic cardiovascular diseases or at a heightened cardiovascular risk.

However, safety concerns have persisted for years regarding the pancreatic effects of GLP-1 RAs. Based on observational data, a 2011 report highlighted an increased risk of pancreatitis and pancreatic cancer in patients using incretin therapy (Elashoff et al., 2011), prompting a warning from the Food and Drug Administration (FDA) regarding the pancreatic safety of GLP-1 RAs (Administration, 2013). A review of case reports (Franks et al., 2012) further heightened concerns about the potential adverse effects of GLP-1RAs on the pancreas, resulting in elevated pancreatic enzymes and AP. A meta-analysis of large randomized controlled trials examining the association between incretin-based therapies and AP revealed an 82% (95% CI, 1.17–2.82) higher likelihood of developing AP when using these drugs compared to conventional therapy (Roshanov and Dennis, 2015). While several recently published meta-analyses of CVOTs have shown that no such association was observed between GLP-1RAs and pancreatitis (Singh et al., 2020; Cao et al., 2020). Nevertheless, significant shortcomings existed in such studies, including relatively short mean follow-up times (of less than 2 years in the RCTs), selected patient cohorts, and limited sample sizes.

In this study, we conducted a review of published literature and an analysis of the US Food and Drug Administration Adverse Event Reporting System (FAERS) data to investigate the incidence of AP undergoing GLP-1 RAs. Our aim was to provide a comprehensive clinical depiction of AP induced by GLP-1 RAs and ascertain the presence of a safety signal between AP and GLP-1 RAs in real-world settings.

2 Methods

2.1 Case series

We conducted a comprehensive literature search using Google Scholar, Scopus, PubMed, and Web of Science, focusing on English-language publications up to 31 December 2023. The following search terms were used: (exenatide OR liraglutide OR albiglutide OR dulaglutide OR lixisenatide OR semaglutide OR tirzepatide OR GLP-1 RAs OR Glucagon-like peptide-1 receptor agonist) AND (acute pancreatitis) AND (case report OR case series). The eligibility criteria included any case report or case series that documented instances of AP during the administration of GLP-1 RAs. Patient demographics such as age and gender, along with dosage, treatment duration, presenting symptoms, imaging results, causality assessment (using Naranjo scale) (Naranjo et al., 1981), acute pancreatitis management, and outcomes were extracted from the examination of medical files.

2.2 Phamacovigilance analysis

This retrospective pharmacovigilance analysis is based on real-world data sourced from individual case safety reports (ICSRs) submitted to the FAERS. FAERS compiles information on adverse events, medication errors, and product quality complaints leading to adverse events. It serves as a cornerstone of the FDA's post-marketing safety surveillance initiative for pharmaceuticals and therapeutic agents, operating as a classic spontaneous reporting system. The database captures a wide array of data including demographics, drug details, indications, outcomes, adverse reactions, sources, and therapies. Data submitting to ICSRs with GLP-1 RAs as suspected drugs were extracted from the FAERS database spanning the period between January 2005 and September 2023. Utilizing the Medical Dictionary for Regulatory Activities (MedDRA) version 26.0, we identified 25 preferred terms (PTs) (Supplementary Table S1) to gather pertinent cases linked to "acute pancreatitis" (Standardized MedDRA Queries (SMQ): 20000022) and closely related clinical conditions. To ensure data integrity, we conducted a thorough review to eliminate potential duplicates, defined as records sharing at least three out of four key fields: event date, age, sex, and reporter's country. Additionally, incorrect data were excluded, such as cases where the GLP-1 RA initiation date was later than the onset date of pancreatitis.

2.3 Statistical analysis

The clinical profile, such as age, sex, primary data source, outcomes, reported year, source region, and indication, were detailed individually for each GLP-1 RAs. Disproportionality analysis and Bayesian analysis were employed, utilizing the reporting odds ratio (ROR), proportional reporting ratio (PRR), Bayesian confidence propagation neural network (BCPNN), and multi-item gamma Poisson shrinker (MGPS) algorithms to identify associations between different GLP-1 RAs and AP events. The equations and criteria for these algorithms (Chen et al., 2020) are detailed in Supplementary Table S2. If any of the four algorithms met the predefined criteria, a positive signal of AP was identified. Analyses were performed using SPSS 23.0 (IBM, Armonk, NY, United States) statistical software.

3 Results

3.1 Case series

During the study period, thirty-nine patients experienced new-onset AP while using GLP-1 RAs (Table 1). More specifically, among these cases, 19 (48.7%) were associated with liraglutide, 9 (23.1%) with dulaglutide, 4 (10.3%) each with exenatide and semaglutide, while 1 (2.6%) case each was linked to lixisenatide, albiglutide, and

TABLE 1 Summary of case reports of GLP-1 receptor agonists-induced acute pancreatitis reported in the literature.

References	Country	Age (year) and gender	Indication	Medication	Dose	Duration	Complaints	Imaging findings	Naranjo result	Treatment	Outcome
Denker and Dimarco (2006)	United States	69 M	T2DM	Exenatide	5 mg bid	Within 24 h	Midepigastric abdominal pain radiating to the back	CT: no evidence of cholelithiasis	Probable	Discontinued exenatide, antoprazole and IV fluids	Recovered
Tripathy et al. (2008)	India	52 F	T2DM	Exenatide	5 mg bid	1 day	Abdominal pain, nausea, vomiting and fever	Ultrasound: significant abnormality	Probable	Discontinued exenatide, NPO, intensive antibiotic therapy, IV fluids	Recovered
Ayoub et al. (2010)	United States	64 F	T2DM	Exenatide	5 mg bid	2 days	Epigastric pain aggravated by food	CT: a enlarged pancreas, particularly at the head and body, with surrounding edema	Probable	Discontinued exenatide, NPO, IV fluids, pain medications and pantoprazole	Recovered
Iyer et al. (2012)	United States	76 F	T2DM	Exenatide and sitagliptin	5 mg qd	3 years	Severe abdominal pain, vomiting, and fever	CT: generalized peripancreatic stranding and dissecting fluid, then developed into extensive pancreatic parenchymal necrosis with a large amount of gas tracking throughout the pancreatic band	Possible	Discontinued exenatide and sitagliptin, supportive care	Died
Lee et al. (2011)	United States	60 F	T2DM	Exenatide 10 μg bid for approximately 4 years and then switched to liraglutide	Liraglutide 1.8 mg qd	23 days	Midepigastric pain radiating to the back	CT: pancreatic calcification	Probable	Discontinued liraglutide and IV fluids	Recovered
Bourezane et al. (2012)	France	63 M	T2DM	Liraglutide	0.6 mg and gradually increased to 1.8 mg qd for 1 month	330 days	Midepigastric pain radiating to the back, flank, chest and lower abdomen	CT: infiltration of peripancreatic fat and presence of fluid collections	Probable	Discontinued liraglutide, insulin, IV fluids and analgesics	Improved
Knezevich et al. (2012)	United States	53 M	T2DM	Liraglutide	Increased from 0.6 to 1.2 mg qd	2 months	Intolerable abdominal pain in the right upper quadrant and left upper quadrant	CT: peripancreatic inflammation	Probable	Discontinued all oral medications, IV fluids and analgesics	Recovered
Taunk et al. (2012)	United States	74 M	T2DM	Liraglutide	0.6 mg bid	1 month	Abdominal pain and vomiting	—	—	Discontinued liraglutide	Recovered

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TABLE 1 (*Continued*) Summary of case reports of GLP-1 receptor agonists-induced acute pancreatitis reported in the literature.

References	Country	Age (year) and gender	Indication	Medication	Dose	Duration	Complaints	Imaging findings	Naranjo result	Treatment	Outcome
Nakata et al. (2012)	Japan	75 F	T2DM	Liraglutide	0.6 mg qd	9 months	Nausea	CT: swelling of the pancreatic tail	Probable	Discontinued liraglutide	Recovered
Famularo et al. (2012)	Italy	67 M	T2DM	Liraglutide	1.2 mg qd	5 months	Nausea, vomiting, and constant pain in the epigastrium	MRI: a moderately enlarged and edematous pancreas	Probable	Discontinued liraglutide and IV fluids	Recovered
Jeyaraj et al. (2014)	India	51 F	T2DM	Liraglutide	0.6 mg for 1 week and increased to 1.2 mg for 7 weeks	8 weeks	Severe abdominal pain, nausea and vomiting	CT: mild enlargement of the pancreas with reduced parenchymal enhancement	Probable	Discontinued liraglutide, antibiotics, IV fluids and insulin	Recovered
Ghabra and Alkhouli (2018)	United States	27 F	T2DM	Liraglutide	—	2 weeks	Epigastric pain radiating into the back, diarrhea	—	—	Discontinued liraglutide, antiemetics, IV fluids and analgesics	Improved
Quesada-Vázquez (2018)	UAE	44 F	Obesity	Liraglutide	1.2 mg qd	6 months	Epigastric pain radiating to the back	—	Probable	Discontinued liraglutide	Recovered
Farooqui et al. (2019)	Qatar	64 F	T2DM	Liraglutide	—	4 weeks	Epigastric pain, nausea	MRI: no significant pathology or obstruction	Probable	Discontinued liraglutide	Recovered
Al-Salameh et al. (2019)	United States	53 F	T2DM	Liraglutide	1.2 mg and increased to 1.8 mg qd for 2 days	—	Epigastric abdominal pain, nausea, and non-bilious emesis	Ultrasound and CT: no evidence of biliary pathology	Probable	Discontinued liraglutide and supportive care	Recovered
Fatakhova et al. (2019)	United States	40 F	Obesity	Liraglutide	—	4 weeks	Sharp epigastric pain radiating to the back, nausea	CT: cholelithiasis without evidence of cholecystitis	Possible	-	Improved
Gameil and Elsebaie (2020)	Egypt	53 M	T2DM	Liraglutide	0.6 mg increased to 1.2 mg and later 1.8 mg qd	3 months	Mild abdominal discomfort and repeated vomiting	CT: a diffuse enlarged pancreas with heterogeneous enhancement of the parenchyma, irregular contour with peripancreatic edema, and fat strands	Probable	Discontinued liraglutide, soft enteral feeding, antibiotics and insulin	Recovered
Dolan et al. (2020)	United States	31 F	T2DM	Liraglutide	3 mg qd	10 months	Sharp midepigastric pain radiating to the back and left upper abdomen	CT: mild interstitial pancreatitis	Probable	Discontinued liraglutide, pain management, fluid resuscitation, and early enteral feeds	Improved

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TABLE 1 (*Continued*) Summary of case reports of GLP-1 receptor agonists-induced acute pancreatitis reported in the literature.

References	Country	Age (year) and gender	Indication	Medication	Dose	Duration	Complaints	Imaging findings	Naranjo result	Treatment	Outcome
Chua and Ng (2021)	Singapore	57 F	T2DM	Liraglutide	0.6 mg	5 days	Abdominal pain in the epigastric region, nausea and vomiting	CT: peripancreatic fluid and fat stranding around the tail of the pancreas	Probable	Discontinued liraglutide, analgesics, IV fluids and soft diet.	Recovered
Fernandez et al. (2021)	United States	48 M	T2DM	Liraglutide and empagliflozin	-	2 months	Acute abdominal pain, nausea and vomiting	CT: large peripancreatic fluid collection	Possible	Discontinued liraglutide, IV fluids and antibiotics	Improved
AlSaadoun et al. (2022)	SAU	25 F	Obesity	Liraglutide	2.4 mg	2 months	Sharp epigastric abdominal pain, nausea and non-bloody, nonbilious emesis	Ultrasound: negative for cholelithiasis, cholecystitis, or biliary ductal dilatation	Probable	Discontinued liraglutide, bowel rest, analgesics, IV fluids, antibiotics, and clexane	Improved
Easow et al. (2022)	India	69 M	T2DM	Liraglutide	1.2 mg	3 years	Abdominal pain in the epigastric region and vomiting	MRI: a stone of 8 mm in the ampulla of Vater producing dilation of the pancreatic duct	Possible	Discontinued liraglutide	Improved
Javed et al. (2023)	United States	73 M	T2DM	Liraglutide	—	20 months	Abdominal pain in the epigastric region, dry heaves and subjective fevers	CT: diffuse edematous inflammation of pancreatic head, body, and tail	Probable	Discontinued liraglutide and IV fluids	Recovered
Jain et al. (2016)	United States	59 M	T2DM	Albiglutide	30 mg qw	26 days	Epigastric pain, nausea	CT: no pancreatic findings	Probable	Discontinued albiglutide, IV fluids, pain medications and insulin	Improved
Bhat and Goudarzi (2021)	United States	69 M	T2DM	Dulaglutide	0.75 mg and increased to 1.5 mg qw for 3 days	3 months	Diffuse abdominal pain, nausea and vomiting	CT: an enlarged pancreas with peripancreatic stranding and slightly diminished enhancement	Probable	Meropenem for necrotizing pancreatitis	Recovered
Cheng et al. (2021)	United States	61 M	T2DM	Dulaglutide	1.5 mg qw	5 months	Acute epigastric pain	Ultrasound: no cholelithiasis, no acute cholecystitis	Probable	Discontinued dulaglutide, IV fluids and analgesics	Recovered
Abdelmasih et al. (2022)	United States	77 M	T2DM	Dulaglutide	1.5 mg and increased to 3 mg qw for 2 weeks	-	Epigastric pain, nausea, and vomiting	CT: confirmed pancreatitis	Probable	Discontinued dulaglutide	Recovered
Babajide et al. (2022)	United States	61 M	T2DM	Dulaglutide	0.75 mg qw	6 months	Upper abdominal pain, nausea and vomiting	CT: increased peripancreatic fat stranding, fluid	Probable	Discontinued dulaglutide and IV fluids	Recovered

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TABLE 1 (*Continued*) Summary of case reports of GLP-1 receptor agonists-induced acute pancreatitis reported in the literature.

References	Country	Age (year) and gender	Indication	Medication	Dose	Duration	Complaints	Imaging findings	Naranjo result	Treatment	Outcome
Yau et al. (2022)	United States	46 M	T2DM	Dulaglutide	—	—	Severe right upper quadrant abdominal pain, nausea	CT: focal hypoattenuation/edema of the pancreatic head with surrounding fat stranding	Probable	Discontinued dulaglutide and IV fluids	Recovered
Khan et al. (2023)	PAK	37 M	T2DM	Dulaglutide	0.75 mg and increased to 1.5 mg qw for 2 weeks	—	Abdominal pain, nausea and vomiting	CT: fat stranding around the pancreas	Probable	IV fluids and as-needed pain medication	Improved
Shahbazi et al. (2023)	United States	56 M	T2DM	Dulaglutide	0.75 mg and increased to 1.5 mg recently	4 weeks	Abdominal pain and nausea	CT: extensive interstitial edema around the pancreatic tail along with peripancreatic fat stranding	Probable	Discontinued dulaglutide, IV fluids, rectal bisacodyl, and linaclotide	Improved
Manuel et al. (2023)	United States	57 M	T2DM	Dulaglutide	1.5 mg and increased to 3 mg qw for 3 months	2 years	Abdominal pain	CT: a hazy inflammatory stranding around the pancreatic uncinate process	Probable	Discontinued dulaglutide, IV fluids and pain management	Improved
Kumar Kulkarni et al. (2023)	United States	68 F	T2DM	Dulaglutide	—	4 years	Severe epigastric pain, nausea and vomiting	CT: acute pancreatitis and no gallstones or bile duct dilatation	Probable	Discontinued dulaglutide, IV fluids, NPO diet and morphine	Improved
Chis and Fodor (2018)	Romania	67 M	T2DM	Lixisenatide	10 mg qd	3 months	Intense epigastric pain, nausea and vomiting	CT: the peripancreatic fatty tissue and pancreatic edema	Probable	Discontinued lixisenatide, IV fluids, proton pump inhibitor and antispasmodic drugs	Recovered
Nohomovich et al. (2023)	United States	60 + F	T2DM	Semaglutide	—	6 weeks	Abdominal pain	CT: enlargement of the pseudocyst to approximately 7 cm in size with ascites	Probable	Discontinued semaglutide, ampicillin-sulbactam and surgery to drain the pseudocyst	Improved
Patel et al. (2023)	United States	61 F	T2DM	Semaglutide	0.5 mg qw	-	Sudden onset abdominal pain	CT: no acute abnormality	Probable	Discontinued semaglutide	Recovered
Ebiai et al. (2023)	United States	60 F	T2DM	Semaglutide	0.5 mg qw for 24 months and increased to 1.0 mg qw for 3 weeks	24 months	Severe abdominal pain, nausea and vomiting	CT: pancreatic fat stranding	Probable	—	—

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TABLE 1 (*Continued*) Summary of case reports of GLP-1 receptor agonists-induced acute pancreatitis reported in the literature.

References	Country	Age (year) and gender	Indication	Medication	Dose	Duration	Complaints	Imaging findings	Naranjo result	Treatment	Outcome
Kumar Kulkarni et al. (2023)	United States	50 M	T2DM	Semaglutide	—	6 months	Acute severe epigastric pain, nausea and vomiting	CT: acute interstitial edematous pancreatitis without cholelithiasis or choledocholithiasis	Probable	Discontinued semaglutide, IV fluids, NPO diet and morphine	Improved
Casanovas et al. (2023)	United States	38 F	Pre-diabetes	Tirzepatide	Increased to 7.5 mg for 1 day before symptoms appeared	2 months	Epigastric pain diarrhea, nausea and vomiting	CT: edematous changes in the pancreatic head and uncinate process region	Probable	Discontinued tirzepatide	Improved

Abbreviations: T2DM, type 2 diabetes mellitus; CT, computed tomography; IV, intravenous; NPO, nil per os; MRI, magnetic resonance imaging.
UAE: United Arab Emirates; SAU: Saudi Arabia; PAK: Pakistan.

tirzepatide. The median age at the onset of AP was 60 years (range: 27–77 years), with 20 (51.8%) being male. All patients in the study were identified as having either type T2DM or obesity, with the exception of one patient who had been diagnosed with prediabetes. Notably, 10 cases (25.6%) involved an escalation in drug dosage within 3 months preceding the event. The median time to onset was 2.5 months (range 0 days–3 years). The predominant presenting symptom was epigastric abdominal pain accompanied by nausea and vomiting. Most cases exhibited evidence of pancreatitis on CT scans. Using the Naranjo scale, 33 cases (84.6%) were deemed to have a probable causal relationship between GLP-1 RAs and AP, while 4 cases (10.3%) were classified as possible. None of the patients from the case series was rechallenged with GLP-1 RAs due to safety concerns. Two cases involved cholelithiasis, and two patients received treatment with either empagliflozin or sitagliptin, which could potentially contribute to or confound pancreatitis. The standard management approach for these patients involved discontinuation of GLP-1 RAs and supportive care, including intravenous fluids and pain management. The majority of patients recovered without complications following this treatment regimen, except for one patient who experienced a fatal outcome.

3.2 Descriptive analysis from FAERS

In total, the FAERS database archived 6,751 reports related to acute pancreatitis induced by GLP-1 RAs from January 2005 to September 2023. Specifically, 2,539 ICSRs (37.6%) were associated with exenatide, 1981 (29.3%) with liraglutide, and 1,352 (20.0%) with dulaglutide. The demographic and clinical characteristics of all ICSRs are outlined in Table 2. The median age of patients across all ICSRs was 57 years (range: 14–99, n = 2,815), similar to that of each specific GLP-1 RA. Female patients accounted for the highest proportion of ICSRs (45.8%), and 6,634 (98.3%) cases were classified as serious. The majority of reports (63.4%) were submitted by healthcare professionals and originated from North America (87.8%). In terms of outcomes, other adverse events (51.5%) were the most prevalent, followed by hospitalization (40.4%), life-threatening (2.8%) and death (2.7%). AP events were predominantly reported for unknown indications (53.8%) and T2DM (43.0%). The events manifested soon after the initiation of GLP-1 RA treatment, with a median onset time of 92 days (range: 0–3,312, n = 1,591) across all ICSRs that provided both drug initiation and AP onset times. Notably, 30.9% of these reports were gathered within the initial month, and almost half (48.5%) were compiled within the first 3 months after eliminating invalid reports. The number of acute pancreatitis adverse events steadily increased from 16 in 2005 to 459 in 2023 (Q1-Q3), peaking in 2011, reflecting the growing clinical utilization of GLP-1 RAs (Figure 1).

3.3 Signal values associated with different GLP-1 RAs

We identified signals of AP events associated with all GLP-1 RAs using the criteria established by the four algorithms, and the results are summarized in Table 3. Each GLP-1 RA satisfied all four criteria,

TABLE 2 Clinical characteristics of patients with GLP-1 receptor agonists-associated acute pancreatitis collected from the FAERS database (January 2005 to September 2023).

Variables	Exenatide n = 2,539	Lixisenatide n = 45	Liraglutide n = 1,981	Albiglutide n = 43	Semaglutide n = 653	Dulaglutide n = 1,352	Tirzepatide n=216	Total n = 6,751
Age median (range)	57 (18–99) n = 955	58.5 (26–79) n = 24	56 (14–96) n = 1,058	59 (35–79) n = 15	59 (17–83) n = 335	58 (20–88) n = 561	52 (21–82) n = 79	57 (14–99) n = 2,815
Sex								
Male	1,192 (46.9)	13 (28.9)	739 (37.3)	20 (46.5)	305 (46.7)	549 (40.6)	49 (22.7)	2,837 (42.0)
Female	1,253 (49.4)	16 (35.6)	917 (46.3)	17 (39.5)	307 (47.0)	523 (38.7)	105 (48.6)	3,095 (45.8)
Not reported	94 (3.7)	16 (35.6)	325 (16.4)	6 (14.0)	41 (6.3)	280 (20.7)	62 (28.7)	819 (12.1)
Primary source								
Healthcare professional	1,295 (51.0)	36 (80.0)	1,690 (85.3)	40 (93.0)	529 (81.0)	716 (53.0)	23 (10.6)	4,281 (63.4)
Consumer	1,223 (48.2)	8 (17.8)	268 (13.5)	3 (7.0)	120 (18.4)	634 (46.9)	193 (89.4)	2,419 (35.8)
Not specified	21 (0.8)	1 (2.2)	23 (1.2)	—	4 (0.6)	2 (0.1)	—	51 (0.8)
Outcomes								
Non-serious	41 (1.6)	1 (2.2)	34 (1.7)	1 (2.3)	12 (1.8)	24 (1.8)	1 (0.5)	114 (1.7)
Hospitalization	998 (39.3)	21 (46.7)	864 (43.6)	20 (46.5)	240 (36.8)	539 (39.9)	69 (31.9)	2,730 (40.4)
Disability	41 (1.6)	1 (2.2)	11 (0.6)	2 (4.7)	2 (0.3)	5 (0.4)	—	62 (0.9)
Life-threatening	84 (3.3)	1 (2.2)	55 (2.8)	—	19 (2.9)	34 (2.5)	—	192 (2.8)
Death	101 (4.0)	—	44 (2.2)	3 (7.0)	17 (2.6)	25 (1.8)	2 (0.9)	179 (2.7)
Other	1,274 (50.2)	21 (46.7)	973 (49.1)	17 (39.5)	363 (55.6)	725 (53.6)	144 (66.7)	3,474 (51.5)
Source region								
Africa	6 (0.2)	—	1 (0.1)	—	—	2 (0.1)	—	9 (0.1)
Asia	41 (1.6)	7 (15.6)	34 (1.7)	—	7 (1.1)	22 (1.6)	5 (2.3)	106 (1.6)
Europe	229 (9.0)	11 (24.4)	191 (9.6)	—	60 (9.2)	90 (6.7)	—	582 (8.6)
North America	2,195 (86.5)	25 (55.6)	1713 (86.5)	41 (95.3)	576 (88.2)	1,231 (91.1)	211 (97.7)	5,927 (87.8)
Oceania	46 (1.8)	—	6 (0.3)	—	5 (0.8)	1 (0.1)	—	56 (0.8)
South America	15 (0.6)	2 (4.4)	33 (1.7)	—	5 (0.8)	5 (0.4)	—	59 (0.9)
Country not specified	7 (0.3)	—	3 (0.2)	2 (4.7)	—	1 (0.1)	—	12 (0.2)
Indication								
Diabetes mellitus	1,039 (40.9)	17 (37.8)	1,061 (53.6)	20 (46.5)	199 (30.5)	531 (39.3)	70 (32.4)	2,903 (43.0)
Other	16 (0.6)	1 (2.2)	129 (6.5)	-	38 (5.8)	10 (0.7)	13 (6.0)	218 (3.2)
Unknown	1,484 (58.4)	27 (60.0)	791 (39.9)	23 (53.5)	416 (63.7)	811 (60.0)	133 (61.6)	3,630 (53.8)
Time to onset median (range), days	212 (0–2,642) n = 676	12 (8–16) n = 2	81 (0–3,312) n = 522	48 (0–434) n = 6	45 (0–1829) n = 179	45 (0–1829) n = 179	31 (0–516) n = 43	92 (0–3,312) n = 1,591

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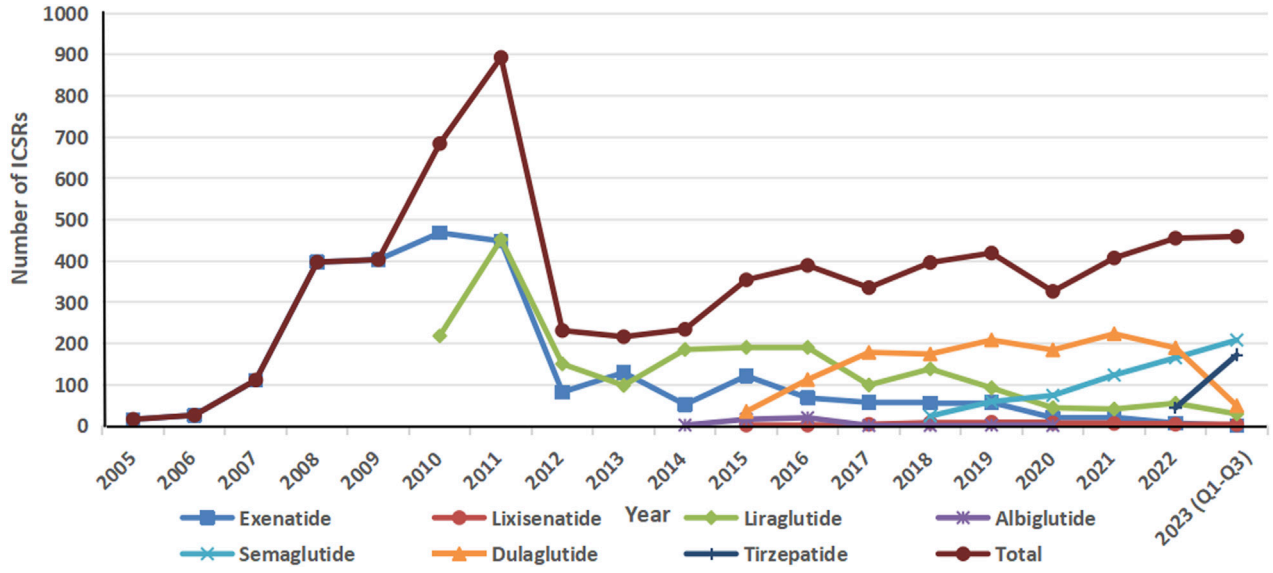

FIGURE 1
Distribution of Individual Safety Reports having GLP-1 RAs as suspect drugs by year (2005–2023 (Q1-Q3))

as did the overall group of GLP-1 RAs. Notably, among all GLP-1 RAs, liraglutide stood out for its association with AP events related to acute pancreatitis. This is highlighted by its notably highest values across various statistical parameters, including an IC at 4.17 (IC025 3.98), an ROR at 20.13 (95% CI 19.21–21.09), and an EBGM at 18.04 (EBGM05 17.35). Following liraglutide, exenatide, semaglutide, dulaglutide, lixisenatide, and albiglutide exhibited progressively lower values, while tirzepatide demonstrated the lowest association.

We further examined adverse events related to AP at the PT level and listed all signal-based ROR criteria in Table 4. As depicted in Table 4, exenatide exhibited the broadest spectrum, with a total of 8 potential signals indicating GLP-1 RA-induced AP, ranging from pancreatic abscess (ROR 4.20, 95% CI 1.03–17.03) to pancreatic phlegmon (ROR 84.53, 95% CI 21.86–326.90). Conversely, lixisenatide, albiglutide, and tirzepatide showed the fewest PTs, with only two signals detected for each drug. Among all ICSRs, cases involving pancreatitis and acute pancreatitis were the most frequently reported PTs for all drugs.

4 Discussion

In conclusion, we found significant over-representation of signals for acute pancreatitis (SMQ: 20000022) over other adverse reactions for all GLP-1 RAs. Though the disproportionality analysis and Bayesian analysis as a rapid and effective method for signal detection, our study represents the largest post-marketing surveillance to date of these GLP-1 RAs. We have provided valuable and timely evidence for clinical evaluation, aiming to mitigate the potential harm associated with acute pancreatitis following treatment with GLP-1 RAs.

Overall, from the first quarter of 2005 to the third quarter of 2023, there were 6,751 reports describing acute pancreatitis associated with GLP-1 RAs in the FAERS database. Both the pharmacovigilance findings and the case series indicated that liraglutide and dulaglutide were the leading suspected GLP-1 RAs, and pharmacovigilance analysis showed that exenatide had the highest number of ICSRs associated with AP. The median age of patients was 57 years (range: 14–99 years) in our pharmacovigilance analysis and 60 years (range: 27–77 years) for the cases of GLP-1 RAs-induced AP published in the case reports, which is in line with earlier observational studies on drug-induced AP (Gagnon et al., 2020; Chadalavada et al., 2020). Our pharmacovigilance results suggest that AP associated with GLP-1 RAs was more frequently reported in females, while the case series results did not show the same trend. However, the validity of this finding cannot be conclusively confirmed, given the multitude of factors that can influence the spontaneous reporting of adverse events. Additionally, the gender of 12.1% of the ICSRs was not reported, which further complicates the analysis. Nevertheless, there is some evidence suggesting that females may experience this condition more frequently (Barreto et al., 2011; Kaufman, 2013). We also observed that the median time to onset of GLP-1 RAs-associated acute pancreatitis was 92 (range: 0–3,312) days across ICSRs that provided both drug initiation and AP onset times, and 2.5 months of the case series, indicating a longer onset duration compared to other gastrointestinal adverse events triggered by GLP-1 RAs (Zhou et al., 2022).

In our study, excluding the initial 3 years since the launch of exenatide, the reported cases have averaged nearly 400 per year since 2015. However, there was a notable surge in cases during 2010 and 2011, with 684 cases reported in 2020 and 893 cases in 2021. This surge may be attributed to the FDA mandating manufacturers of incretin-based medications to revise their product labels in 2009, providing information regarding the potential risk of pancreatitis (Nelson et al., 2014). Approximately 87.8% of the reports were derived from North America, which may be attributed to FAERS being established in the United States. Furthermore, 40.4% of ICSRs involved hospitalized patients, 2.7% resulted in patient mortality, 0.9% led to disability, and 2.8% caused life-threatening reactions, while only 1.7% classified as non-serious outcomes. Additionally, within the case series results, one patient (2.6%) died, while 2.7% of

TABLE 3 Associations of GLP-1 receptor agonists with acute pancreatitis.

GLP-1 RAs	N	ROR (95% CI)	PRR (χ^2)	IC (IC025)	EBGM (EBGM05)
Exenatide	2,539	13.00 (12.48, 13.54)	12.48 (25294.99)	3.56 (3.42)	11.79 (11.39)
Lixisenatide	45	5.83 (4.34, 7.83)	5.73 (176.08)	2.52 (1.87)	5.72 (4.47)
Liraglutide	1,981	20.13 (19.21, 21.09)	18.88 (32075.86)	4.17 (3.98)	18.04 (17.35)
Albiglutide	43	4.73 (3.50, 6.39)	4.67 (124.18)	2.22 (1.64)	4.66 (3.62)
Semaglutide	653	8.23 (7.61, 8.90)	8.02 (3,966.43)	2.98 (2.76)	7.91 (7.41)
Dulaglutide	1,352	6.69 (6.34, 7.07)	6.56 (6,192.43)	2.67 (2.53)	6.38 (6.10)
Tirzepatide	216	2.94 (2.57, 3.36)	2.92 (272.29)	1.54 (1.35)	2.91 (2.60)
Total	6,751	11.62 (11.32, 11.93)	11.25 (53134.84)	3.26 (3.18)	9.61 (9.40)

Abbreviations: GLP-1, RAs; GLP-1, receptor agonists; N, the number of reports of GLP-1, RAs associated-acute pancreatitis; ROR, reporting odds ratio; CI, confidence interval; PRR, proportional reporting ratio; χ^2, chi-squared; IC, information component; EBGM, empirical Bayes geometric mean.

ICSRs from our FAERS analysis had a fatal outcome, underscoring the seriousness of acute pancreatitis and the necessity for specialized attention.

Our study detected a notable signal between different GLP-1 RAs and AP in the FAERS database throughout the study duration. Meanwhile, liraglutide exhibited the strongest association with acute pancreatitis, evidenced by the highest values of IC, ROR, and EBGM. Following liraglutide, exenatide emerged as the second-highest in terms of this association. Despite exenatide showing a higher reported number of reactions compared to liraglutide (2539:1981), the associations with acute pancreatitis events were weaker, which was also observed in cases of pancreatic cancer (Cao et al., 2023). It is suggested that patients at risk of pancreatitis avoid using any GLP-1 RAs, particularly liraglutide and exenatide. And the association of tirzepatide with acute pancreatitis events appears to be the weakest, possibly due to its later launch on the market.

AP ranks as the primary cause of hospital admissions for gastrointestinal disease (Mossad et al., 2017) and the fifth leading cause of in-hospital mortality in the United States (Sorribas et al., 2023). Addressing the underlying causes of pancreatitis is essential to prevent its recurrence. Gallstones and alcohol abuse stand out as the primary triggers for AP, while genetic factors, medications, and smoking also play contributing roles (Lee and Papachristou, 2019). Additionally, T2DM poses a significant risk for AP, particularly among younger diabetic patients (Lankisch et al., 2015). Moreover, worsening glycemic control escalates the likelihood of AP (Cho et al., 2023). Although drugs only account for 0.1%–2% of AP cases, their impact can be life-threatening (Wolfe et al., 2020). Therefore, managing drug-induced AP necessitates discontinuing the causative medication and providing supportive care. The GLP-1RAs should not be restarted if pancreatitis is confirmed (Wharton et al., 2022), and none of the patients from the case series were rechallenged with GLP-1 RAs for safety reasons. Failure to identify the responsible drug can lead to significant delays in treatment, potentially resulting in critical outcomes (Jones et al., 2015). Unraveling a causal relationship between GLP-1 agonists and AP is intricate, particularly as patients with T2DM are already three times more predisposed to pancreatitis compared to their non-diabetic counterparts (Girman et al., 2010). Therefore, it's imperative to examine all plausible factors and to rely on a diagnosis of exclusion when attributing AP to drug-induced causes.

Three out of thirty-nine patients (7.7%) from the case series were diagnosed with obesity. Obesity doesn't just pose a risk for local and systemic complications in acute pancreatitis; it also elevates mortality rates associated with this condition (Martínez et al., 2006). Currently, the FDA has approved three GLP-1 RAs for obesity treatment: liraglutide, semaglutide and tirzepatide. Notably, the dosage for obesity treatment is considerably higher than that for diabetes management. Take semaglutide as an example; the maintenance dose for the treatment of obesity is 2.4 mg subcutaneously once a week, whereas for diabetes, the maximum dose is 1 mg subcutaneously once a week. Whether this elevated dosage could potentially increase the risk of acute pancreatitis in obese patients compared to those with diabetes is a subject that necessitates further investigation.

In this study, we applied four algorithms to analyze the association between GLP-1 RAs and acute pancreatitis. Each method has distinct advantages and limitations. BCPNN and MGPS are Bayesian approaches known for their higher specificity (Bate et al., 1998; DuMouchel, 1999). They are particularly useful when working with sparse data or for pattern recognition in higher dimensions, making them applicable in a variety of scenarios. However, they are less sensitive compared to frequentist methods and can be less transparent to those unfamiliar with Bayesian statistics (Almenoff et al., 2006). On the other hand, PRR and ROR are frequentist approaches that are simpler to apply and interpret (Evans et al., 2001; van Puijenbroek et al., 2002). They have the advantage of higher sensitivity, making them useful for early detection of adverse drug events (Li et al., 2008). However, these methods are less specific and can sometimes produce false positives, particularly in rare drug-event combinations. The consistency of signals across all four methods strengthens our findings and minimizes the influence of biases inherent to any single algorithm. The convergence of these results enhances confidence in the association between GLP-1 RAs and acute pancreatitis, ensuring a comprehensive and reliable evaluation of the data.

Additionally, clinicians should view the statistical associations observed in this study as hypothesis-generating rather than conclusive evidence of a cause-and-effect relationship. The primary metrics used in this study, including the reporting odds ratio (ROR) and Bayesian confidence propagation neural network (BCPNN) indicators, are designed to identify disproportionalities in reporting patterns. These tools help detect potential safety signals but do not account for confounding variables such as baseline

TABLE 4 Signal strength for GLP-1 receptor agonists based on PT level in FAERS.

PT	Exenatide		Lixisenatide		Liraglutide		Albiglutide		Semaglutide		Dulaglutide		Tirzepatide	
	N	ROR (95% CI)	N	ROR (95% CI)	N	ROR (95% CI)	N	ROR (95% CI)	N	ROR (95% CI)	N	ROR (95% CI)	N	ROR (95% CI)
Oedematous pancreatitis	—		—		3	4.58 (1.47, 14.29)	—		4	8.16 (3.04, 21.92)	4	3.17 (1.18, 8.52)	—	
Pancreatic abscess	2	4.20 (1.03, 17.03)	—		—		—		—		—		—	
Pancreatic phlegmon	3	84.53 (21.86, 326.90)	—		—		—		—		—		—	
Pancreatic pseudocyst	19	10.80 (6.81, 17.14)	—		13	14.28 (8.21, 24.84)	—		—		—		—	
Pancreatitis	1963	14.03 (13.39, 14.70)	28	5.05 (3.48, 7.33)	1,525	21.48 (20.38, 22.64)	33	5.08 (3.60, 7.16)	545	9.62 (8.83, 10.48)	1,151	8.02 (7.56, 8.51)	190	3.63 (3.15, 4.19)
Pancreatitis acute	577	10.93 (10.05, 11.89)	17	8.40 (5.21, 13.54)	481	17.86 (16.29, 19.58)	10	4.19 (2.25, 7.80)	90	4.24 (3.45, 5.22)	184	3.39 (2.93, 3.93)	—	
Pancreatitis haemorrhagic	19	15.24 (9.55, 24.30)	—		—		—		—		—		—	
Pancreatitis necrotising	51	8.51 (6.43, 11.26)	—		28	9.01 (6.19, 13.10)	—		17	7.22 (4.47, 11.65)	16	2.64 (1.64, 4.32)	6	2.81 (1.26, 6.26)
Pancreatitis relapsing	23	13.47 (8.83, 20.54)	—		—		—		—		—		—	
Total	2,657		45		2050		43		656		1,355		196	

Abbreviations: PT, preferred term.

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patient characteristics, comorbidities, or concomitant medication use. Consequently, the presence of a signal should be interpreted as an indication of potential risk that needs to be further evaluated in the context of robust, well-controlled clinical studies.

Despite the advantages of real-world studies and data mining techniques in this research, there are numerous limitations to consider. Firstly, the spontaneous reporting system is affected by limitations within the FAERS database, including duplicate reports, reporting accuracy and quality, incomplete or insufficient details regarding drug administration (such as site, route, dose and timing), and the lack of important patient characteristics (such as medical history and comorbidities). Secondly, reports from FAERS lack medical confirmation, potentially introducing reporter bias (Nomura et al., 2015). As a result, data mining alone does not provide sufficient evidence to establish causality and primarily emphasizes the need for practitioner vigilance. It is important to note that all signal detection can only suggest a statistical correlation, and further investigation and research are needed to determine if there is a real causal relationship. Lastly, despite individually reviewing ICSRs in our study and considering data on other drugs that could potentially induce adverse reactions, the possibility of notoriety bias cannot be dismissed. Despite these inherent limitations in spontaneous reporting, the FAERS database remains a valuable resource. Data mining remains a critical tool for the ongoing assessment and management of risks associated with commercially available pharmaceutical products.

5 Conclusion

In conclusion, a notable reporting signal for acute pancreatitis exists across all GLP-1 RAs in the FAERS database, particularly associated with exenatide and liraglutide. Clinicians must be vigilant and monitor this potentially serious adverse event. Moreover, we anticipate further pharmacovigilance studies, cohort analyses, and clinical trials in the future to develop evidence-based treatment strategies for patients experiencing GLP-1 RA-induced AP.

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

HG: Data curation, Formal Analysis, Methodology, Software, Writing–original draft, Writing–review and editing. QG: Data curation, Formal Analysis, Software, Writing–review and editing. ZL: Formal Analysis, Software, Writing–review and editing. ZW: Supervision, Writing–review and editing.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2024.1461398/full#supplementary-material

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EDITED BY
Daniele Maria-Ferreira,
Instituto de Pesquisa Pelé Pequeno Príncipe, Brazil

REVIEWED BY
Layanne Cabral Da Cunha Araujo,
State University of Ceará, Brazil
Marta G. Novelle,
Complutense University of Madrid, Spain

*CORRESPONDENCE
Nikolina Canová,
ncano@lf.cuni.cz

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Effects of celastrol on the heart and liver galaninergic system expression in a mouse model of Western-type diet-induced obesity and metabolic dysfunction-associated steatotic liver disease and steatohepatitis

Nikolina Canová [1]*, Jana Šípková[1], Mahak Arora[1], Zuzana Pavlíková[2,3], Tomáš Kučera[2], Ondřej Šeda[4], Tijana Šopin[4], Tomáš Vacík[4] and Ondřej Slanař[1]

[1]Institute of Pharmacology, First Faculty of Medicine, Charles University and General University Hospital, Prague, Czechia, [2]Institute of Histology and Embryology, First Faculty of Medicine, Charles University, Prague, Czechia, [3]Department of Anthropology and Human Genetics, Faculty of Science, Charles University, Prague, Czechia, [4]Institute of Biology and Medical Genetics, First Faculty of Medicine, Charles University and General University Hospital, Prague, Czechia

Background: The complexity of the galaninergic system is still not fully understood, especially under specific pre-existing comorbidities related to metabolic dysfunction. A plant-derived triterpenoid celastrol was demonstrated to exert a complex effect on the galaninergic system and to have hepatoprotective and anti-obesity properties. However, the exact molecular mechanisms responsible for these effects remain unclear. Specifically, there are no data on the impact of celastrol on the heart and liver galaninergic system. Therefore, this study aimed to investigate the effects of celastrol on the galaninergic system expression in the heart and liver of mice suffering from diet-induced obesity and metabolic dysfunction-associated steatotic liver disease and steatohepatitis (MASLD/MASH).

Methods: The male mice C57BL/6J were fed a Western-type high-fat diet for 16 and 20 weeks to induce obesity and MASLD/MASH. Celastrol was administered along with a specific diet for the last 4 weeks to evaluate its impact on the progression of these conditions. Moreover, the inhibitor of sterol regulatory element-binding protein 1/2 (SREBP1/2), fatostatin, was also tested to compare its influence on the galaninergic system with celastrol.

Results: The study demonstrates that celastrol treatment was safe and led to a reduction in food and energy intake, body fat and liver weights, and MASLD-to-MASH progression and improved glucose tolerance, serum biochemistry markers, and hepatic lipid peroxidation in mice. Quantitative gene expression originally showed significant regulation of galanin and all three of its receptors (GalR1/2/3) in the heart ventricles and only GalR2 in the liver of obese mice. Celastrol influenced the gene expression of galanin receptors: it downregulated *Galr1* in the heart and upregulated *Galr2* in the liver and *Galr3* in the heart ventricles, potentially affecting energy metabolism, oxidative stress, and

inflammation. Fatostatin suppressed gene expression of all the detected members of the galaninergic system in the heart ventricles, depicting the role of SREBP in this process.

Conclusion: These findings suggest that celastrol may beneficially modulate the galaninergic system under obesity and MASLD-to-MASH progression, indicating its potential as a therapeutic agent for disorders associated with metabolic dysfunction.

KEYWORDS

celastrol, fatostatin, galanin receptor, heart, obesity, MASLD, MASH, mouse

1 Introduction

Neuropeptide galanin is an important member of the so-called galaninergic system. Although 4 decades have passed since its discovery (Tatemoto et al., 1983), there are still numerous biological processes where the role of galanin is not yet fully understood (Jiang and Zheng, 2022; Zhu et al., 2022). The described pleiotropic effects of galanin as a neurotransmitter include its involvement in the regulation of sleep and arousal processes, behavioral processes, anxiety, learning and memory, pain and nociception, and other processes. The galaninergic system has also been found to play an important role in many peripheral organ functions, specifically in the heart and cardiovascular system, pancreas, and gastrointestinal system, as well as in bone, connective tissue, and skin (Lang et al., 2015; Šípková et al., 2017a). The diverse effects of galanin are evident not only in typical physiological conditions but also in pathological contexts (Gopalakrishnan et al., 2021).

The pleiotropy and complexity of galanin-mediated signalization are based on the existence of three different G-protein-coupled receptors (GPCRs), namely, GalR1, GalR2, and GalR3, which transduce the biological signal through different pathways (Jiang and Zheng, 2022). In addition, new ligands with partial homology to the galanin molecule were discovered over the years: GALP (galanin-like peptide) and alarin. According to the current knowledge, only GALP is capable of activating galanin receptors, namely, GalR2/GalR3, while alarin is not, despite their partial homology. Specific receptors for alarin are not known (Fang et al., 2020; Abebe et al., 2022). The newest member of the galaninergic system is spexin, a small peptide with pleiotropic functions that can activate human GalR2 and GalR3 receptors (Behrooz et al., 2020).

There are multiple studies describing the important role of the galaninergic system in metabolism, food intake, and obesity. The hypothalamic activity of galanin through GalR1 stimulation leads to increased fat intake. Moreover, there is a capability of stimulating positive feedback, which can lead to excessive fat intake and obesity (Marcos and Coveñas, 2021). This dysregulation may be followed by glucose intolerance, leading to type 2 diabetes mellitus (T2DM) and metabolic syndrome (Fang et al., 2012). Similarly, fat intake and feeding behavior can also be modified by the activity of GALP (Takenoya et al., 2018). Finally, the role in regulating food intake, satiety status, and, subsequently, obesity risk was confirmed for spexin as well (Behrooz et al., 2020). Spexin was also shown to mitigate high-fat diet (HFD)-induced murine hepatic steatosis both *in vivo* and *in vitro* (Jasmine et al., 2016). The complex role of the galanin family peptides and their receptors is also modified by other regulatory pathways and external factors, like acute and chronic stress (Sciolino et al., 2015; Šípková et al., 2017b). The inter-species differences also should not be neglected as the results of experiments in various animal models may not provide consistent results (Kuramochi et al., 2006; Hirako et al., 2017), raising the question of extrapolation of these data to humans.

Multiple studies have confirmed the presence of galanin receptors in the hearts of different vertebrates, including laboratory mice, rats, and guinea pigs. All the types of galanin receptors were discovered in the heart tissue quite early (Wang et al., 1997a; Wang et al., 1997b), but the exact role of galaninergic signalization in the heart is still not fully understood. In guinea pigs, galanin signalization was involved in positive inotropic action and a prolonged effective refractory period (Kocic, 1998). Galaninergic signalization may also be involved in the pathophysiological response to myocardial injury; for example, the myocardial galanin content was increased after cardiac ischemia and reperfusion in rats (Ewert et al., 2008). This phenomenon could be theoretically used for the treatment of cardiac muscle ischemic injury in the future (Pisarenko et al., 2017).

Celastrol, 3-hydroxy-9β,13α-dimethyl-2-oxo-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid (Figure 1), is a pentacyclic triterpenoid (Cascão et al., 2017). It was isolated from the root of *Tripterygium wilfordii*, which is a plant widely used in traditional Chinese medicine with reported anti-inflammatory and anticancer effects (Lee et al., 2006). When tested as a potential therapeutic agent, it was found that celastrol is a potent leptin sensitizer and anti-obesity substance in mice (Liu et al., 2015; Ye et al., 2024). Wang et al. stated that celastrol promoted white adipose tissue browning and also protected against HFD-induced obesity by the activation of the hypothalamus–sympathetic axis (Wang et al., 2021). Kyriakou et al. found that celastrol-induced weight loss is mediated by the inhibition of leptin-negative regulator protein in the hypothalamus (Kyriakou et al., 2018). Another effect was mentioned by Abu Bakar et al., who found that celastrol interferes with mitochondrial metabolism and increases pyruvate dehydrogenase complex activity while down-regulating pyruvate dehydrogenase kinase 4 expression (Abu Bakar et al., 2022). Moreover, Fang et al. suggested that celastrol leads to weight loss by inhibiting the expression of galanin and GalR1 and GalR3 receptors in the hypothalamus of mice fed on HFD (Fang et al., 2019). In the aforementioned study (Fang et al., 2019), celastrol also led to a decrease in the plasma levels of GALP, indicating that the effect of celastrol on the galaninergic system is quite complex. Finally, celastrol has been shown to have hepatoprotective properties for liver diseases, including metabolic dysfunction-associated fatty liver disease and steatohepatitis (MASLD/MASH) (Li M. et al.,

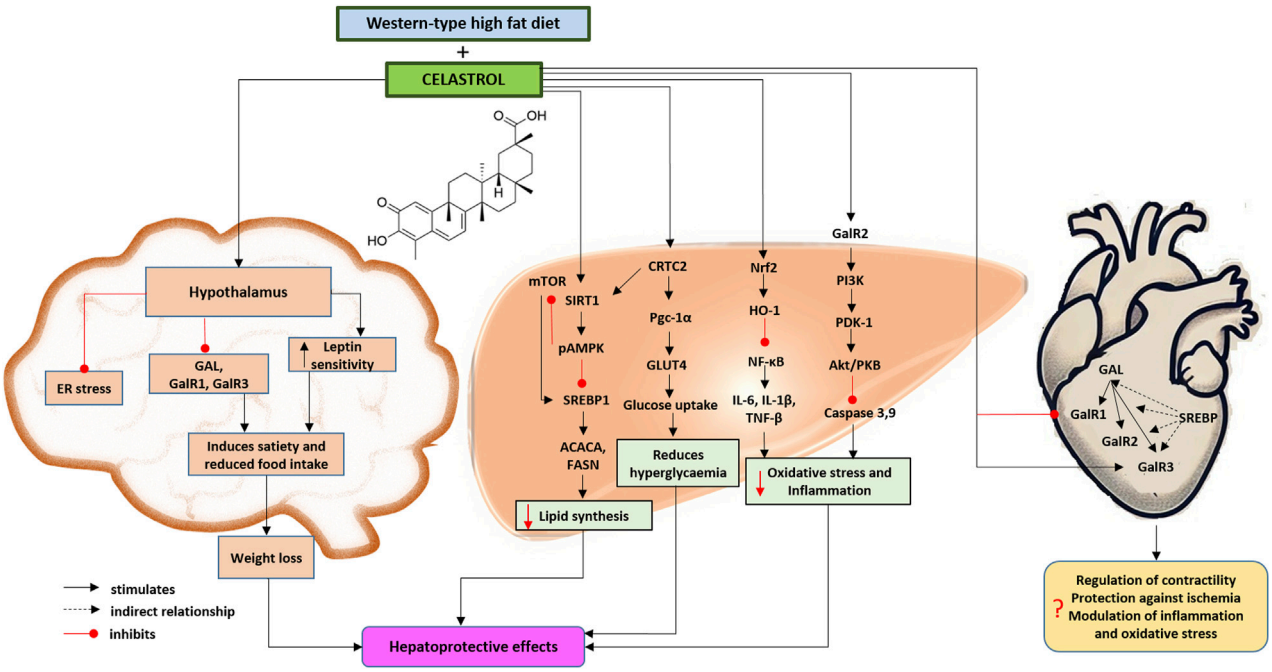

FIGURE 1
Proposed effects of celastrol on the brain, the heart and liver galaninergic system in a mouse model of Western-type diet-induced obesity and metabolic dysfunction-associated steatotic liver disease and steatohepatitis based on the presented results and herein discussed literature (Chen et al., 2015; Zhang et al., 2016; Fang et al., 2019; Palkeeva et al., 2019; Martinelli et al., 2021; Boal et al., 2022; Li M. et al., 2022; Serebryakova et al., 2023; She et al., 2023). Chemical structural formula of celastrol was drawn by using ChemDraw Professional Software (version 23.1.1.3, 64-bit). The heart was painted by OpenAI (ChatGPT 4o, 2024). Abbreviations: ACACA, acetyl-coenzyme A carboxylase alpha; CRTC2, CREB (cAMP response element-binding protein)-regulated transcription coactivator 2; ER, endoplasmic reticulum; FASN, fatty acid synthase; GAL, galanin; GalR1/2/3, galanin receptor 1/2/3; HO-1, heme oxygenase 1; GLUT4, glucose transporter-4; IL-1β, interleukin 1 beta; IL-6, interleukin 6; mTOR, mechanistic target of rapamycin kinase; NF-κB, nuclear factor kappa B; Nrf2, also Nfe2l2, nuclear factor, erythroid derived 2, like 2; pAMPK, phosphorylated adenosine monophosphate-activated protein kinase; PDK-1, phosphoinositide-dependent protein-kinase 1; PKB, protein kinase B; Pgc-1α, peroxisome proliferative activated receptor, gamma, coactivator 1 alpha; PI3K, phosphatidylinositol 3-phosphate; SIRT1, sirtuin 1; SREBP1, sterol regulatory element-binding protein 1; TNF-α, tumor necrosis factor alpha.

2022). The exact molecular mechanisms that are responsible for these effects remain unclear. Specifically, there are no data on the effect of celastrol on the heart and liver galaninergic system. Several authors have mentioned the possibility of using galanin or the GALP agonist/antagonist in the therapy of obesity and MASH (He et al., 2023). However, the complexity of the galaninergic system is still not fully understood, especially under specific pre-existing comorbidities. Further research is needed for the potential clinical use of galanin and celastrol in the pharmacological treatment of obesity and its related metabolic and cardiovascular diseases in humans.

Therefore, the main goal of this study was to evaluate the effect of celastrol treatment on the heart and liver galaninergic systems in the mouse model of Western-type diet-induced obesity and MASLD/MASH (Figure 1).

2 Materials and methods

2.1 Materials

Unless otherwise stated, all the high-quality standard chemicals were purchased from Sigma-Aldrich (Merck, Germany) or P-Lab (Czech Republic).

2.2 Declaration on experimental animals

All experimental animals were kept under conventional conditions with free access to water and granular food, regulated room temperature, and a 12-hour light regime in an accredited facility of the Institute of Pharmacology and the Center for Experimental Biomodels (CEB) of the First Faculty of Medicine, Charles University, Prague. The work with experimental animals was conducted following the Animal Protection Law of the Czech Republic (501/2020) and the Directive 2010/63/EU of the European Parliament and the Council. It was approved by the Expert Commission for Work with Experimental Animals of the First Faculty of Medicine, Charles University, and the Ministry of Education and Sports of the Czech Republic under the project No. MSMT - 11956/2021-4.

The male mice from inbred strain C57BL/6J (CEB, Prague, Czech Republic) aged at least 5–6 weeks were used for *in vivo* experiments. They were allowed to acclimatize for 1 week before feeding them with a defined diet. All the animals had unlimited access to a specific diet and drinking water. Due to the mutual aggression of the male mice and for the purpose of monitoring the food and fluid intake, the mice were housed individually in separate cages throughout the experiment.

TABLE 1 Induction of MASLD/MASH and division of mice into the experimental groups.

Set (Total duration)	Treatment groups (abbreviation, number of mice)	Induction period (diet)	Treatment (intraperitoneal dose)	Frequency of CEL dosing and treatment period
Set 1 (16 weeks)	Negative control (STD, n = 3)	12 weeks (STD + water)	+ DMSO (1 mL/kg)	Each second day for the next 4 weeks
	Positive control (WD, n = 7)[a]	12 weeks (WD/FG)	+ DMSO (1 mL/kg)	
	CEL treatment (WD + CEL, n = 8)		+ Celastrol (200 μg/ml/kg)	
Set 2 (20 weeks)	Negative control (STD, n = 3)	16 weeks (STD + water)	+ DMSO (1 mL/kg)	
	Positive control (WD, n = 8)	16 weeks (WD/FG)	+ DMSO (1 mL/kg)	
	CEL treatment (WD + CEL, n = 8)		+ Celastrol (200 μg/ml/kg)	

CEL, celastrol; STD, standard diet; WD/FG, Western-type diet with fructose and glucose in drinking water.
[a]One animal was excluded from all evaluations due to a cyst of the right kidney and severe cirrhosis with hyperbilirubinemia (Supplementary Figure S3).

2.3 Induction of obesity and metabolic dysfunction-associated fatty liver disease/steatohepatitis in mice and their treatments

A special atherogenic Western-type high-fat diet (WD) in the form of 10-mm pellets (4,575 kcal/kg) containing 1.25% cholesterol (E15723-34, Ssniff Spezialdiäten, Germany, through Anlab, Czech Republic) was used for the induction of obesity and MASLD/MASH, as described previously (Arora et al., 2023). In addition, the mice received fructose (23.1 g/L = 86.62 kcal/L) and sucrose (18.9 g/L = 74.47 kcal/L), FG, in drinking water. The negative control group received a pelleted standard diet (STD; 3,226 kcal/kg; Altromin 1324, from Velaz, Lysolaje, Czech Republic). The access to food was unrestricted in all the studied groups. After the induction period for 12 or 16 weeks, the *in vivo* experiments were performed in two independent sets, namely, set 1 and set 2, representing the early stage and late stage of MASH, respectively (Table 1). The mice on WD/FG of each set were randomly divided into two groups: the positive control group (WD) and the celastrol treatment group (WD+CEL). Mice were then kept on the established diet and concurrently treated intraperitoneally on each second day with either the vehicle (DMSO, 1 mL/kg) or CEL (Tripterin, 20 mg, # HY-13067, MedChemExpress, United States, through Scintila, Czech Republic) at the dose of 200 μg/ml/kg for 4 additional weeks (Table 1).

This study was preceded by a pilot study aiming to identify the MASLD/MASH development and the gene expression of the galanin family system members (galanin, galanin-like peptide, galanin receptor 1, galanin receptor 2, and galanin receptor 3) in mouse heart ventricles depending on the duration of the WD/FG feeding for 12–21 weeks. In this pilot study, we found that, under the given conditions, MASLD in mice begins to transit into MASH at week 12 on a Western diet and that by the end of week 16, MASH is already fully developed (data not shown). Therefore, we determined that the 4-week administration of CEL from week 12 could prevent the progression of MASLD to MASH (i.e., the transition of MASLD to MASH representing the early stage of MASH) and treatment from week 16 rather than the treatment of already-advanced diet-induced MASH (i.e., the late stage of MASH).

Although there is extensive research on the functions of sterol regulatory element-binding proteins (SREBPs) and their impact on lipid metabolism, the direct effect of SREBPs on galanin and galanin receptors is not well-documented in the available literature. Therefore, we evaluated fatostatin (FAT) for comparative analysis to reveal the role of SREBPs in the galaninergic system. Unlike CEL, FAT primarily affects fat metabolism and reduces adipose tissue and hepatic fat accumulation by inhibiting the activation of SREBP1/2 without any direct impact on appetite or food intake (Kamisuki et al., 2009; Zhao et al., 2022). For these purposes, we treated additional mice (n = 8) on WD/FG diet with FAT (an intraperitoneal dose of 10 mg/kg, dissolved in DMSO) each second day during weeks 12–16 (corresponding to set 1 of *in vivo* experiments).

2.4 Oral glucose tolerance test

An oral glucose tolerance test (OGTT) was performed 36 h before mouse euthanasia to measure the glucose concentration in the blood of experimental mice, as we described previously (Arora et al., 2023) with minor modifications. OGTTs were conducted on mice that had been fasted for 8 h (Andrikopoulos et al., 2008). In relation to oral glucose application (2 g/kg of body weight), blood samples were collected from the tail vein at 0, 30, 60, and 120 min to measure blood glucose levels using the Accu-Chek® Instant glucometer (Czech Dia, Roche). The area under the curve (AUC) of blood glucose was then calculated using the trapezoidal method (AUC = 1/4 * fasting glycemia + 1/2 * 30-min glycemia + 3/4 * 60-min glycemia + 1/2 * 120-min glycemia) (Li L. et al., 2021).

2.5 Blood, heart, liver, and fat sampling

The animals were fasted 12 h before terminal sampling. Initially, mice were anesthetized by intraperitoneal application of a ketamine (100 mg/kg) and xylazine (5 mg/kg) mixture (Bioveta, Czech Republic). Blood was terminally withdrawn by retro-orbital puncture using heparinized glass capillaries. Blood samples were left for 30 min at room temperature to clot and then centrifuged at 4,000 rpm for 10 min at 4°C. Serum aliquots were stored at −20°C until biochemical measurements. The mice were further dissected to

extract the hearts, livers, and intra-abdominal and epididymal fat, which were washed in cold phosphate-buffered saline (PBS), dried on sterile gauze, and weighed. If necessary, the heart ventricles and individual liver lobes were separated to be utilized for respective analyses, as described below.

2.6 Biochemical analysis

Determination of serum concentrations of lipids (total cholesterol and triglycerides), glucose, albumin, liver enzymes (ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase), and nitrogen metabolites (urea and creatinine) was performed using the customized commercial kits in the routine Central laboratory of the Institute of Medical Biochemistry and Laboratory Diagnostics of the General University Hospital in Prague. The triglyceride content was estimated in liver caudate lobes exactly by following the instructions in the Triglyceride Quantification Colorimetric/Fluorometric Kit manual (# MAK266-1KT, Sigma-Aldrich, through Merck, Germany) and expressed as micrograms per μg of lysate protein, as measured using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific™).

2.7 Determination of liver oxidative stress parameters

First, a liver left lateral lobe was homogenized (10% w/v) in the cooled lysis buffer (0.2 M Tris-HCl, pH 7.4, 0.002 M EDTA-Na_2, and 0.025 M sucrose) and centrifuged at 4,000 rpm for 15 min at 4°C. Then, the supernatant was used for the analysis of oxidative stress markers. To determine the severity of liver oxidative stress caused by the metabolic disease, total lipid peroxidation was estimated by evaluating conjugated dienes, thiobarbituric acid reactive substances (TBARS), and nitrites, as previously described (Farghali et al., 2009; Arora et al., 2023). The total amount of protein in the liver homogenates was determined using the Bio-Rad protein assay (Bio-Rad, Prague, Czech Republic).

2.8 Quantitative RT-PCR for gene expression

Two different qRT-PCR methods were used depending on the tissue collected: heart ventricles or liver lobes. RNA from heart ventricles (right and left together) was isolated using the QIAzol Lysis Reagent (QIAGEN, CA, United States). Extracted RNA was purified using the RNeasy Plus Mini Kit, as per the manufacturer's protocol. Quantitative and qualitative analyses of RNA for quality determination were performed using the Agilent 2100 Bioanalyzer system (Agilent, CA, United States). The RNA integrity number (RIN) was used as an integrity parameter. Only samples showing RIN above 7.5 were used for further analysis (Schroeder et al., 2006). Total RNA (1 μg) was reverse-transcribed with oligo-dT primers using SuperScript IV (Invitrogen, Carlsbad, CA, United States). For validation, the following sets of TaqMan probes (Thermo Fisher Scientific; Waltham, MA, United States) were used: galanin (*Gal*, TaqMan Assay Mm00439056_m1), galanin-like peptide (*Galp*, TaqMan Assay Mm00626135_m1), galanin receptor 1 (*Galr1*, Mm00433515_m1), galanin receptor 2 (*Galr2*, Mm00726392_s1), and galanin receptor 3 (*Galr3*, Mm00443617_m1). The qRT-PCR reaction was performed in triplicate with TaqMan Gene Expression Master Mix (Applied Biosystems), according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, United States) using the Applied Biosystems 7900HT Real-Time PCR System. Cycle threshold (Ct) values were normalized using glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) (TaqMan chemistry, Applied Biosystems) as a standard.

Isolated liver left median lobes were stored in the RNAlater™ stabilization solution and maintained at −20°C for further qRT-PCR analysis. TRI reagent (Sigma-Aldrich, Prague, Czech Republic) was used to homogenize livers that were then treated consequently with chloroform, ice-cold isopropanol, and ice-cold 75% ethanol and centrifuged to obtain RNA pellets (Arora et al., 2023). Concentrations of extracted RNA were measured using a NanoReady Micro UV-Vis Spectrophotometer (LifeReal) and were reverse transcribed using a LunaScript RT SuperMix (New England Biolabs), following the manufacturer's protocol. Furthermore, cDNA was subjected to quantitative PCR using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad), following the Luna Universal qPCR Master Mix (New England Biolabs) protocol, along with the primers listed in Table 2. The data were analyzed using CFX Maestro™ software (Bio-Rad Laboratories). Relative quantification was performed using Livak's–Schmittgen's ΔΔCt method (Livak and Schmittgen, 2001).

2.9 Western blot

As described previously (Arora et al., 2023), the liver samples were homogenized and lysed in RIPA lysis buffer with added protease and phosphatase inhibitors. Equal amounts of lysate protein, specifically 30 μg, as determined by the Pierce™ BCA Protein Assay Kit (Thermo Scientific™), and PageRuler Prestained Protein Ladder (# 26616, Thermo Fisher Scientific, Czech Republic) were subjected to Mini-PROTEAN®TGX Stain-free™ 4%–20% precast gels (# 456-1093, Bio-Rad, Czech Republic) and then transferred electrophoretically onto a methanol-activated Wet Immobilon E (0.45 μm) nitrocellulose membrane. The membranes were blocked by incubating with Tris-buffered saline containing 5% bovine serum albumin (BSA) and 0.1% sodium azide for 30 min at room temperature. Subsequently, the membranes were incubated with primary antibodies overnight at 4°C. The primary antibodies comprised the rabbit anti-SREBP1 polyclonal antibody (1:1,000 dilution, # PA1-337, Invitrogen, through Thermo Fisher Scientific, Czech Republic), rabbit anti-GalR2 polyclonal antibody (1: 400 dilution, # bs-11527R, Bioss Antibodies, through iBioTech, Czech Republic), rabbit anti-HRPT1 monoclonal antibody (1: 1,000 dilution, # A8783, ABclonal Technology), and rabbit anti-beta2-microglobulin monoclonal antibody (1: 1,000 dilution, # 59035, Cell Signaling Technology™). On the next day, the membranes were washed in TBST buffer and incubated with goat anti-rabbit polyclonal IgG and horseradish peroxidase-conjugated antibody (1: 5,000-1: 15,000 dilutions, # ADI-SAB-300-J, Enzo® Life Sciences, through iBioTech, Czech Republic) in 5% non-fat milk

TABLE 2 List of the primers used for the qRT-PCR analysis of liver expression of selected genes.

Target gene	Forward primer (5′-3′)	Reverse primer (3′-5′)
Nrf2	CGCCAGC-TACTCCCAGGTT	GGATATCCAGGGCAAGCG
Ppargc1a	GCTGGTTGCCTGCATGAGT	CCAACCAGAGCAGCACACTCT
Srebf1	ATTGAGAAGCGCTACCGGTCT	TGTGCACTTCGTAGGGTCAGG
Mtor	GTTTGTGGCTCTGAATGACCAG	GCTCCTTGATTCTCCCAATGC
Crtc2	CAACAATGTCACCCACCTTGTC	GGGCAATCGCTGGTCAGTAG
Sirt1	TCTATGCTCGCCTTGCGG	GACACAGAGACGGCTGGAACT
Hmox1	AGCCGTCTCGAGCATAGCC	ATCCTGGGGCATGCTGTC
Acaca	GCTTACAGGATGGTTTGGCCT	CAAATTCTGCTGGAGAAGCCAC
Sod2	CGCTTACAGATTGCTGCCTG	GGTAGTAAGCGTGCTCCCACA
Fasn	TCCTGGAACGAGAACACGATCT	GAGACGTGTCACTCCTGGACTTG
Tnfa	GCCTCTTCTCATTCCTGCTTGT	CTGATGAGAGGGAGGCCATTT
Il1b	TGCCACCTTTTGACAGTGATG	TGATGTGCTGCTGCGAGATT
Gal	GAGCCTTGATCCTGCACTGAC	GGGTCCAACCTCTCTTCTCCTT
Galr1	CTTACGTGGTGTGCACTTTCG	GGCAGCCAGGATATGCCA
Galr2	GACTGTAGTAGCTCAGGTAG	CGTTCATTTCCTCATCTTCC
Glar3	CACCATGTATGCCAGCAGCTT	ACCGTGCCGTAGTAGCTTAGGT
Hprt1	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG

Abbreviations: *Ppargc1a*, also Pgc-1α, peroxisome proliferative activated receptor, gamma, coactivator 1 alpha; *Crtc2*, CREB, cAMP response element-binding protein, regulated transcription coactivator 2; *Fasn*, fatty acid synthase; *Acaca*, acetyl-coenzyme A carboxylase alpha; *Srebf1*, sterol regulatory element-binding transcription factor 1; *Nrf2*, also Nfe2l2, nuclear factor, erythroid derived 2, like 2; *Mtor*, mechanistic target of rapamycin kinase; *Hmox1*, heme oxygenase 1; *Sirt1*, sirtuin 1; *Sod2*, superoxide dismutase 2, mitochondrial; *Tnfa*, tumor necrosis factor alpha; *Il1b*, interleukin 1 beta; *Gal*, galanin; *Galr1/2/3*, galanin receptor 1/2/3; *Hprt1*, hypoxanthine phosphoribosyltransferase 1.

blocking solution at room temperature for 1 h. The protein bands on the membranes were visualized via SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific™ through GeneTiCA s.r.o., Prague, Czech Republic) by using the ChemiDoc™ MP imaging system (Bio-Rad, Czech Republic). The band intensities of SREBP1 and GalR2 were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, United States) and normalized to the respective house-keeping protein (HRPT1 and β2-microglobulin) and, subsequently, to the corresponding negative control group.

2.10 Histology

The heart and liver tissues were fixed in 4% paraformaldehyde and then embedded in paraffin, cut into 7–8-μm-thick sections, and stained with hematoxylin–eosin (HE) or Sirius red. Liver samples of the right median lobe were scored for MASLD and fibrosis. MASLD was scored according to the grading system specifically established by Liang et al. for rodent MASLD models using samples stained with HE (Liang et al., 2014). In brief, steatosis was determined by analyzing hepatocellular vesicular steatosis, namely, macrovesicular steatosis, microvesicular steatosis, and hepatocellular hypertrophy (each scored 0–3). Macrovesicular and microvesicular steatosis were evaluated separately, according to its severity, based on the percentage of total area affected. Hepatocellular hypertrophy, which is defined as the enlargement of cells to more than 1.5 times the normal diameter of hepatocyte, was also assessed based on the percentage of total area affected as well. Inflammation was assessed by counting the number of inflammatory foci present per field, with a focus being a cluster of five inflammatory cells. Five different fields were counted, and their average was then rated into the four categories (score 0, 1, 2, and 3) (Liang et al., 2014). Two key features of MASH, steatosis (score 0–9) and inflammation (score 0–3), were used to calculate the total MASLD score (ranging from 0 to 12 score). If the total steatosis score was 0, MASLD was not diagnosed, regardless of inflammation. MASLD was diagnosed if steatosis was present. Finally, MASH was diagnosed if both steatosis and any inflammation were observed (Liang et al., 2014). Liver fibrosis was identified using 8-μm slides stained with Sirius red (SR) dye and scored according to Kleiner et al. (2005). This liver fibrosis classification system recognizes five stages, namely, stage 0 (no fibrosis), stages 1A/numerically 1, 1B/1.33, and 1C/1.67 (representing mild and moderate perisinusoidal fibrosis and portal/periportal fibrosis, respectively); stage 2 (both perisinusoidal and portal/periportal fibrosis); stage 3 (bridging fibrosis); and stage 4 (cirrhosis). Liver tissue sections were analyzed using a Leica DMLB microscope equipped with a Leica MC170 HD camera. One representative HE-stained section was scored for steatosis and inflammation (Liang et al., 2014), and one representative SR-stained section was scored for fibrosis in each specimen (Kleiner et al., 2005). To exclude differences in individual subjective scoring,

all liver samples were scored by the same trained "blinded" histologist throughout the study (Arora et al., 2023).

Some samples of the heart were immediately fixed in 4% paraformaldehyde, cryoprotected with sucrose, embedded into the optimal cutting temperature compound, frozen at −20°C, and stored until further use. For the indirect immunofluorescence method, 7-μm-thick cryosections were used. After thawing and washing in PBS, non-specific antibody-binding sites were blocked with 5% goat serum in PBS. In a pilot study, we used three different primary antibodies: polyclonal rabbit anti-GalR1 (#AGR-011), anti-GalR2 (#AGR-012), and anti-GalR3 (extracellular) (#AGR-013) (all from Alomone Labs, Israel), to screen for positivity in mouse hearts. Interestingly, immunoreactivity was detected only for GalR1. Therefore, other sections were incubated only with polyclonal rabbit anti-GalR1 antibody (Cat. No. LS-C831302, LS Bio, through EXBIO Prague, Czech Republic) diluted 1: 1,000 in PBS + 1.5% normal goat serum overnight at 4°C. For visualization, a secondary goat anti-rabbit IgG biotin antibody (Agilent) diluted 1: 400 in the PBS + 5% normal goat serum was applied to sections for 30 min at room temperature. Visualization was carried out using the avidin-biotinylated peroxidase complex (VECTASTAIN ABC Elite Kit, Vector Laboratories) and finally DAB (Agilent) as a substrate.

2.11 Determination of TNF-α

TNF-α was estimated using customized ELISA kits, as described in the Supplementary Material.

2.12 *In vitro* MASLD model

The palmitic acid (PA)-induced primary hepatocyte lipotoxicity model, as we introduced previously (Arora et al., 2023), is described in detail in the Supplementary Material.

2.13 Statistical analysis

Normal distribution of the data was checked using the Shapiro–Wilk test. To compare the differences between groups, one-way or two-way ANOVA with the *post hoc* Bonferroni test, whenever appropriate, was used. To compare histopathological scores between the STD, WD, and WD+CEL groups, the Kruskal–Wallis test with the *post hoc* Dunn test was used. Student's *t*-test with adjusted p-values was used for pair-wise comparisons. The results of the variables' data are expressed as the mean with a respective standard deviation (SD). Unless otherwise indicated in the legend of a specific figure, the numbers (n) of all values scored correspond to the number of mice in each group, exactly as presented in Table 1. The differences were considered statistically significant if $p < 0.05$. In the graphs and the result section of *in vivo* experiments, there are significant differences only between negative (STD) and positive (WD) controls and between positive controls (WD) and CEL treatments (CEL+WD) presented. Statistical analyses and data visualization were performed using GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California, United States).

3 Results

3.1 The effect of celastrol treatment on mouse's body, fat and liver weight, and food intake

We successfully adopted WD/FG-induced obesity and MASLD/MASH in C57BL6J male mice (Arora et al., 2023), as evidenced by the progressive increase in body weights (Supplementary Figure S1), liver weight, and fat-to-body weight ratio in the positive control groups of both sets of experiments (Figure 2). CEL treatment (i.e., CEL + WD group) significantly decreased mouse body weights after 1 week of treatment when compared to the body weights of the positive control (i.e., WD group) in both sets of experiments. This weight loss persisted until the end of the experiment (Figures 2A, B). CEL significantly reduced food consumption and energy intake compared to positive controls, which was more pronounced during the first 2 weeks of the treatment (Supplementary Figures S2; Figures 2C, D, respectively). CEL significantly decreased the liver weight in both sets of experiments (Figure 2E). The absolute amount of white intra-abdominal plus epididymal fat tissue and fat-to-body weight ratios was also significantly decreased by CEL throughout the study (Figure 2F). As the absolute heart weights were not modified throughout the groups and sets, the heart-to-body weight ratio was significantly decreased in both positive controls and completely restored by CEL during set 1 (Figure 2G).

3.2 The effect of celastrol treatment on mouse glycemia and serum liver and kidney biochemistry markers

At the end of experimental set 1 (i.e., week 16), mice fed WD/FG showed an elevated overall OGTT curve, as evidenced by the significantly increased glycemic AUC that was substantially reduced by CEL (Figures 3A, C). The same significance was also detected for 12-hour fasting glucose levels (Figure 3D). At the end of experimental set 2 (i.e., week 20), only 12-hour fasting glycemia was remarkably elevated in positive controls (Figures 3B, D).

CEL treatment significantly reduced or displayed a tendency to drop (not statistically significant, n.s.) all liver enzyme activities at week 16 or week 20 (Figures 4A–C). Serum albumin comprises an essential endogenous protein synthesized by the liver. Moreover, the decrease in the serum albumin concentration is suggested to be an essential clinical predictor for MASLD-associated hepatic damage (Kawaguchi, K. et al., 2021). In our study, WD/FG-induction displayed a progressive significant decrease in serum albumin levels, which were highly significantly further decreased by the CEL treatment (Figure 4D). Additional decline could be caused by the very high affinity of CEL to serum albumin, which can interfere with the colorimetric assay method (especially with using bromocresol green, as in our case) to estimate the albumin concentration (Zhang et al., 2009; Fan et al., 2022). A significant reduction of serum urea was also seen in all positive controls against negative controls, probably due to decreased liver synthesis (Arora et al., 2023). Serum creatinine levels decreased with time (e.g., animal age) but were not affected by WD/FG. The CEL

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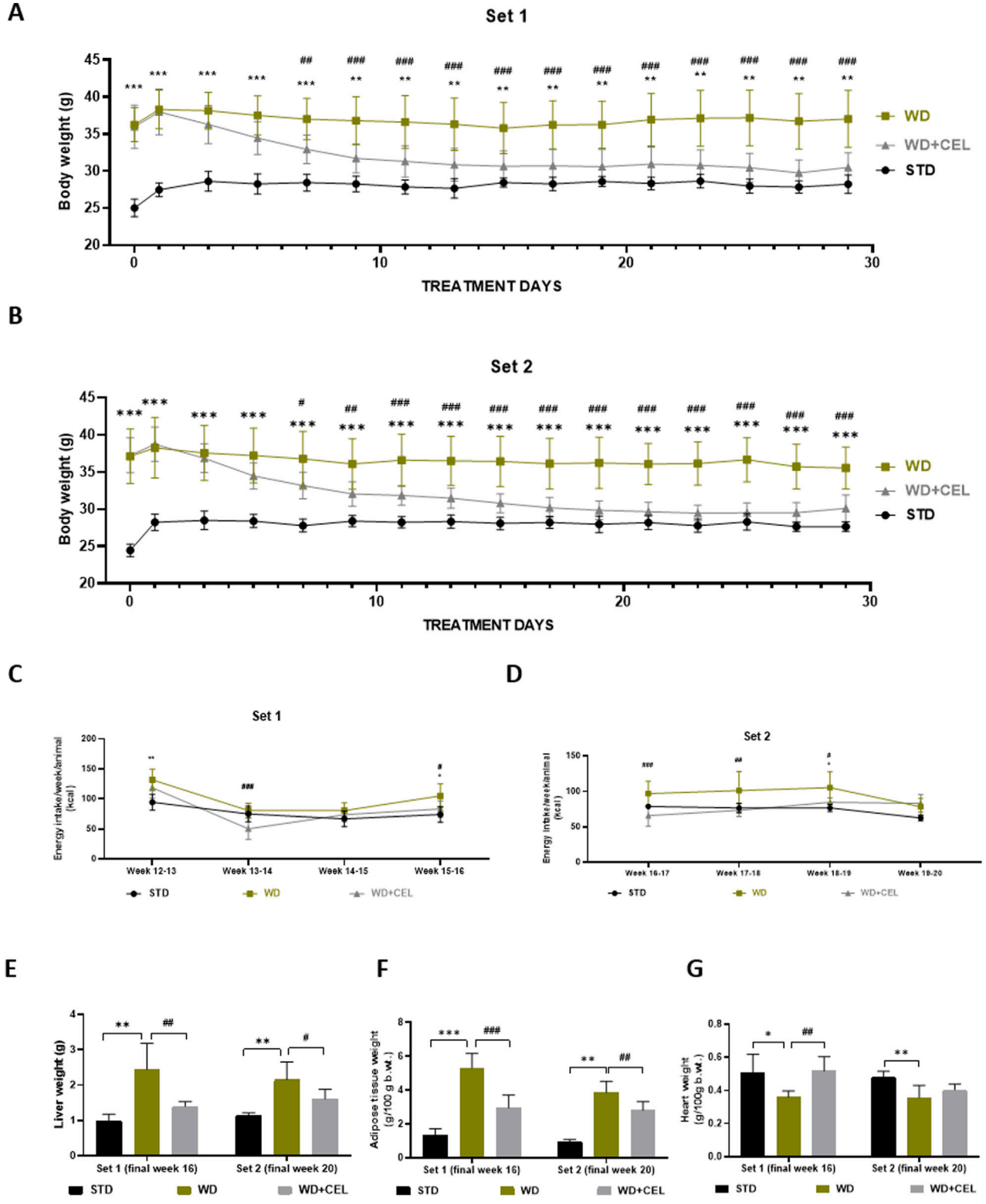

FIGURE 2
Effect of 4-week celastrol (CEL) treatment on mouse body and liver weight, energy consumption, and adipose tissue-to-body and heart-to-body weight ratios in each set of *in vivo* experiments. **(A)** Each second-day body weights of the experimental set 1 mice during weeks 12–16 (note that the first measurement was taken after 12-h fasting). **(B)** Each second-day body weights of the experimental set 2 mice during weeks 16–20 (note that the first mouse weighing was done after 12-h fasting). **(C)** Final weekly energy consumption per mice of set 1 (measured during weeks 12–16). **(D)** Final weekly energy consumption per mouse of set 2 (measured during weeks 16–20). **(E)** Liver weight. **(F)** Ratio of abdominal plus epididymal fat weight to total body weight. **(G)** Heart-to-body weight ratio. Data are expressed as means ± SD (n = 3 for negative controls, n = 7–8 for positive controls and CEL treatment), where **p < 0.01, ***p < 0.001, and ****p < 0.001 when comparing respective positive control (WD group) against negative control (STD

(*Continued*)

FIGURE 2 (Continued)
group) and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, and $^{\#\#\#\#}p < 0.001$ when comparing CEL treatment (WD+CEL group) against positive control, as assessed by one-way **(E–H)** or two-way **(A–D)** ANOVA with the *post hoc* Bonferroni test.

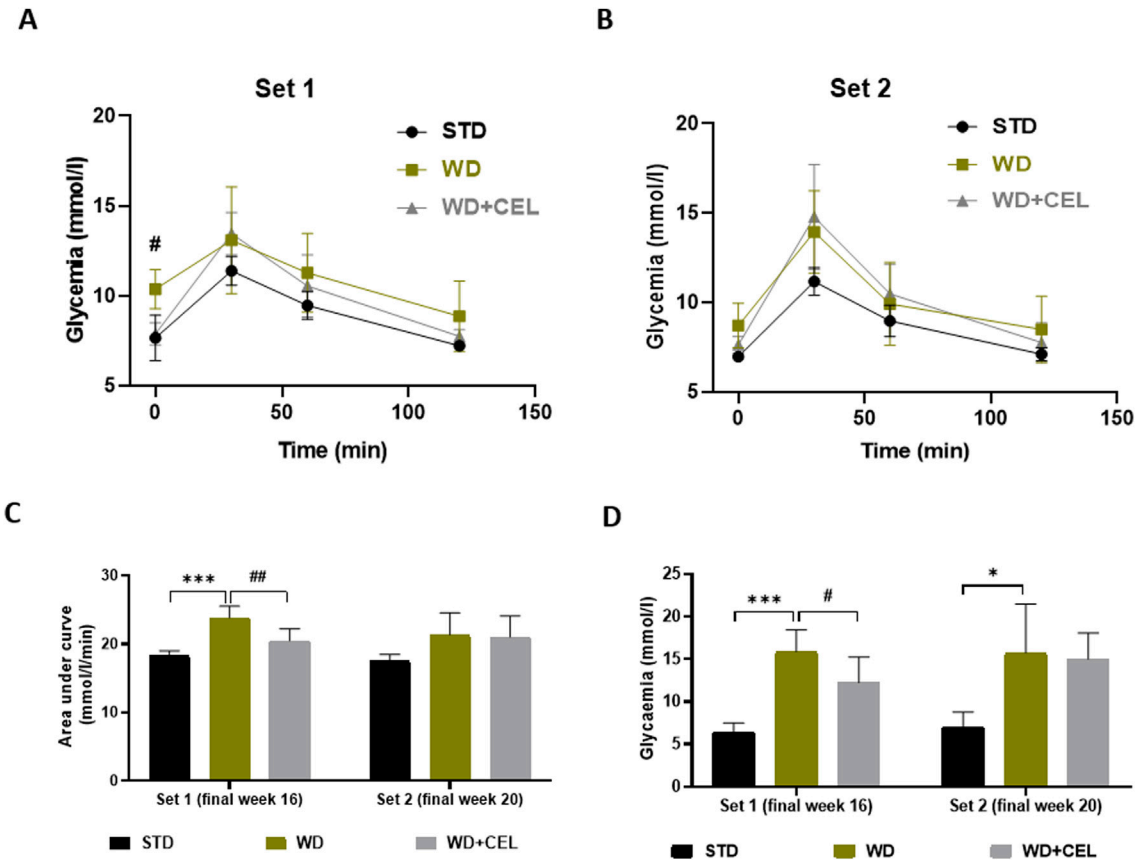

FIGURE 3
Effect of celastrol (CEL) on serum glucose levels in each experimental set of WD/FG-induced obesity and MASLD/MASH in mice. **(A)** Oral glucose tolerance test (OGTT) glucose levels at week 16 of set 1. **(B)** OGTT glucose levels at week 20 of set 2. **(C)** Area under the curve of OGTT glycemia calculated using the trapezoidal rule. **(D)** Terminal 12-h fasting serum glucose levels. Data are expressed as means ± SD (n = 3 and 7–8/group as noted in Table 1), where $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to the respective positive control (WD group) against negative control (STD group) and $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to CEL treatment (WD+CEL group) against positive control, as assessed by one-way **(C, D)** or two-way **(A, B)** ANOVA with the *post hoc* Bonferroni test.

treatment did not produce any further alterations in serum urea and creatinine levels at any time point, indicating its safety for the kidney (Figures 4E, F).

3.3 The effect of celastrol treatment on serum lipids, liver triglyceride content, oxidative stress markers, and TNF-alpha

In both sets of *in vivo* experiments, the atherogenic WD/FG diet significantly enhanced the concentrations of serum total cholesterol when compared to negative controls. CEL treatment decreased this serum lipid marker significantly only at the end of set 1 (Figure 5A). Although serum triglyceride levels were not affected by either WD/FG or WD/FG + CEL (Figure 5B), the liver TG content was highly significantly increased in positive controls and, conversely, remarkably ($p < 0.05$) reduced after CEL treatment in both sets (Figure 5C).

Animals in the positive control (i.e., WD) group displayed significantly enhanced concentrations of liver TBARS, conjugated dienes, nitrites, and TNF-α at the end of week 16 (i.e., set 1) when CEL treatment was able to significantly reduce only TBARS. At the end of week 20 (i.e., set 2), WD/FG alone significantly increased only the conjugated diene content in the liver, while CEL had no additional effect on any of the liver oxidative stress markers and TNF-α (Figures 5D–F; Supplementary Figure S3, respectively).

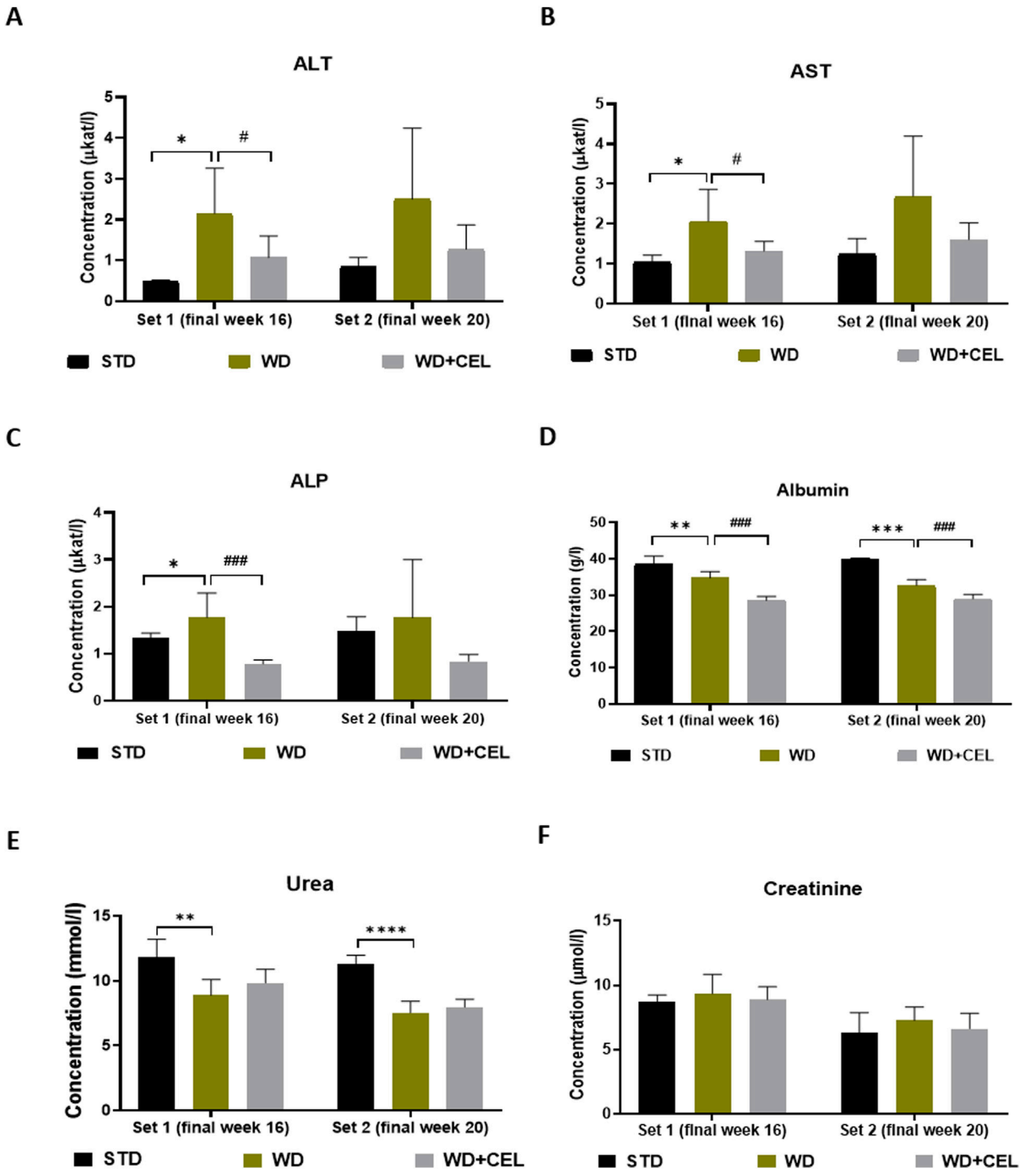

FIGURE 4
Effect of celastrol (CEL) treatment on biochemical serum markers of liver and kidney functions in each experimental set of WD/FG-induced obesity and MASLD/MASH mouse model. **(A)** serum ALT, **(B)** serum AST, and **(C)** serum ALP catalytic activity concentrations, **(C)** serum ALP levels, **(D)** serum albumin levels, **(E)** serum urea levels, and **(F)** serum creatinine levels. Data are expressed as means + SD (n = 3 and 7–8/group, as noted in Table 1), where *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.001$ compared to the positive control (WD group) against the respective negative control (STD group) and $^{###}p < 0.001$ compared to CEL treatment (WD+CEL group) against the positive control, as assessed by one-way ANOVA with the *post hoc* Bonferroni test.

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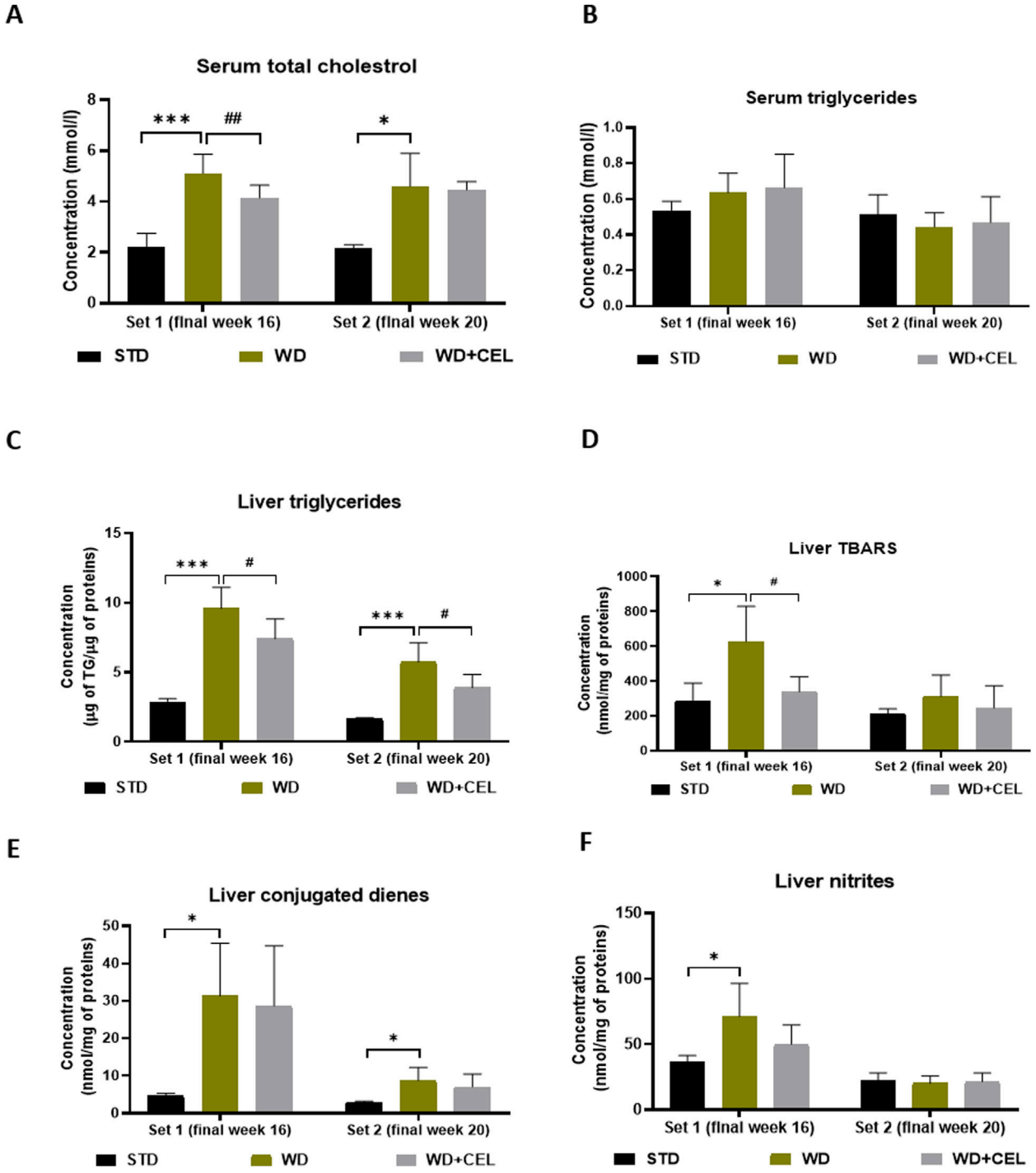

FIGURE 5
Effect of celastrol (CEL) treatment on concentrations of mouse serum total cholesterol **(A)**, serum triglycerides **(B)**, liver triglyceride (TG) content **(C)**, liver TBARS **(D)**, liver conjugated dienes **(E)**, and liver nitrites **(F)** in both sets of the *in vivo* experiment. Data are expressed as means + SD (n = 3 and 7–8/group, as noted in Table 1), *$p < 0.05$ and ***$p < 0.001$ compared to the respective positive control (WD group) against the negative control (STD group) and $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to CEL treatment (WD + CEL group) against the positive control, as assessed by one-way ANOVA with the *post hoc* Bonferroni test.

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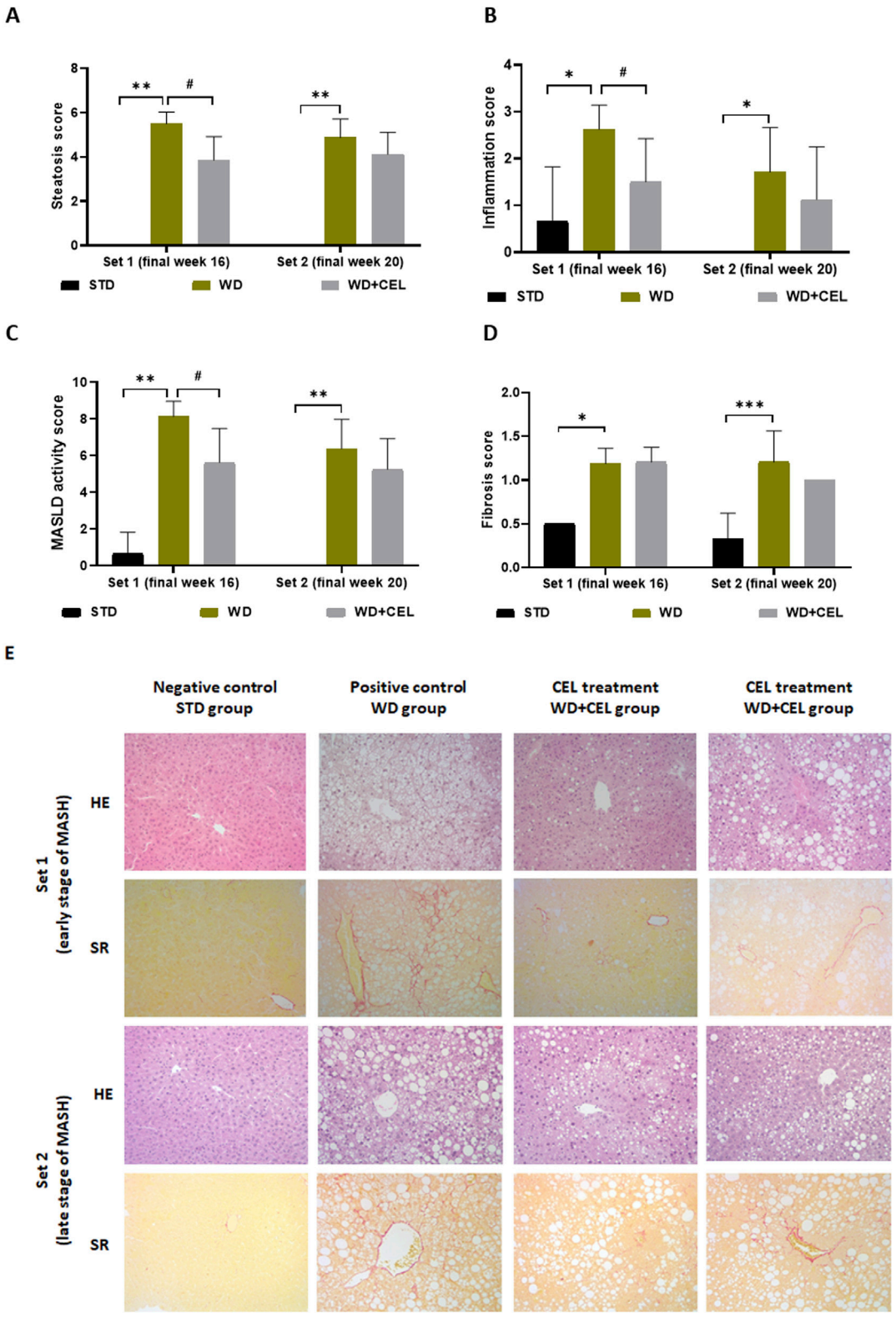

FIGURE 6
Impact of celastrol treatment on mouse MASLD/MASH histopathological scoring and staging of the experimental set 1 and set 2 (representing the end of weeks 16 and 20, respectively), including **(A)** total cell macrovesicular, microvesicular, and hypertrophy liver steatosis score, **(B)** total liver inflammation score, **(C)** MASLD activity score, and **(D)** liver fibrosis score. The data are expressed as the means + SD, where *$p < 0.05$, ***$p < 0.01$, and **$p < 0.01$ when comparing the positive control (WD group, n = 7–8) to the respective negative control (STD group, n = 3) and $^{\#}p < 0.05$ when comparing CEL treatment (WD+CEL, n = 8) to the positive control, as assessed by the Kruskal–Wallis test with *post hoc* Dunn's multiple comparison test.
(*Continued*)

FIGURE 6 (Continued)
Note: some data are missing for negative controls as the scoring values were zero for all three samples. Similarly, some SDs are missing as the data were completely equal for all livers in the respective group, that is, the SDs were zero. **(E)** Example images of liver sections used for histopathological evaluation after staining with hematoxylin–eosin (HE) or Sirius red (SR) at a magnification of ×200.

3.4 The effect of celastrol treatment on the liver morphology of diet-induced MASLD/MASH

Macroscopically, there was a noticeable difference between the negative and positive control livers, which were hypertrophied and much lighter due to fat accumulation (Supplementary Figure S4). The positive controls displayed highly significantly elevated total steatosis, inflammation, MASLD activity, and fibrosis scores in mouse livers of both sets, confirming MASH. However, the overall fibrosis caused in mouse fed on WD/FG was low and reached only that of stage 1B in average (i.e., 1.67), representing moderate perisinusoidal fibrosis at the microscopic level. At the end of week 16, CEL significantly reduced liver steatosis, inflammation, and the total MASLD activity score and ameliorated liver morphology. CEL treatment displayed a similar pattern of slight reduction (n.s.) in all histological scores and overall liver morphology at the end of week 20 (Figure 6), when the liver became harder with the disorganization of typical microarchitecture, prevailing macrovesicular steatosis and increasing the number of fibroblast-like cells. Moreover, the development of liver tumors was noted in one positive control case of set 2 (Supplementary Figure S4).

3.5 The effect of celastrol treatment on MASLD/MASH-related liver gene expression in diet-induced obesity of mice

Some genes illustrated variation during the progression from steatosis to hepatitis at different time points, while other genes indicated the same pattern in both sets. The expression of the gene *Ppargc1a*, which encodes a transcriptional coactivator PGC-1α affecting energy metabolism, was downregulated by WD/FG at both sets; however, it was significant only at the end of week 16. On the other hand, CEL significantly upregulated *Ppargc1a* gene expression only at the end of week 20 (Figure 7A). Similar trends of the atherogenic diet and CEL were observed for the *Crtc2* gene-encoding CRTC2 protein, which is also involved in glucose metabolism, lipogenesis, and other various cell processes (Figure 7B). As CRTC2 can regulate mTOR signaling pathway transduction (Zheng et al., 2023), we also screened for *Mtor* gene expression. Similar to *Crtc2* at week 16, *Mtor* gene expression was significantly downregulated by WD/FG. On the other hand, it was significantly upregulated by WD/FG at week 20, which was not affected by CEL (Figure 7C).

Gene expression levels of *Acaca* and *Fasn* that code for important lipogenic enzymes promoting *de novo* lipogenesis and adipogenesis were induced in mouse livers of positive controls of both sets. However, CEL-treatment normalized *Acaca* and *Fasn* mRNA levels only at the end of week 16. At week 20, CEL further enhanced upregulated *Acaca* gene expression (Figures 7D, E). Surprisingly, unlike *Acaca* and *Fasn*, the expression of the *Srebf1* gene was significantly increased only in set 2, and CEL significantly increased its expression in both experimental sets (Figure 7F).

Expression of genes involved in cell survival, senescence, and/or oxidative status (i.e., decreasing oxidative stress), such as *Sirt1*, *Nrf2*, *Hmox*, and *Sod2*, produced highly variable results. For example, *Sirt1* was significantly downregulated by an atherogenic diet at week 16 and remarkably upregulated by CEL at week 20 (Figure 7G). Although *Sirt1* should play a key role in activating the Nrf2/ARE (antioxidant response element) signaling pathway and protecting against oxidative stress (Zhuang et al., 2021), the *Nrf2* gene was highly significantly upregulated by WD/FG throughout the experiment; however, CEL decreased its mRNA levels remarkably only during set 1 (Figure 7H). Nrf2, a crucial transcription factor, plays a pivotal role in determining the liver's antioxidant capacity and detoxification status (Shin et al., 2013). Nrf2, among others, coordinates gene expression of *Hmox1*, which was affected in the same way as *Nrf2* only at the end of week 16. Later on, *Hmox1* mRNA was further increased by CEL (Figure 7I). Only at the end of week 20, CEL also very significantly increased the otherwise unaffected expression of the *Sod2* gene, which codes the critical antioxidant enzyme SOD-2 (Figure 7J).

For the evaluation of the inflammatory pathway, we assessed the liver expression of genes coding TNF-α and IL-1β cytokines. In contrast to liver TNF-α protein levels (Supplementary Figure S3), *Tnfa* mRNA levels were not affected by WD/FG; however, it was significantly downregulated by CEL only at week 16 (Figure 7K). Gene expression of *Il1b* was significantly upregulated by the atherogenic diet during both sets, and CEL treatment significantly reversed it only at the end of set 1 (Figure 7L).

3.6 The effect of celastrol treatment on the gene expression of galaninergic system members in the liver and heart ventricle tissues of Western diet-induced obese mice

We realized that the quantitative gene expression of the members of the galanin family in mouse heart ventricles was the strongest for *Galr2*, followed, in a descending manner, by *Gal*, *Galr1*, and *Galr3*. *Galp* gene expression was very low and under or at the limit of detectability in all heart ventricle samples; therefore, it was not possible to meaningfully analyze this. In mouse liver tissue, only *Galr2* gene expression could be quantitatively evaluated because the expression of other genes (i.e., *Gal*, *Galr1*, and *Galr3*) was very low.

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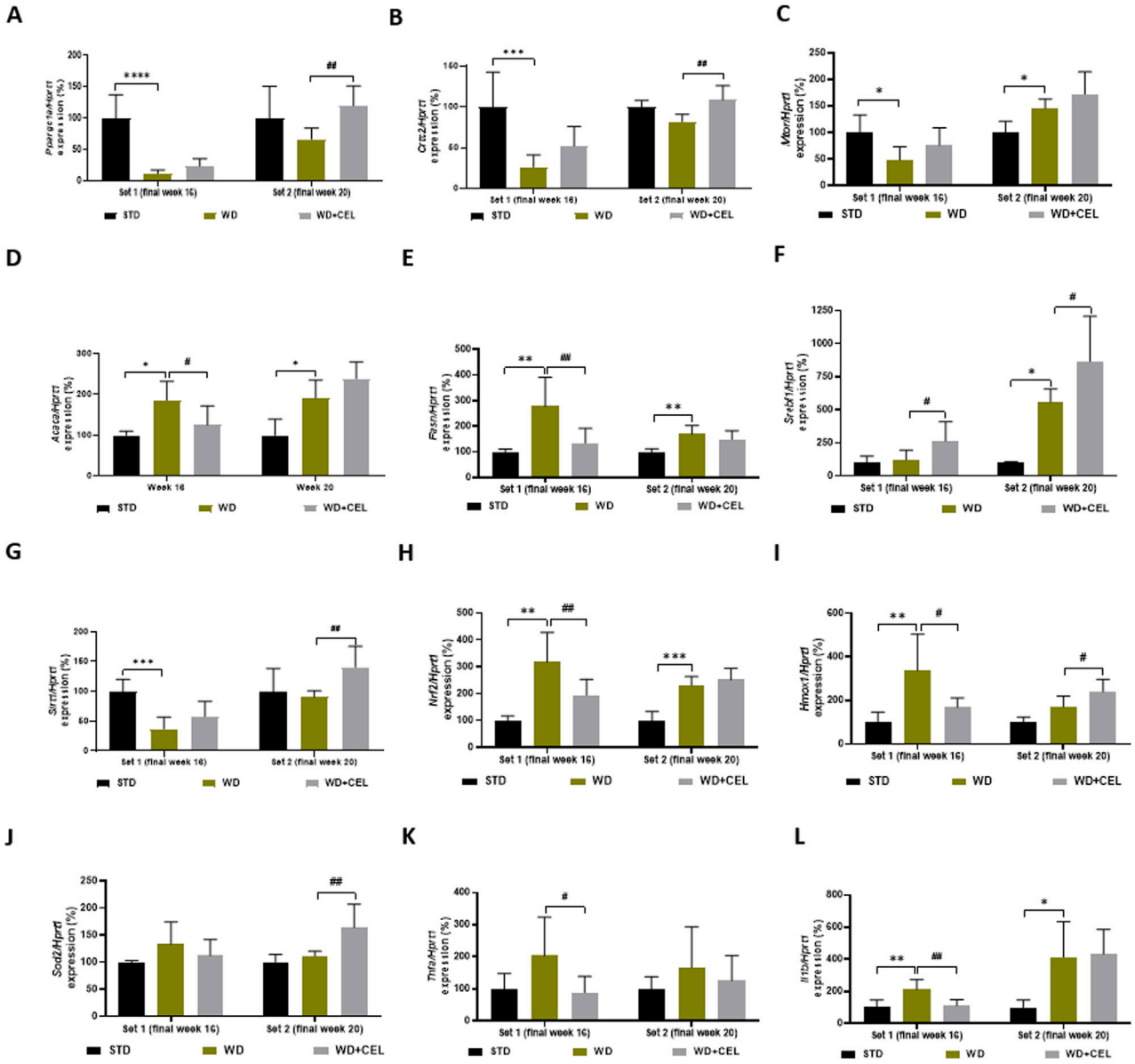

FIGURE 7
Effect of celastrol treatment on liver expression of selected genes evaluated in each experimental set of WD/FG-induced obesity and the MASLD/MASH mouse model, where **(A)** *Ppargc1a* (peroxisome proliferative activated receptor gamma, coactivator 1 alpha), **(B)** *Crtc2* (CREB, cAMP response element-binding protein, regulated transcription coactivator 2), **(C)** *Mtor* (mechanistic target of rapamycin kinase), **(D)** Acaca (acetyl-coenzyme A carboxylase alpha), **(E)** *Fasn* (fatty acid synthase), **(F)** *Srebf1* (sterol regulatory element-binding transcription factor 1), **(G)** *Sirt1* (sirtuin 1), **(H)** *Nrf2* (also Nfe2l2, nuclear factor, erythroid derived 2, like 2), **(I)** *Hmox1* (heme oxygenase 1), **(J)** *Sod2* (superoxide dismutase 2, mitochondrial), **(K)** *Tnfa* (tumor necrosis factor alpha), and **(L)** *Il1b* (interleukin 1 beta) represent specific gene expressions. Data are expressed as means + SD (in percentage of average negative control ΔΔCt value calculated against the expression of housekeeping gene *Hprt1*, hypoxanthine phosphoribosyltransferase 1), where *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ compared to the respective positive control (WD group, n = 6–8) against the negative control (STD group, n = 3) and $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ when comparing CEL treatment (WD + CEL, n = 7–8) against the positive control, as assessed by one-way ANOVA.

As for specific qRT-PCR results in mouse heart ventricles, there was a highly detectable expression of the *Gal* gene increasing with age in negative controls, which was further significantly upregulated by the Western-type diet, with the peak expression at weeks 16–19 (Figure 8A). CEL had no additional effect, while FAT significantly downregulated *Gal* expression (Figure 8B). The expression of the *Galr1* gene was lower than that of *Gal*; however, it exerted a similar pattern (Figure 8C). CEL and FAT addition to WG/FG highly significantly decreased the *Galr1* gene expression, which was slightly increased (n.s.) or significantly upregulated by the Western-type diet at the end of weeks 16 and 20, respectively (Figure 8D). In the heart ventricles, there was very high expression of the *Galr2* gene, which was relatively stable, concerning the mouse age and significantly upregulated by WD/FG, with the peak expression at weeks 16–20. CEL had no additional effect on this, while FAT significantly downregulated enhanced *Galr2* mRNA levels at the end of week 16 (Figures 8E, F). Interestingly, *Galr2* gene expression in the mouse liver of set

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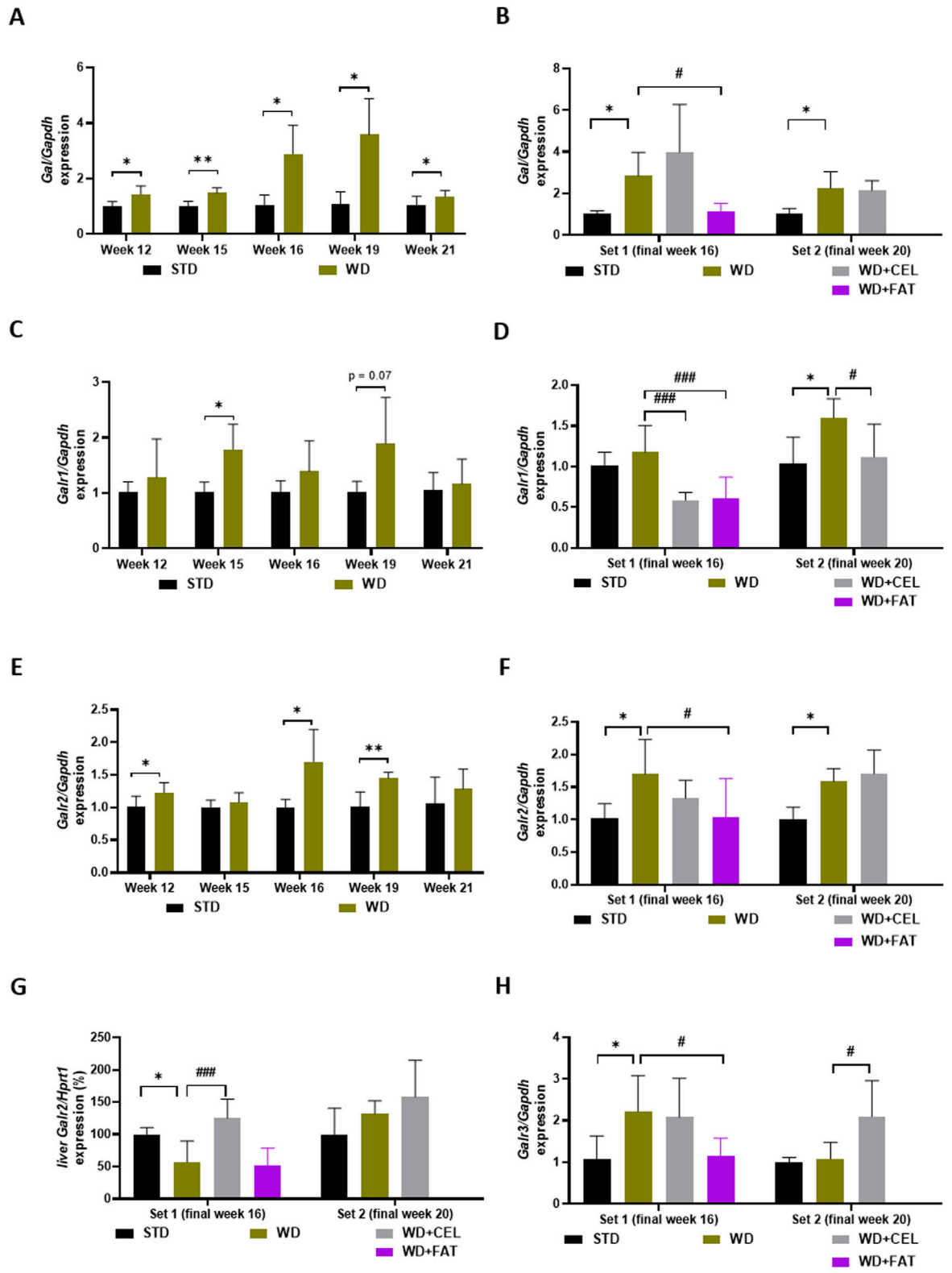

FIGURE 8
Effect of celastrol (CEL) and fatostatin (FAT) treatment on gene expression of members of the galanin system in mouse heart ventricles **(A–F, H)** and livers **(G)** in WD/FG-induced obesity and MASLD/MASH model, where **(A, B)** *Gal* (galanin gene), **(C, D)** *Galr1* (galanin receptor 1 gene), **(E–G)** *Galr2* (galanin receptor 2 gene), and **(H)** *Galr3* (galanin receptor 3 gene) represent the specific gene expression of the exploratory pilot study **(A, C, E)** and the *in vivo* experiments covering set 1 and set 2 lasting for 16 and 20 weeks, respectively **(B, D, F–H)**. Data are expressed as means + SD (as average of negative control ΔΔCt value calculated against the expression of housekeeping gene), where *p < 0.05, **p < 0.01, and ***p < 0.001 when comparing the
(*Continued*)

FIGURE 8 (Continued)
respective positive control (WD group, n = 4 for hearts sampled at weeks 15 and 19, n = 6 for hearts sampled at weeks 12 and 16, n = 7 for livers from set 1, and n = 8 for others) against the respective negative control (STD group, n = 3 or n = 5 for only hearts sampled at week 21) and $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ when comparing CEL or FAT treatment (WD+CEL or WD+FAT, n = 6 for hearts only, n = 8 for livers of both sets) against the respective positive control, as assessed by Student's t-test [for graphs **(A, C, E)**] or one-way ANOVA [for graphs **(B, D, F–H)**].

1 was affected in a completely different manner: it was significantly downregulated by WD/FG, highly significantly increased by CEL, and unchanged by FAT when compared to that in positive controls (Figure 8G). Finally, there was a very low expression of the *Galr3* gene, generally in the heart, which was nearly undetectable until week 15, with a peak at week 19 and a remarkable drop at week 21 in both the negative and positive controls. At the end of week 16, *Galr3* mRNA levels were borderline detectable and significantly enhanced by WD/FG in heart ventricles, which remained unaffected by CEL; however, they were significantly downregulated by FAT treatment. At the end of week 20, *GalrR3* gene expression was significantly upregulated by the addition of CEL to the Western-type diet, of which *Galr3* mRNA levels were the same as for the negative control group (Figure 8H).

3.7 The effect of celastrol treatment on the MASLD/MASH-related liver protein expression of SREBP1 and GalR2

In liver homogenates, we detected two Western blot lines of positive bands (Figure 9A), which represent the immature and mature forms of SREBP1 (Tiscione et al., 2019). Interestingly, the protein expression of mature (cleaved, active, and nuclear) SREBP1-m was not affected in set 1; however, it significantly decreased in set 2 of the *in vivo* experiment and returned toward negative control levels under CEL treatment (Figure 9A). The amount of its precursor (immature and uncleaved) SREBP1-p highly significantly decreased both in positive and CEL-treated groups (Figure 9B), suggesting SREBP1-p consumption due to its activation through the cleavage in livers of mice on the WD/FG diet. Therefore, we calculated the SREBP1 activity as the ratio of SREBP1-m/SREPBP1-p (relative protein expressions), which was significantly increased in positive controls in both sets. CEL had no additional effect on this SREBP1 activity (Figure 9C).

In accordance with *Galr2* mRNA expression, CEL significantly upregulated GalR2 protein expression, which was apparently reduced in positive control mouse livers of only experimental set 1 (Figure 9D).

3.8 The immunohistochemical detection of GalR1 in the heart tissue

We performed advanced immunohistochemical detection of GalR1 in the hearts of mice on STD, WD/FD, and mice on the Western-type diet treated with CEL or FAT in two individual experimental sets. GalR1 was detected in all samples, both in the atria and in the ventricles. The intensity of the immunohistochemical reaction was similar in the hearts of mice from two different sets and different experimental groups (Figures 10A–F); however, there was considerably higher immunoreactivity in positive controls and lower immunoreactivity in heart sections of CEL- and FAT-treated mice (Figures 10H–K). The reaction product was mostly diffusely distributed in the cytoplasm of cardiomyocytes. We often found a prominent reactivity at the intercalated discs (Figure 10G). This staining pattern was not characteristic for any of the groups. Due to the variable GalR1 positivity, which is diffused in the cytoplasm compared to the concentrated one in the intercalary discs, it was not possible to perform a relevant quantitative analysis.

3.9 The effect of celastrol and M871 on palmitic-induced hepatocyte lipotoxicity *in vitro*

Pre-treatment of primary hepatocytes with CEL at a nontoxic concentration (500 nM) had no effect on palmitic acid (PA)-induced lipotoxicity, as evidenced by cell viability, ALT release, and Red Oil O stain accumulation (for *in vitro* methods and Supplementary Figure S5, see Supplementary Material). In combination with PA and/or M871, CEL exhibited a noteworthy reduction in nitrite production by hepatocytes (Supplementary Figure S5C). The GalR2 inhibitor, an M871 compound (100 nM), significantly increased PA-induced cytotoxicity and nitrite levels (as an oxidative stress indicator). On the other hand, M871 rather decreased lipid accumulation in hepatocytes (Supplementary Figures S5D–F).

4 Discussion

At the beginning of the Discussion section, it is important to stress that the aim of the presented study was not to prove or develop a mouse MASLD/MASH model, as we already did this in our previous study (Arora et al., 2023). Therefore, and to reduce the number of mice to the minimum, we used only three mice in the negative controls (STD group) as a type of background for the whole experiment, which caused some results in the positive control (WD group) not reaching statistically significant values. This may appear to be the main shortcoming of our study. Another shortcoming of our study is the fact that the impact of CEL has been exclusively studied only in male subjects, although MASLD prevalence is increasing in women with polycystic ovary syndrome and estrogen deficiency, especially after menopause, leading to higher risks of MASH and advanced fibrosis compared to men of the same age (Eng et al., 2023). However, as our main aim was to evaluate the effect of celastrol treatment (WD + CEL group) against WD/FG-

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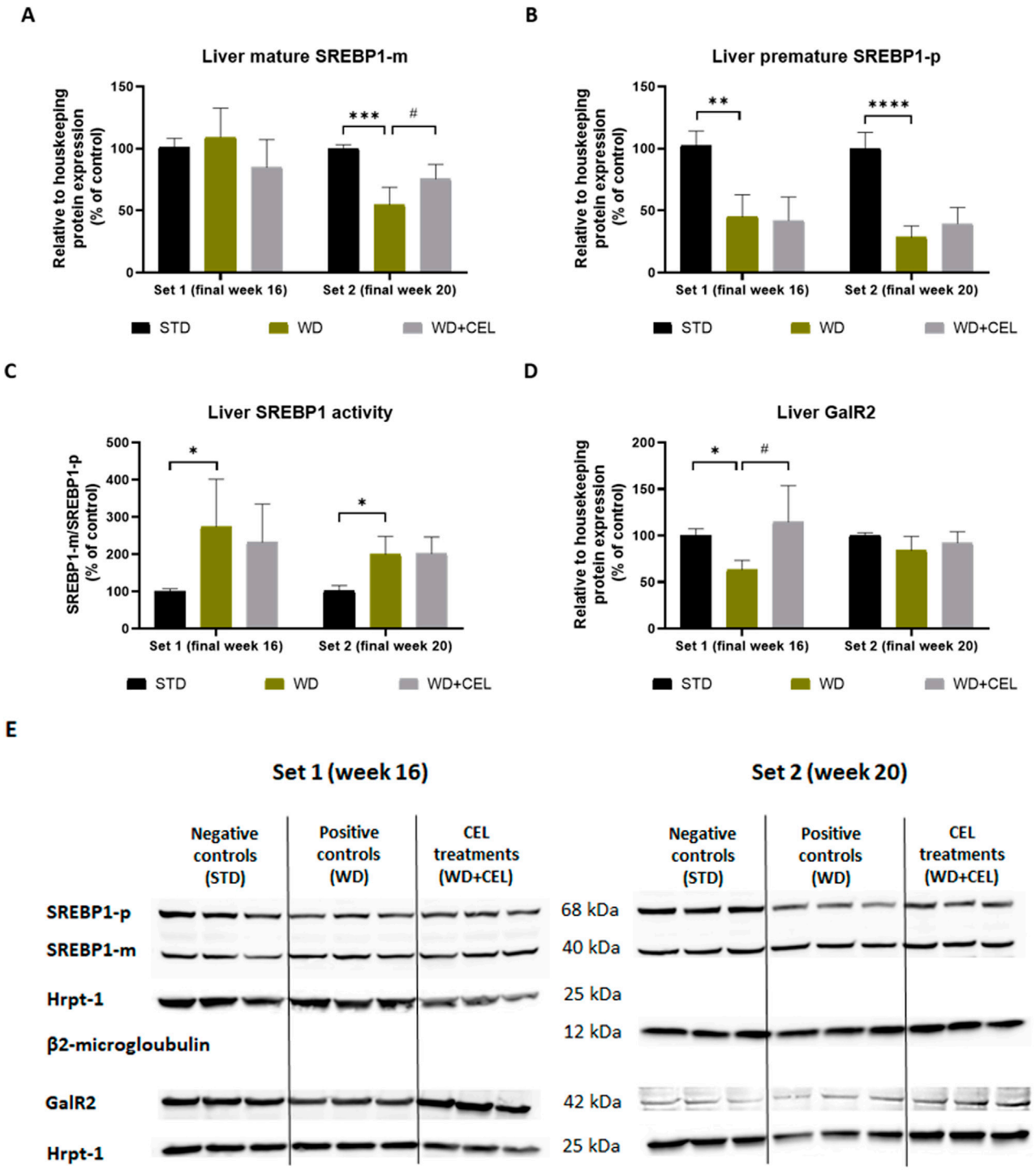

FIGURE 9
Effect of celastrol (CEL) treatment on liver protein expression of mature SREBP1 (SREBP1-m), SREBP1 precursor (SREBP1-p), and galaninergic receptor type 2 (GalR2) in WD/FG-induced obesity and the MASLD/MASH model of the *in vivo* experiments covering set 1 and set 2 lasting for 16 and 20 weeks, respectively. **(A)** Quantification of SREBP-m protein expression. **(B)** Quantification of SREBP-p protein expression. **(C)** SREBP1 activity calculated as the ratio of SREBP1-m/SREPBP1-p relative protein expressions. **(D)** Quantification of GalR2 protein expression. **(E)** Example of Western blot analyses of SREBP-p, SERBP-m, GalR2, Hrpt-1, and beta2-microglobulin proteins. Data in the graphs are expressed as means + SD (as an average of negative control value calculated against the expression of housekeeping protein), where *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ when comparing the respective positive control (WD group, n = 5 and n = 6 for set 1 and set 2, respectively) against the respective negative control (STD group, n = 3) and $^{\#}p < 0.05$ when comparing CEL treatment (WD + CEL group, n = 5 and n = 6 for set 1 and set 2, respectively) against the respective positive control, as assessed by one-way ANOVA.

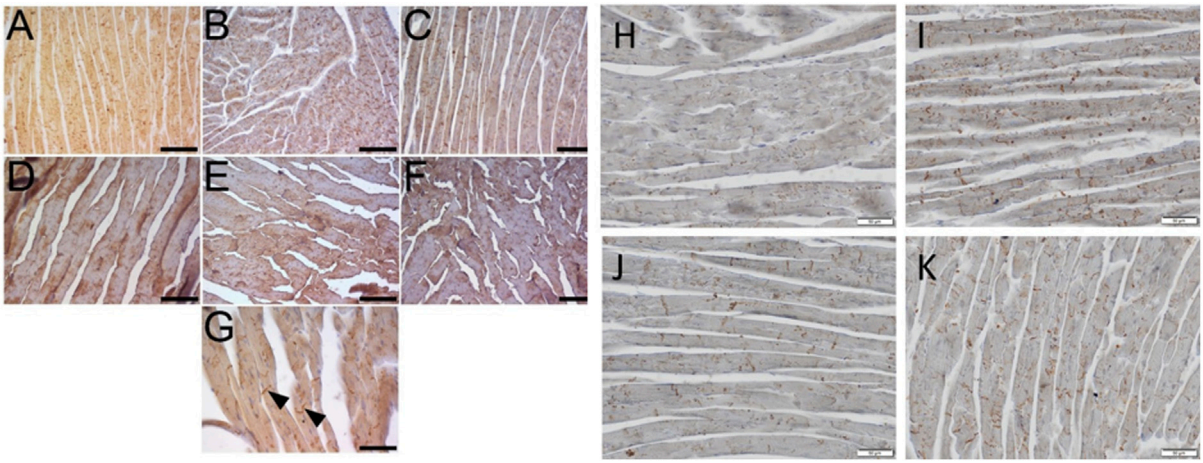

FIGURE 10
Immunohistochemical detection of GalR1 in hearts of mice on standard diet (STD, negative controls) and Western-type diet (WD, positive controls) for overall 16 or 20 weeks and mice on WD treated for the last 4 weeks with celastrol (CEL) or fatostatin (FAT). Example images taken by a Leica DMLB microscope equipped with a Leica MC170 HD camera show the results of immunoperoxidase reaction to detect GalR1 in the heart sections. All images show the left ventricular wall, where **(A–C)** represent myocardia after 20 weeks (corresponding to set 2), and **(D–K)** represent myocardia after 16 weeks (corresponding to set 1) of *in vivo* experiments. Mouse hearts of STD **(A, D, G, H)**, WD **(B, E, I)**, WD + CEL **(C, F, J)**, or WD + FAT **(K)** groups. GalR1 immunoreactivity is visible in cardiomyocytes as a diffuse reaction product in the cytoplasm **(A–F)** and a concentrated reaction product in intercalated discs of cardiomyocytes **(G–K)**, best seen in transversal sections (arrows) **(G)** and at higher magnification **(H–K)**. Scale bar in **(A–F)** = 200 µm, in **(G)** = 100 µm, and **(H–K)** = 50 µm.

induced obesity and MASLD/MASH, we preferably statistically analyzed the differences between WD and WD+CEL groups of male mice, which we discuss in the following text.

In the current study, we originally demonstrated that celastrol has beneficial effects on the Western-type diet-induced obesity and progression of MASLD to MASH in mice via reduced liver inflammation, enhanced antioxidant defense, and improved lipid and glucose metabolism in at least one set of *in vivo* experiments. Other studies documented similar ameliorative effects of CEL on HFD-induced obesity and fatty liver in mice (Li M. et al., 2022; Zhang et al., 2016; Ye et al., 2024). The body weight-lowering effect of CEL in our study could be at least partially explained by decreased food and energy intake. It can be interpreted by the suppression of blood GAL and GALP levels and Gal/GalR1/GalR3, NPY, and leptin expressions in the hypothalamus of obese mice, where CEL works as a leptin sensitizer (Fang et al., 2019; Liu et al., 2015; Marcos and Coveñas, 2021; Takenoya et al., 2018; Ye et al., 2024). However, we have shown that the body, liver, and fat tissue weights of CEL-treated mice on the diet were significantly lower at the end of the experimental sets, although the reduced energy end/or food intake already did not differ from that of positive controls that also had considerably reduced WD consumption, namely, in set 2. Potentially, central (hypothalamic) resistance to the long-term effect of celastrol could also develop as the development of tissue galanin resistance with persistent galanin levels in obese subjects has been described before (Marcos and Coveñas, 2021). Therefore, factors other than central anorexigenic effects could also contribute to CEL complex ameliorative properties on the body, liver, and fat tissue weights and energy metabolism (i.e., improved glucose tolerance and reduced fasting glucose levels, reduced serum total cholesterol, and decreased liver triglyceride content).

To understand the progression of metabolic disease represented by MASLD/MASH, it is crucial to assess the expression of central metabolic, oxidative status, and inflammatory genes through time. This assessment provides valuable insights into the complex impact of celastrol treatment at various stages of MASH pathogenesis in the WD/FG-induced obesity experimental model. Therefore, mRNA levels (i.e., gene expression) of the selected genes involved in MASLD/MASH progression were determined in mouse livers by employing quantitative RT-PCR: *Ppargc1a* (peroxisome proliferative activated receptor, gamma, coactivator 1 alpha), *Crtc2* (CREB, cAMP response element-binding protein, regulated transcription coactivator 2), *Mtor* (mechanistic target of rapamycin kinase), *Fasn* (fatty acid synthase), *Acaca* (acetyl-coenzyme A carboxylase alpha), *Srebf1* (sterol regulatory element-binding transcription factor 1), *Nrf2* (also Nfe2l2, nuclear factor, erythroid derived 2, like 2), *Hmox1* (heme oxygenase 1), *Sirt1* (sirtuin 1), *Sod2* (superoxide dismutase 2, mitochondrial), *Tnfa* (tumor necrosis factor alpha), and *Il1b* (interleukin 1 beta). CEL produced metabolic benefits through the activation of PGC-1α, leading to enhanced energy expenditure and browning of white adipose tissue by regulating mitochondrial function and biogenesis (Fang et al., 2019; Li M. et al., 2022). Throughout our *in vivo* experiment, CEL upregulated *Ppargc1a* gene expression that was suppressed by WD/FG, resembling liver-specific-deficient PGC-1α mice with manifested hepatic steatosis (Rodgers et al., 2005). We have observed similar patterns in *Crtc2* and *Sirt1* gene regulation by the Western-type diet and CEL. CRTC2 is a transcriptional coactivator of CREB (cAMP response element-binding protein), which plays a role in glucose, lipid, and energy metabolism through increased PGC-1α transcription and regulation of several pathways, including the cAMP and mTOR pathways, and SRBEP1/2 (Zheng et al., 2023). There are no specific studies on *Crtc2* gene expression in response to CEL, while celastrol's impact on SIRT1 expression was described to contribute to its protective effects against fatty liver (Zhang et al., 2016). SIRT1 deactivated CRTC2 and SREBP-1c,

decreasing early gluconeogenesis and lipogenesis, and activated PGC-1α, reducing adiposity and lipogenesis while promoting fatty acid oxidation (Elibol and Kilic, 2018). Reciprocally, CREB and PGC-1α can upregulate SIRT1 (Molla et al., 2020), indicating that PGC-1α, CRTC2, and SIRT1 are interconnected players in metabolic regulation, impacting processes like gluconeogenesis and lipid metabolism. Therefore, we can assume that the hepatoprotective and beneficial metabolic effects of CEL in our study can, to some extent, be mediated by the CRTC2-PGC-1α-SIRT1 pathway. Furthermore, WD/FG increased serum total cholesterol, adipose tissue weight, liver triglyceride content, and steatosis score that were accompanied by significant upregulation of lipogenic genes like *Acaca* and *Fasn*. CEL significantly decreased all these lipid metabolism parameters and both genes in the set 1 experiment, which is in accordance with other animal studies (Hu et al., 2018; Zhang et al., 2016). Later, as MASH progressed, the effect of CEL was not as evident and *Acaca* gene expression even increased. *Fasn* and *Acaca* are the target genes for sterol regulatory element-binding protein 1 (SREBP1) transcription factor, which is involved in glucose metabolism; fatty acid, cholesterol, and TG synthesis; and adipogenesis and is encoded by the *Srebf1* gene (Soyal et al., 2015). CEL can also target SIRT1 to promote AMPK-α phosphorylation and inhibit Srebp-1c-mediated lipid synthesis against oxidative stress and inflammation (Li M. et al., 2022). Unpredictably, CEL significantly upregulated increased *Srebf1* mRNA in our *in vivo* experiments. We assumed that this was a compensatory mechanism when CEL might inhibit the activation of SREBP1 protein through the induction of the CRTC2-PGC-1α-SIRT1 pathway as CRTC2 modulated hepatic SREBP-1c cleavage (Zhang et al., 2018). This would be confirmed by the fact that CEL decreased the expression of liver protein SREBP-1c, while the *Srebf1* mRNA expression in HFD-induced steatotic liver remained unchanged (Zhang et al., 2016). CEL either did not affect or normalized significantly downregulated protein expression of maturated (active, cleaved, and nuclear) SREBP1-m in set 1 and set 2, respectively. Moreover, CEL had no additional effect on SREBP1 activity, which is calculated as the ratio of SREBP1-m to premature SREPBP1-p, which was significantly increased by feeding mice WD/FG in both sets. Therefore, we may summarize that CEL does not produce its ameliorative effect on MASH primarily through the modulation of SREBP1 activity under our *in vivo* experimental setting.

Due to the histological scoring system's limitations and variability in set 1, we could not clearly demonstrate a difference between the early and late stages of the positive control MASH at weeks 16 and 20, respectively. However, at week 20 and later on (Arora et al., 2023), we observed a smaller, firmer liver with less fat, confirmed biochemically and microscopically, indicating disease progression characterized by the decreasing amount of steatotic hepatocytes, the disorganized microarchitecture, and the development of liver cirrhosis and tumors. Given the considerably inconsistent findings regarding histopathological outcomes, gene mRNA (namely, *Mtor* and *Galr2*), and oxidative stress across the experimental sets, both our research team and other scientists posit that MASLD, the transition from MASLD to MASH (i.e., early-stage MASH), and advanced MASH (i.e., later-stage MASH) represent distinct and complex phenotypes (Martin et al., 2021; Arora et al., 2023). These conditions appear to involve unique signaling pathways in the liver affected by CEL in a considerably different manner in early-stage and later-stage MASH, with CEL showing more beneficial effects in the earlier stage of MASH (i.e., set 1). For example, *Nrf2*, *Hmox1*, *Sod2*, and *Galr2* gene expressions showed variable responses to CEL in the liver. The heightened expression of antioxidant molecules was attributed to their defensive and adaptive response against the substantial reactive oxygen species production and oxidative stress triggered by the Western diet. Moreover, the same study demonstrated that mitochondria generate less cellular oxidative stress, resulting in decreased TBARS at the stage of MASH formation (Staňková et al., 2021). Another intriguing study investigated the effects of a prolonged HFD on mRNA expression across a critical set of genes (including metabolic and antioxidant ones) in C57BL/6J mice. Although SREBP expression remained unchanged in this mouse strain, PGC-1α decreased and Nrf2 showed significant enhancement, mirroring our findings (Kim et al., 2004). Similarly, TBARS, conjugated dienes, nitrites, and gene expression of *Nrf2*, *Hmox1*, and also slightly of *Sod2* were concomitantly upregulated by WD/FD with down-regulating or, more appropriately, normalizing the effect of CEL at the end of set 1. On the other hand, CEL enhanced gene expression of antioxidant molecule genes in the situation when they were induced by long-term feeding with the Western-type diet at a much lower extent accompanied by decreasing oxidative stress in the liver of positive control mice at set 2. CEL has been found to activate SOD2 and Nrf2-HO1, contributing to its hepatoprotective effects in the liver of HFD obese mice (Luo et al., 2017; Zhang et al., 2016; Li M. et al., 2022). Moreover, CEL was shown to induce HO-1 through the upregulation of Nrf2 in hepatoma cells (Tseng et al., 2017). In our study, CEL significantly decreased both the liver inflammatory score and pro-inflammatory gene expression (*Tnfa* and *Il1b*) at the end of week 16, similar to that in various other studies with mouse models of fatty liver (Zhang et al., 2016; Li M. et al., 2022). Notably, macrophage infiltration and expression of macrophage M1 biomarkers (e.g., IL-6, IL-1β, TNF-α, and iNOS mRNA) were decreased after 3 weeks of CEL treatment in the livers of C57BL/6 mice induced with HFD (Luo et al., 2017), which can, at least partially, explain our findings. Wang et al. (2020) also demonstrated potent anti-inflammatory properties of CEL that remarkably suppressed the protein levels of pro-inflammatory cytokines (IL-1β, IL-6, IL-18, and TNF-α) while increasing the levels of anti-inflammatory cytokines (IL-10 and IL-13) in the serum and fibrotic livers of rats in a dose-dependent manner, while the lowest daily dose of 250 μg/kg CEL was not significantly effective. The relatively low CEL dose used in our study could explain why we did not achieve a significant reduction in mildly increased serum and liver TNF-α with CEL administration. Finally, our results on the anti-inflammatory effect of CEL in the early stage of MASH could be supported by the study demonstrating that transcription analysis provided a more promising means for identifying an immune profile as there are large discrepancies and lack of correlation between transcription and protein expression data when using the multiomics approach (Jacobsen et al., 2024). Taken together, we suppose that CEL is less effective on late-stage MASH in the second set of our *in vivo* experiments because other molecular signaling cascades and genes are involved in this stage of the disease.

The involvement of galanin in liver fibrosis and inflammation is multifaceted, with varying research findings (He et al., 2023). Galanin was found to exert its anti-inflammatory effects primarily through these receptor subtypes, particularly GalR2/3, while GalR1 is believed to be pro-inflammatory and pro-fibrogenic

in the liver (Lang and Kofler, 2011; Mills et al., 2021; He et al., 2023). The hepatic expression of the *Galr2* gene, which was the only detected member of the galanin family at measurable mRNA levels in the mouse liver, was also variable throughout our study. Recently, it was demonstrated that human patients with MASLD have increased levels of serum galanin and that daily 5-week-lasting treatment with CEL ameliorates HFD/high cholesterol-induced MASH in mice (He et al., 2023). Moreover, in the same study, it was shown that murine macrophages express GalR2, proposing a new role for galanin in inhibiting the pro-inflammatory phenotype of macrophages and inducing their M2-polarization (He et al., 2023). Notably, the same authors revealed that galanin can inhibit primary rat hepatic stellate cell (HSC) activation and suppress the pro-fibrogenic characteristics of HSCs by activating the GalR2 receptor, whose expression is induced in activated HSCs but undetectable in quiescent HSCs (He et al., 2016). In our *in vitro* experiment, celastrol did not reduce palmitic acid-induced lipotoxicity in primary hepatocytes even at the highest tested non-toxic concentration. The selective GalR2 inhibitor, M871, significantly enhanced PA-induced cytotoxicity and nitric oxidative stress alongside rather reduced lipid accumulation in hepatocytes. M871 showed its tendency to reverse the effects of CEL. These results suggest that GalR2 plays an important role in hepatoprotection independent of lipid accumulation and that, in the liver, CEL might positively affect GalR2 expression in cells other than primary (untransformed) hepatocytes (e.g., macrophages/Kupffer cells, HSCs, fibroblasts, and hepatoma cells), at least in the early stages of MASH. Spexin-mediated mitigating effects on obesity and MASLD in mice on HFD and in PA-induced HepG2 cells were eradicated by M871, proving that spexin, a known GalR2/3 agonist, produces its beneficial effect through the activation of GalR2 (Wang et al., 2022). Moreover, anti-apoptotic and anti-proliferative effects of galanin and GALP were shown to be mediated through GalR2 (Berger et al., 2004; Tofighi et al., 2008), which can explain no liver cancer development in mice under the CEL treatment. Interestingly, CEL in our study also exerted highly similar ameliorative effects as spexin *in vivo*, including changes in *Ppargc1*, *Fasn*, *Srebf1*, and *Sirt1* expressions (Wang et al., 2022). Therefore, we can speculate that CEL could exert its MASH ameliorative effects through increased GalR2 gene and protein expression in the liver, namely, in set 1 of our *in vivo* experiments. Interestingly, fatostatin did not affect liver *Galr2* gene expression, suggesting that SREBP activity has no effect on *Galr2*/GalR2 expression and *vice versa* in the liver. The liver activity of SREBP1 in our *in vivo* experimental settings both did not correlate with GalR2 protein expression and was not influenced by CEL.

Notably, the distribution of galanin receptor subtypes in rodents differs: GALR1 is exclusively expressed in the central and peripheral nervous systems, while GALR2 and GALR3 are widely distributed in both the central and peripheral tissues of rats (Mills et al., 2021; Waters and Krause, 2000). Previously, the expression of the Galr1 gene was detected in the brain, spinal cord, heart, and skeletal muscle; however, no mouse *Galr1* mRNA was detected in the liver, kidney, testis, lung, and spleen (Wang et al., 1997b). According to RNA profiling data sets generated later by the Mouse ENCODE project, there is some extent of expression of *Galr2*, negligible expression of *Galr3,* and no expression of *Gal, Galp*, or *Galr1* RNAs in the adult liver and heart under physiological conditions of 8-weak-adult C57BL/6J mice. Interestingly, *Galp* and *Galr3* were revealed in embryonal liver but not in adult ones, and all three *Galr* RNAs were detected in adult genital fat pads, with the most pronounced one being *Galr2* (Yue et al., 2014; National Library of Medicine–National Centre for Biotechnology Information, 2024). ENCODE data showed GalR2 to be the only receptor subtype in adult mouse hearts, cardiomyocytes, and H9C2 cardiomyoblasts (Boal et al., 2022). In terms of *Galr2* gene expression, our observations align with the highest levels of its mRNA detected in genes related to the galanin system, both in the liver and heart of adult C57BL/6J mice, regardless of their age. Conversely, we observed an age-related increase in gene expressions of *Gal*, *Galr1*, and *Galr3* (in order of decreasing quantity) in the heart ventricles, with a peak occurring between weeks 16 and 20 on the controlled STD. According to the available literature, *Gal* and *Galr3* expressions have not been detected in the mouse heart until the completion of this manuscript. Furthermore, we had originally demonstrated that WD/FG significantly increased mRNA levels of *Gal*, *Galr1*, *Galr2*, and *Galr3* in mouse heart ventricles compared to those in negative controls in our experiments, suggesting that other members except for GalR2 might play a role in cardiovascular physiology and pathophysiology related to metabolic diseases. We had previously described the gene and protein expressions of galanin, GALP (Škopek, 2013), and galanin receptors (GalR1, GalR2, and GalR3) in rat hearts and their modulation under stress (Škopek, 2013; Šípková et al., 2017b). Moreover, galanin's cardioprotective and crucial role in regulating cardiac autophagy and apoptosis in hypertrophied hearts, following myocardial infarction in mice, was demonstrated (Martinelli et al., 2021). A recently published review based on the experimental data on many studies summarized that galanin plays a role in the development of insulin resistance and diabetic heart conditions; however, it also helps alleviate hyperglycemia and improves insulin sensitivity, enhances glucose utilization, and promotes mitochondrial growth in the cardiac muscle through GalR2. These findings depict GalR2 as a potential therapeutic target for treating diabetic cardiomyopathy (She et al., 2023). Moreover, genetic suppression of *Galr2 in vivo* (by using *Galr2* knockout mice) and *in vitro* (by using siRNA transfection) promoted cardiac hypertrophy, fibrosis, and mitochondrial oxidative stress and eradicated the beneficial effects of galanin on mouse heart and primary cardiomyocytes, respectively. Therefore, the authors concluded that targeting GalR2 might offer novel therapeutic strategies for heart diseases (Boal et al., 2022). Stimulation of GalR2 signaling by selective galanin ligands was also presented to be involved in the attenuation of myocardial ischemic/reperfusion injury and the improvement of cardiac function in rats *in vivo*, which was completely reversed by M871 (Palkeeva et al., 2019; Serebryakova et al., 2023). In our *in vivo* experiments, CEL did not affect both *Gal* and *Galr2* expressions that were upregulated by WD/FG, probably as a result of an adaptive mechanism to metabolic stress, thus preserving the protective effect of locally produced galanin on the heart through GalR2. Moreover, CEL was histologically demonstrated to be safe not only for the heart but also for the other major organs including the liver, spleen, lungs, kidneys, and brain of HFD-induced obese mice (Fan et al., 2022).

Contrarily to the stimulation of GalR2, which activates phospholipase C-induced signaling pathways, GALR1 and

GALR3 primarily decrease cAMP-mediated signaling pathways, hence interposing the inhibitory effects of galanin (Šípková, 2017a). The data on the role of GalR1 and GalR3 in heart diseases are controversial or missing (She et al., 2023). We present evidence of GalR1 expression in the mouse heart, both at the mRNA level and at the protein level detected by immunohistochemistry. In our scenario, the failure of immunostaining for the other proteins of interest (i.e., GalR2 or GalR3) might be attributed to the fixation of heart tissue by paraformaldehyde or/and the possibility that, under the experimental conditions, the endogenous GalR2 and GalR3 adopted a unique configuration different from the immunogens used to generate the antibodies (Lu and Bartfai, 2009; Michalickova et al., 2023). In general, there is a lack of sensitive and selective antibodies capable of recognizing specific endogenous G-protein-coupled receptors like mouse GalRs (Lu and Bartfai, 2009), although previously we managed to detect immunoreactivity of all three types of receptors in rat heart sections using the completely same antibodies against mouse GalRs (Šípková et al., 2017b). Therefore, when screening the effect of different modalities on galaninergic receptors, gene expression analysis is preferably used. In our experiment, GalR1 immunoreactivity was positive in cardiomyocytes of both heart atria and ventricles and was concentrated in the intercalated discs, mimicking our previous findings in rat hearts (Šípková et al., 2017b). Intercalated discs serve as intricate structures that facilitate both mechanical and electrical connections between cardiomyocytes, showing that galanin collaborates with other transmitters to regulate cardiac function as previous research indicates that galanin, through its interaction with GalR1, decreases acetylcholine release and suppresses vagal bradycardia (Herring et al., 2012). The use of the preferable GaR1 antagonist M40 resulted in enhanced cardiac function and reduced remodeling in rats with heart failure. The potential mechanism underlying this cardioprotective effect involves upregulation of cardiac SERCA2 (sarco/endoplasmic reticulum calcium ATPase 2) and a decrease in plasma IL-6 (Chen et al., 2015). Moreover, we had previously detected both mRNA and immunoreactivity of GalR1 in endothelial cells and discussed that utilizing GalR1 antagonists in clinical settings might yield antiangiogenic effects to counteract detrimental angiogenesis (Michalickova et al., 2023).

Another review primarily outlined the favorable impacts of CEL on cardiovascular disease (CVD), derived from *in vitro* and *in vivo* preclinical research and potential underlying mechanisms, including CEL´s inhibitory effect on the central galaninergic system (Li Z. et al., 2022). As CEL in our study significantly reduced *Galr1* gene expression in the heart ventricle, we can assume that it can contribute to CEL's overall favorable metabolic effect and possibly also the cardioprotective effect. This pattern of CEL can be at least partially (via decreasing fat intake, body weight, and adiposity) related to the modulation of SREBP activity as FAT produced the same Galr1 down-regulating effect in the mouse heart. It was demonstrated *in vitro* that mouse *Galr1* gene expression is upregulated by cAMP through a CREB-dependent mechanism (Zachariou et al., 2001) and that CREB is a co-activator of SREBP-mediated transcription of reporter genes (Oliner et al., 1996). These and our findings (i.e., enhanced *Galr1* expression in hearts of obese mice on WD/FG) suggest that increasing activation of SREBPs by HFD in obese mice (Soyal et al., 2015; Zhang et al., 2016) can induce *Galr1* gene expression in the heart. It seems to be favorable that CEL does not completely suppress the *Galr1* gene and GalR1, as we showed, because mice with a completely knockout (KO) *Galr1* gene have modified intake of only HFD (not STD) and experience glucose metabolism impairment, while *Galr1* heterozygotes do not differ from wild-type mice (Zorrilla et al., 2007). Interestingly, both changes in food behaviors under challenging HFD and glucose intolerance were also described for *Gal*-KO mice (Ahrén et al., 2004; Adams et al., 2008), suggesting the role of galanin and GalR1 in adjusting food intake and metabolic responses to variations in dietary fat while also influencing glucose regulation in mice (Zorrilla et al., 2007).

On the contrary, the *Galr3*-KO (backcrossed on the C57BL/6 background) mouse strain displayed physiological breeding and physical development alongside a similar trend (n.s.) of higher plasma TG and cholesterol levels compared with age-matched wild-type mice. Notably, male *Galr3*-KO mice exhibited an anxiety-like phenotype and reduced social affiliation (Brunner et al., 2014). However, the role of GalR3 in CVDs remains unclear as the expression of Galr3 is generally very low (i.e., as in our study) or missing depending on the age, tissue, and species, as we discussed above. Furthermore, CEL significantly increased mouse heart *Galr3* expression only when it was not upregulated in positive controls. On the other hand, FAT significantly downregulated WD/FG-enhanced *Galr3* mRNA, like in the cases of *Gal*, *Galr1*, and *Galr2* genes, indicating the role of SREBP and fat metabolism in the gene expression of members of the galaninergic system in the heart of obese mice. To the best of our knowledge, this significant downregulating effect of FAT on the galaninergic system in the heart has not yet been described anywhere. However, both SREBP1 and GalR1/GalR2 played pivotal roles within the adipogenic signaling pathways induced by the HFD in mice. Notably, cinchonine, a natural compound, exerted simultaneous inhibitory effects on these pathways (i.e., it significantly downregulated gene expression of *Srepbf1*, *Galr1*, and *Galr2* in fat tissue), resulting in reduced adipogenesis and mitigated obesity (Jung et al., 2012). However, the specific regulatory relationship between SREBPs and the galaninergic system remains unclear based on the current research. Therefore, more extensive studies are required to elaborate on the SREBP–galaninergic system interactions in various organs (including the heart) and species. Taken together, we can assume that the peripheral effects of CEL on the heart are produced mainly by decreasing the expression of *Galr1* in WD/FG-induced obese mice. It can be supported by the fact that CEL was likewise demonstrated to both effectively inhibit fat intake and promote weight loss by downregulating the expression of galanin and its receptors (specifically GalR 1 and 3) in the mouse hypothalamus during HFD conditions (Fang et al., 2019).

5 Conclusion

According to our results, CEL may have a beneficial effect on the galaninergic system modulation in the heart and liver of obese male mice on the Western-type atherogenic diet. Along with reducing the fat intake, weight gain, and amelioration of MASLD to MASH progression in mice, CEL also affected the expression of galanin and its receptors in the heart ventricles and liver lobes, which may be

involved in the regulation of energy metabolism, oxidative stress, and inflammation. Therefore, CEL may be a potential therapeutic agent for these metabolic dysfunction-associated disorders in humans. However, the exact mechanisms and implications of celastrol's action on the galaninergic system are not fully understood and require further investigation. Future research should also evaluate its impact on female animal models of MASLD/MASH, particularly those with deficits or alterations in sex hormone levels, highlighting the need for personalized treatment approaches for women.

Data availability statement

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

Ethics statement

The animal study was approved by the Expert Commission for Work with Experimental Animals of the First Faculty of Medicine, Charles University, and Ministry of Education and Sports of the Czech Republic under the project No. MSMT-11956/2021-4. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

NC: conceptualization, data curation, formal analysis, investigation, methodology, project administration, resources, supervision, visualization, writing–original draft, and writing–review and editing. JŠ: conceptualization, formal analysis, investigation, methodology, and writing–original draft. MA: conceptualization, formal analysis, funding acquisition, investigation, methodology, project administration, visualization, and writing–original draft. ZP: data curation, formal analysis, investigation, methodology, visualization, and writing–original draft. TK: formal analysis, methodology, supervision, visualization, and writing–original draft. OŠe: data curation, formal analysis, funding acquisition, investigation, methodology, and writing–original draft. TŠ: formal analysis, investigation, methodology, and writing–original draft. TV: formal analysis, methodology, supervision, and writing–original draft. OSl: conceptualization, formal analysis, funding acquisition, supervision, and writing–review and editing.

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Conflict of interest

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

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2025.1476994/full#supplementary-material

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EDITED BY
Daniele Maria-Ferreira,
Instituto de Pesquisa Pelé Pequeno Príncipe, Brazil

REVIEWED BY
Fengjie Sun,
Georgia Gwinnett College, United States
Ju Wang,
Sapienza University of Rome, Italy

*CORRESPONDENCE
Feiyan Lou,
lfy13486371389 2023@163.com

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The potential of Sijunzi decoction in the fight against gastrointestinal disorders: a review

Liangjun Yang[1], Zheng Fang[1], Jiajie Zhu[1], Xiaofang Li[2], Bo Yang[3], Haiyan Liu[1] and Feiyan Lou[4]*

[1]Department of Gastroenterology, Tongde Hospital of Zhejiang Province (Zhejiang Academy of Traditional Chinese Medicine), Hangzhou, China, [2]Science and Technology Innovation Center, Guangzhou University of Chinese Medicine, Guangzhou, China, [3]Department of Pharmacy, Tongde Hospital of Zhejiang Province (Zhejiang Academy of Traditional Chinese Medicine), Hangzhou, China, [4]Endoscopy Center, Tongde Hospital of Zhejiang Province (Zhejiang Academy of Traditional Chinese Medicine), Hangzhou, China

Sijunzi Decoction (SJZD) is a traditional Chinese medicine formula widely used in the treatment of gastrointestinal disorders. Clinical studies have substantiated the efficacy of SJZD in managing conditions such as functional dyspepsia, chronic gastritis, gastric cancer, irritable bowel syndrome, colorectal cancer, and ulcerative colitis. Despite its proven effectiveness, the precise mechanisms by which SJZD operates remain incompletely understood. In this study, we undertake a systematic review of both the clinical applications and the mechanistic underpinnings of SJZD in the context of gastrointestinal disease treatment. Research indicates that SJZD functions through a spectrum of mechanisms including the regulation of intestinal flora, alleviation of inflammation, modulation of immune responses, and facilitation of mucosal repair in the treatment of gastrointestinal ailments. This comprehensive analysis aims to provide a clearer understanding of how SJZD benefits patients with gastrointestinal disorders.

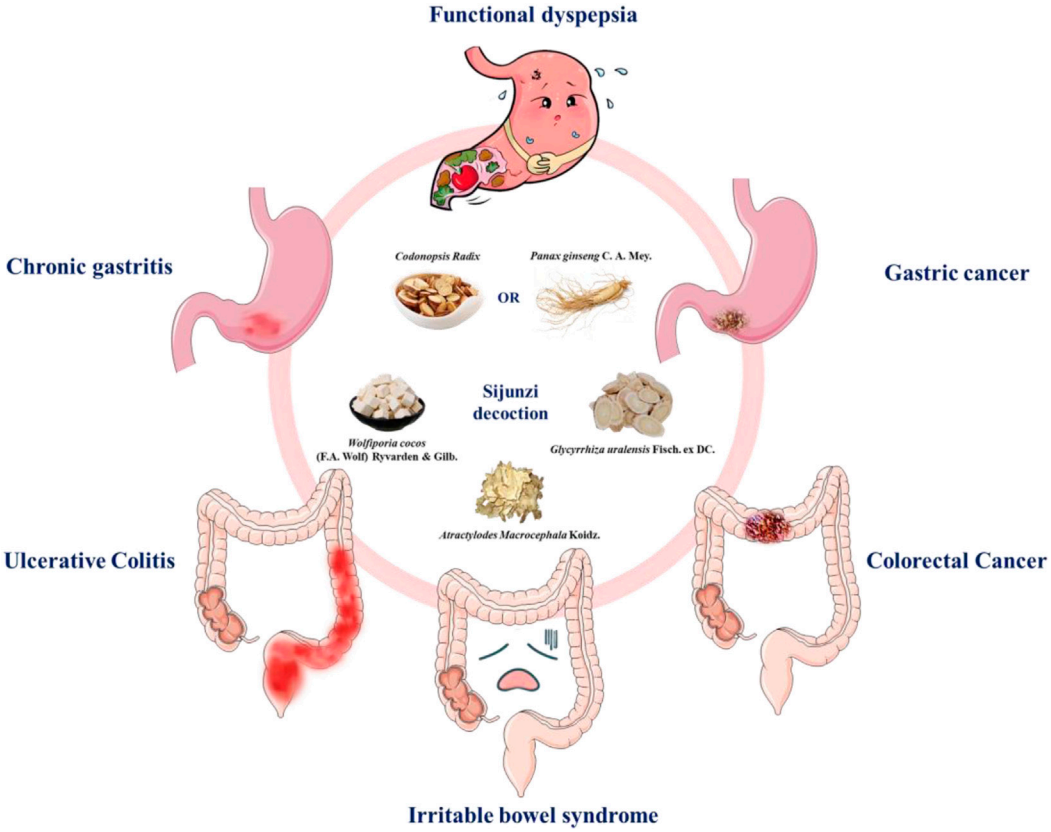
KEYWORDS

Sijunzi decoction (SJZD), gastrointestinal disorders, traditional Chinese, functional dyspepsia, irritable bowel syndrome, tumor

Introduction

The digestive system, which consists of the gastrointestinal tract, liver, pancreas, and gallbladder, facilitates the digestion of food—a crucial process that converts food into nutrients used by the body for energy, growth, and cellular repair. Gastrointestinal diseases, such as functional gastrointestinal diseases, inflammatory bowel disease, and tumors, significantly impact a large segment of the population, leading to a range of uncomfortable symptoms, increased morbidity, and substantial healthcare costs (Peery et al., 2015). Gastrointestinal diseases are prevalent, with an estimated prevalence rate of 16% for functional dyspepsia (FD) (Ford et al., 2020), over 50% for chronic gastritis

Abbreviations: SJZD, Sijunzi Decoction; TCM, Traditional Chinese medicine; FD, Functional dyspepsia; IBS, Irritable bowel syndrome; UC, Ulcerative colitis.



GRAPHICAL ABSTRACT

(Sipponen and Maaroos, 2015), and 9.2% for irritable bowel syndrome (IBS) (Oka et al., 2020). The inflammatory bowel diseases, primarily including Crohn's disease and ulcerative colitis (UC), are chronic inflammatory disorders of the gastrointestinal tract. In China, there has been a significant increase in the prevalence and incidence of inflammatory bowel disease over the past 3 decades, which provides challenges for the medical field (Park and Cheon, 2021). Additionally, among gastrointestinal malignancies in China, colorectal and gastric cancers rank second and fifth in terms of incidence, while they rank third and fourth in mortality, respectively (Han et al., 2024). Overall, digestive diseases remain a major contributor to the global healthcare burden, with little to no decline in prevalence or incidence observed in recent years (Wang et al., 2023). Given the substantial impact of these conditions, intervention in patients with digestive diseases is critically important for alleviating symptoms and enhancing their quality of life. However, developing effective drug-based therapies for gastrointestinal diseases presents considerable challenges, particularly due to the unclear etiology of many of these disorders (Halama and Haberkorn, 2020; Mishra et al., 2020; Singh et al., 2022). Addressing this complexity is essential for improving patient outcomes and managing the growing burden of gastrointestinal diseases in the healthcare system.

Traditional Chinese medicine (TCM), developed in China, is an ancient system that extensively examines human physiology, pathology, disease diagnosis, and treatment, with a rich historical background of effectively addressing digestive disorders. Central to this practice is the use of herbal medicine, which is regarded as a vital approach for managing a range of conditions, including functional gastrointestinal diseases (Tan et al., 2020), inflammatory bowel disease (Zhang S. et al., 2022), and malignancies such as gastric and colorectal cancers (Chen J.-F. et al., 2023; Dai Z. et al., 2024). Sijunzi decoction (SJZD), a classical formula in TCM, is composed of four Chinese herbs: *Panax ginseng* C. A. Mey. (or *Codonopsis radix.*), *Wolfiporia cocos* (F.A. Wolf) Ryvarden and Gilb., *Atractylodes macrocephala* Koidz., and *Glycyrrhiza uralensis* Fisch. ex DC. In the SJZD, *P. ginseng* C. A. Mey. (or *C. radix.*) takes the role of the Emperor herb, being the primary ingredient for replenishing Qi and strengthening the spleen and stomach. *Atractylodes macrocephala* Koidz. acts as the Minister herb, supporting the spleen and drying dampness to amplify *C. radix*'s effects on Qi and spleen reinforcement. *Wolfiporia cocos* (F.A. Wolf) Ryvarden and Gilb. is the Assistant herb, which works in conjunction with *A. macrocephala* Koidz. to further strengthen the spleen and eliminate dampness. Lastly, *G. uralensis* Fisch. ex DC. serves as the Messenger Herb, harmonizing the middle, supplementing Qi, and coordinating the actions of the other herbs within the formula. Together, these four herbs create a balanced and effective composition for tonifying the spleen and stomach.

This formulation has been widely utilized to address spleen deficiency, which presents symptoms such as anorexia, diarrhea, loose stools, and systemic discomforts including fatigue, weakness in the limbs, and cold extremities. A randomized, double-blind,

placebo-controlled, multi-center clinical trial has demonstrated that SJZD can alleviate fatigue symptoms and enhance overall health status in patients with chronic fatigue syndrome (Dai L. et al., 2024). SJZD is primarily used to treat gastrointestinal diseases, including chronic gastritis, functional gastrointestinal disorders, UC, and gastrointestinal carcinomas, based on its effects of strengthening the spleen and replenishing Qi in accordance with TCM theory. Over the past 2 decades, an increasing number of clinical studies have reported the application of SJZD in patients with gastrointestinal diseases. Concurrently, a series of experimental studies have been conducted to elucidate the mechanisms by which SJZD treats gastrointestinal diseases. However, the clinical application, pharmacodynamics, and molecular mechanisms of SJZD in the treatment of gastrointestinal diseases have not yet been systematically analyzed, and the common points of SJZD in gastrointestinal diseases have not been revealed. In this article, we review and summarize previous clinical and experimental studies on SJZD for gastrointestinal disorders, as well as insights into its pharmacological effects, aiming to illustrate the advantages and promote the application of TCM in the treatment of gastrointestinal diseases.

Methodology

In this review, we aim to summarize and evaluate recent evidence primarily drawn from clinical and experimental research, as well as systematic reviews, regarding the efficacy of SJZD in the treatment and management of gastrointestinal disorders. A comprehensive literature search was conducted, focusing mainly on publications from PubMed and ScienceDirect, limited to articles written in English. The keyword "sijunzi" was employed for searches on both databases.

The inclusion criteria encompassed evidence from randomized controlled trials, systematic and narrative reviews, observational studies, nonclinical studies, case reports, and expert opinions published over the past 24 years (2000–2024). In contrast, study protocols, duplicate articles, and conference abstracts were excluded from consideration.

Sijunzi decoction

As an empirical decoction, Sijunzi decoction was recorded in the Taiping Huimin Hejiju Fang of the Song Dynasty (published around 1110 A.D.). It consists of *P. ginseng* C. A. Mey. (Renshen) or *C. radix* (Dangshen), *W. cocos* (F.A. Wolf) Ryvarden and Gilb. (Fuling), *A. macrocephala* Koidz. (Baizhu), and *G. uralensis* Fisch. ex DC. (Gancao).

Panax ginseng C. A. Mey. is one of the most important traditional herbs and healthy foods in East Asian herbal remedies, with a history spanning over 2,000 years. The roots and rhizomes of *P. ginseng* C. A. Mey. are utilized in traditional medicine to treat diseases by replenishing Qi (Zhang et al., 2024). Modern research has identified the main chemical components of ginseng, which primarily include ginsenosides, ginseng polysaccharides, and ginseng polypeptides. Pharmacological studies indicate that ginseng possesses strong anti-inflammatory and immunoregulatory effects in the intestinal system (Zhao et al., 2024). Additionally, a meta-analysis has demonstrated that ginseng consumption is associated with a significantly reduced risk of gastric and colorectal cancer (Jin et al., 2016). This may be due to ginseng's ability to inhibit the proliferation, growth, invasion, and metastasis of tumor cells, induce tumor cell apoptosis, and ultimately suppress tumor initiation and progression (Ni et al., 2022; Zhao et al., 2022).

Codonopsis radix is an herb that has historically been used as a cost-effective substitute for the more expensive *P. ginseng* C. A. Mey. As a widely recognized medicinal plant in the field of TCM, *C. radix* possesses the ability to replenish Qi, strengthen the spleen, and nourish the blood. This herb contains a diverse array of chemical constituents, including flavonoids, polyacetylenes, triterpenes, steroids, alkaloids, resinous substances, and other biologically active compounds. Substantial evidence demonstrates that *C. radix* exhibits diverse biological activities, including immunomodulatory, antitumor, anti-inflammatory, and anti-fatigue properties (Luan et al., 2021). Furthermore, extracts of *C. radix* possess extensive pharmacological properties, such as protecting the gastrointestinal mucosa, exhibiting anti-ulcer effects, demonstrating antitumor activity, regulating endocrine functions, improving hematopoietic activity, and providing cardiovascular protection (Dong et al., 2023).

Wolfiporia cocos (F.A. Wolf) Ryvarden and Gilb, commonly known as *Poria cocos*, is a well-known fungus that has been used as a traditional medicine and functional food in China for over 2,000 years. The primary bioactive constituents of *Poria cocos* include polysaccharides, triterpenoids, steroids, fatty acids, and enzymes. Clinically, it is used to treat spleen-deficiency syndrome, often accompanied by symptoms such as digestive disorders, diarrhea, indigestion, and vomiting. Numerous studies have demonstrated that *Poria cocos* offers several beneficial effects, including intestinal protection, modulation of intestinal flora, anti-inflammatory, and antitumor activity (Zou et al., 2021; Wang et al., 2022). As a result, it is considered effective in the treatment of conditions such as IBS (Yang et al., 2023), colitis (Lan et al., 2023), and gastric cancer (Wang et al., 2022).

The rhizomes of *A. macrocephala* Koidz. have traditionally been utilized to invigorate the spleen and replenish qi for the treatment of various diseases. The primary chemical constituents of *A. macrocephala* Koidz. include terpenoids, polysaccharides, alkynes, flavonoids, and steroids. A significant body of research has highlighted the beneficial effects of *A. macrocephala* Koidz. on gastrointestinal diseases. Studies have confirmed that *A. macrocephala* Koidz. and its extracts possess multiple pharmacological activities, including immune enhancement (Xiang et al., 2020), antitumor (Liu et al., 2022), antioxidant (Bailly, 2021), and anti-inflammatory effects (Gu et al., 2019), which have therapeutic potential for gastrointestinal tumors (Chen T. et al., 2023; Choi et al., 2024b), chronic gastritis (Yang S. et al., 2020), UC (Cheng et al., 2023), and IBS (Choi et al., 2024a).

The dried roots and rhizomes of *G. uralensis* Fisch. ex DC., commonly known as Gan-Cao in Chinese, are widely used as herbal medicine worldwide. It is regarded as an "essential herbal medicine" in TCM due to its ability to reduce toxicity and enhance the effectiveness of other medicinal plants when used in combination. The primary chemical constituents of licorice include triterpenoid saponins, flavonoid glycosides, free phenolic

compounds, polysaccharides, coumarins, and alkaloids. Currently, it is extensively employed in the treatment of respiratory, liver, and gastrointestinal diseases, owing to its hepatoprotective, anti-inflammatory, antiviral, and antioxidant properties (Jiang et al., 2020; Li et al., 2020).

In summary, the herbs in SJZD exhibit a wide range of pharmacological activities, including immunomodulation, antitumor effects, anti-inflammatory properties, and gastrointestinal protection, thereby addressing a spectrum of gastrointestinal disorders and contributing to the overall health.

Sijunzi decoction in upper gastrointestinal disorders

Functional dyspepsia

FD, located in the gastroduodenal region, is among the most common functional gastrointestinal disorders. The prevalence of FD has been steadily increasing, significantly impacting patients' quality of life. The main contributing factors to FD include abnormal gastrointestinal dynamics, heightened visceral sensitivity, *Helicobacter pylori* infection, disturbances in intestinal flora, and psychological influences. This disorder is characterized by a diverse range of clinical manifestations, such as early satiety, postprandial fullness, and epigastric pain or burning, which seriously affect patients' quality of life and impose a heavy social and economic burden. In modern medicine, the treatment options for FD predominantly involve gastrointestinal motility agents, therapies aimed at alleviating visceral hypersensitivity, and medications targeting anxiety and depression. However, the efficacy of these treatments remains unsatisfactory (Lacy et al., 2023). TCM is a promising alternative, offering a holistic approach that may provide specific advantages in addressing the complex nature of FD. Based on the symptomatology of functional dyspepsia in TCM, it is commonly described as "distension and fullness," "stomach pain," and "retention" (Speciality Committee of Digestive DiseasesChinese Association of Integrative Medicine, 2011). Meta-analysis has shown that TCM formulas tend to yield better outcomes in alleviating overall dyspeptic symptoms (Chu et al., 2018). According to TCM, SJZD has been utilized in accordance with Chinese medicine for managing symptoms of indigestion. A meta-analysis has shown that SJZD-based prescriptions could be effective in treating FD, with no significant adverse effects detected (Wang Y. et al., 2021). However, due to the potential for bias, further validation of the benefits of SJZD for FD treatment necessitates the implementation of standardized, large-scale, and rigorously designed RCTs. These findings suggest that SJZD-based therapies hold promising potential for treating FD.

Chronic gastritis

Chronic gastritis is a chronic inflammatory response of the gastric mucosa that can be attributed to various factors such as *H. pylori* infection, medication usage, stress, and autoimmune processes. Generally, it presents as bloating, epigastric pain, indigestion, appetite loss, and other symptoms, considerably compromising patients' quality of life and increasing their susceptibility to developing cancer (Quan et al., 2018). Despite its prevalence, therapeutic approaches for chronic gastritis primarily focus on symptomatic treatment, as no specific Western medicine has been identified, particularly for chronic atrophic gastritis (Yan-Rui et al., 2024). Studies have confirmed that TCM is effective in alleviating the clinical symptoms of patients with chronic gastritis and can also impede the progression of chronic atrophic gastritis towards gastric cancer (Qin et al., 2013; Yang L. et al., 2020). As a classic TCM decoction, a clinical study has proved that SJZD can significantly improve the scores of the histopathology of chronic gastritis and *H. pylori* clearance rate (Gan et al., 2017). Additionally, several scholars have employed a modified version of SJZD for the treatment of chronic atrophic gastritis, and their findings indicate the efficacy of modified SJZD in alleviating fatigue and tiredness symptoms among chronic atrophic gastritis patients (Tian et al., 2019). Furthermore, a meta-analysis has shown that SJZD is an effective treatment option for chronic atrophic gastritis, as it improves clinical outcomes, enhances quality of life, and increases the eradication rate of *H. pylori,* as well as levels of GAS-17, PGI, and PGR in patients with chronic atrophic gastritis (Huang and Shao, 2024).

High gastric acid secretion is correlated with the manifestation of discomforting symptoms in individuals with chronic gastritis. *In vitro* experiments have substantiated the antacid properties of SJZD (Wu et al., 2010), suggesting a potential link to alleviating the distressing symptoms of this condition. Additionally, through network pharmacology analysis, it has been predicted that SJZD may alleviate chronic gastritis by suppressing the inflammatory response of peripheral blood leukocytes. Subsequent experimental studies have further validated the efficacy of SJZD in ameliorating both local gastric inflammation and inflammation in peripheral blood leukocytes (Wang et al., 2020). Gastric precancerous lesions are considered to be crucial steps in the progression from chronic atrophic gastritis to gastric cancer. Early identification, management, and surveillance of gastric precancerous lesions are imperative for preventing gastric cancer. According to proteomics and metabolomics analyses, research has demonstrated that SJZD possesses the capacity to hinder the progression of gastric precancerous lesions by regulating oxidative phosphorylation (Zhu et al., 2024). Additionally, modified formulas derived from SJZD, such as Weipiling decoction (Yang et al., 2022) and Weiwei decoction (Hong et al., 2024), have exhibited specific therapeutic effects in managing precancerous gastric lesions. These results demonstrated that SJZD is effective in the treatment of chronic gastritis.

Gastric cancer

Gastric cancer, a leading cause of cancer-related deaths globally, is ranked as the fifth most prevalent malignant tumor worldwide (Thrift et al., 2023). Despite advancements in stomach cancer treatment in recent years, the mortality rate associated with this disease remains significant. Hence, finding novel therapeutic approaches for gastric cancer holds exceptional significance. TCM is widely used in gastric cancer patients, especially in Asia. A retrospective cohort study conducted in Taiwan from 1997 to

2010 indicates that Chinese herbal medicine enhances the overall survival of gastric cancer patients (Hung et al., 2017). These findings establish a foundation for future research exploring the effectiveness of TCM as a therapeutic approach for gastric cancer.

SJZD has been widely utilized as a therapeutic approach for the long-term treatment of gastric cancer. A retrospective analysis has demonstrated that combining enteral nutrition with SJZD can effectively treat precachexia in cancer patients through the alleviation of inflammatory response, improvement of nutritional status, and enhancement of performance (Li et al., 2021). Additionally, a meta-analysis was conducted to evaluate the efficacy and safety of SJZD combined with enteral nutrition in GC patients, resulting in significant improvements in albumin, prealbumin, transferrin, immunoglobulin, $CD3^+$, $CD4^+$, and $CD4^+/CD8^+$ (Chen et al., 2020). Furthermore, treatment with SJZD inhibited cell growth and triggered apoptosis in gastric cancer cells. This effect was achieved by suppressing the expression of gastric cancer stem cell markers, as well as by reducing the nuclear accumulation and DNA binding activity of β-catenin (Jia et al., 2018; Li Y.-J. et al., 2022). CMTM2, an immune-related gene within the CMTM family, plays a significant role in tumor progression, and prognosis in gastric cancer (Qian et al., 2020). *In vitro* investigations reveal that SJZD markedly attenuates the proliferation, migration, invasion, and cancer stem cell-like characteristics of gastric cancer cells through the upregulation of CMTM2 expression (Li X. et al., 2022). Network pharmacology analysis predicted that SJZD may exert anti-gastric cancer effects via a comprehensive mechanism involving multiple compounds, targets, and pathways. Subsequent experimental findings have supported these predictions, demonstrating that SJZD can effectively suppress tumor growth and induce apoptosis in gastric tumor cells. These effects are mediated by the downregulation of key genes such as VEGFA, iNOS, COX-2, and Bax/Bcl2, as well as the inhibition of p-PI3K and p-AKT expression levels (Ding et al., 2022). Overall, the integrated approach of network pharmacology and experimental validation highlights the potential of SJZD as a therapeutic option for gastric cancer treatment.

Sijunzi decoction in lower gastrointestinal disorders

Irritable bowel syndrome

IBS is a chronic functional gastrointestinal disorder that presents with abdominal pain related to defecation or alterations in bowel habits. IBS is characterized by various gastrointestinal symptoms, including bloating, abdominal pain, urgency, and diarrhea or constipation, along with altered bowel habits, without any organic abnormalities. The conventional management of IBS typically includes lifestyle modifications, dietary interventions, and pharmacological treatments. Pharmacological treatment options include the regulation of gastrointestinal motility, supplementation with probiotics and prebiotics, as well as the use of antidepressants. However, the effectiveness of these treatments is frequently found to be unsatisfactory (Huang et al., 2023). TCM has shown effectiveness in treating IBS, addressing clinical symptoms such as abdominal pain, distension, and bowel habits, while also improving quality of life. As a promising approach, TCM has the potential to target dysmotility, visceral hypersensitivity, and the gut-brain axis by increasing NPY levels and reducing serotonin, making it a viable option for treating IBS (Xiao et al., 2015; Liu et al., 2023).

SJZD has been utilized in the treatment of IBS, specifically IBS with diarrhea (IBS-D). A meta-analysis revealed that the combined use of modified SJZD and Tongxie Yaofang significantly enhanced the overall clinical effectiveness rate for IBS-D when compared to conventional Western medicine treatments (Yan et al., 2023). The gut microbiota, functioning as a distinct organ with well-defined roles, plays a pivotal role in the development and severity of IBS. Alterations in the microbiota composition are significant factors contributing to the persistence of IBS symptoms (Canakis et al., 2020). Experimental evidence has demonstrated that a purified homogeneous polysaccharide obtained from SJZD exhibits immunomodulatory effects by modulating the abundances of nine genera of intestinal bacteria (Gao et al., 2018). To investigate the specific interactions between SJZD and the intestinal microbiota-derived from patients with IBS-D, an *in vivo* cocultivation system was created. The findings demonstrated that SJZD effectively restored the dysbiosis of the intestinal microbiota and improved the disrupted neurotransmitter metabolism associated with the key symptoms of IBS-D (Xia et al., 2022). In summary, SJZD exhibits therapeutic potential for treating IBS-D by modulating gut microbiota, thereby underscoring its efficacy in comparison to traditional treatments.

Colorectal cancer

Colorectal cancer ranks as the third most prevalent cancer and the second leading cause of cancer-related deaths worldwide. While the incidence and mortality rates of colorectal cancer have decreased in recent decades, epidemiological studies indicate a potential increase in its occurrence among individuals under the age of 50 (Thanikachalam and Khan, 2019). Considering that the symptoms of the disease typically only manifest in the advanced stage, the implementation of active screening and treatment represents an effective approach to diagnose and treat the condition. The primary treatments for colorectal cancer currently include surgery, radiotherapy, chemotherapy, immunotherapy, and targeted therapy. However, challenges such as surgical complications, chemotherapy resistance, toxic side effects, and elevated rates of metastasis and recurrence significantly compromise patients' quality of life (Pilkington et al., 2022). Consequently, research into more effective treatment options has become a focal point in this field. Chinese medicine has been utilized in the treatment of colorectal cancer. A cohort study conducted in China revealed that Chinese herbal medicines might mitigate the risk of recurrence and metastasis in patients with stage II and III colorectal cancer (Tang et al., 2022). This emerging evidence highlights an important frontier in the fight against colorectal cancer, suggesting that integrative treatment strategies incorporating TCM could significantly enhance patient prognosis and improve quality of life.SJZD has been used as a classical decoction in the treatment of digestive malignant tumor (Wu and Xuan, 2007). Through network pharmacology analysis, researchers have discovered that SJZD exerts its therapeutic effects on colorectal cancer by modulating multiple targets and pathways. A study specifically investigated the core genes involved in the

therapeutic mechanism of SJZD for colorectal cancer, revealing that SJZD influences protein binding in colon cancer by altering the expression of HSPB1, IGFBP-3, and SPP1 (Du et al., 2022). Furthermore, *in vitro* and *in vivo* experiments have confirmed that SJZD can induce apoptosis and autophagy in colorectal cancer cells through the PI3K/Akt/mTOR pathway (Shang et al., 2023). To identify potential therapeutic targets of SJZD, microarray analysis was conducted on patients with colorectal cancer who were undergoing treatment with SJZD. The results indicated that KLF4, which showed a significant correlation with reduced overall survival and recurrence rates, may serve as a potential therapeutic target of SJZD for the treatment of colorectal cancer (Jie et al., 2017). NK cells play a crucial role in cancer immunosurveillance by targeting and killing tumor cells, holding great potential for the therapy of gastrointestinal cancers (Wang F. et al., 2021). A study has shown that SJZD enhances the expression levels of death receptor 4 and death receptor 5 through modulation of P53 expression. This increase in receptor levels consequently enhances the sensitivity of colon cancer cells to NK cell-mediated killing, resulting in the inhibition of colon cancer growth (Wang et al., 2024). The liver is a frequent site of metastasis for colon cancer. A study has proved that modified SJZD demonstrates the ability to inhibit liver metastasis in colon cancer by activating the innate immune system (Zhou et al., 2019). These findings offer a potential complementary and alternative therapy for colon cancer.

Ulcerative colitis

UC is a multifaceted, chronic immune-mediated inflammatory bowel disease that typically initiates in the rectum and can progress proximally to affect the entire colon (Feuerstein et al., 2019). Common symptoms exhibited by UC patients include frequent bowel movements, presence of mucus and pus, blood in stool, diarrhea, abdominal pain and discomfort, tenesmus, as well as weight loss. Conventional treatments for UC—such as corticosteroids, aminosalicylates, immunomodulatory agents, and biological therapies—are widely utilized in clinical practice. However, these medications have significant limitations, particularly due to their undesirable side effects (Kucharzik et al., 2020). In TCM, UC is classified under the "dysentery" category based on its symptomatology. Clinical research has shown that TCM granules are superior to placebo in inducing clinical remission and promoting mucosal healing among patients with moderately active and 5-ASA-refractory UC (Shen et al., 2021). Recent studies investigating the mechanisms by which TCM treats UC have been steadily increasing. These studies indicate that TCM can effectively inhibit the onset and progression of UC through its anti-inflammatory and antioxidant properties, while also modulating the body's immune response and other related factors (Zheng et al., 2022).

As a classical Chinese herbal formula, SJZD has been extensively utilized and clinically proven effective in the treatment of UC. Research findings have revealed that SJZD restores the microbial homeostasis and intestinal barrier integrity in UC by inhibiting the abundance of the phylum Proteobacteria and the genus Escherichia-Shigella, alleviating colon tissue damage, and enhancing the expression of tight junction proteins (Wu et al., 2023). The mucosal epithelium plays a crucial role in maintaining the balance of the intestinal ecosystem. Experimental studies have shown that SJZD exhibits a protective effect on the intestinal barrier against TNBS-induced colitis in rats and TNBS-damaged Caco-2 cells *in vitro* (Lu et al., 2017). As an essential substrate for nucleotide synthesis in epithelial cells and a major energy source, l-glutamine is widely used in the treatment of gastrointestinal diseases to alleviate mucositis. Research has demonstrated that the combination of l-glutamine with SJZD is more effective than using l-glutamine alone in ameliorating the severity of diarrhea, histopathological damage, and the disruption of villus and crypt structures in the intestinal mucosa following continuous 5-Fu injections in mice (Qu et al., 2020). As an immune-mediated inflammatory disorder affecting the colon, immune dysregulation plays a crucial role in the pathogenesis of UC. To investigate the impact of SJZD on the immune system in UC, a research study was conducted. The findings demonstrated that SJZD effectively ameliorated the DSS-induced inflammatory response in UC rats by increasing the content of secretory IgA in the intestinal mucosa and elevating the levels of IL-2 in the intestinal tissue (Yu et al., 2016). In summary, SJZD primarily exerts its effect in the treatment of UC by promoting mucosal repair and regulating mucosal immune function.

Pharmacological mechanisms of SJZD for the treatment of gastrointestinal disorders

Over the past few decades, a variety of *in vivo* and *in vitro* experimental studies have enhanced our understanding of the mechanisms underlying the effectiveness of SJZD in treating gastrointestinal disorders. This section offers a summary of the different mechanisms of action, which encompass regulating intestinal flora, reducing inflammation, modulating the immune response, and promoting mucosal repair (Figure 1). Table 1 shows detailed information of the pharmacological studies carried out on SJZD.

Regulating the intestinal flora

Microbiota plays a critical role as an important ecosystem in maintaining health by regulating immunity, metabolism, endocrinology, and providing protection against pathogen invasion. However, the intestinal flora is susceptible to dysbiosis caused by various external and internal factors, resulting in gastrointestinal dysfunction. Thus, improving the gut microbiota is essential for understanding the pathogenesis and treatment of gastrointestinal diseases.

Numerous studies have demonstrated that TCM can positively modulate the intestinal environment by influencing gut microbiota metabolites (Yue et al., 2019). To explore the effects of SJZD on gut microbiota, an analysis of ingredients and reaction pathways was conducted to elucidate its impact on gut microorganisms. The findings revealed a significant increase in the abundance of *Bifidobacterium_pseudolongum* with no observed decrease in the abundance of any other species following the administration of SJZD (Zhang Y. et al., 2022). Moreover, SJZD was utilized to

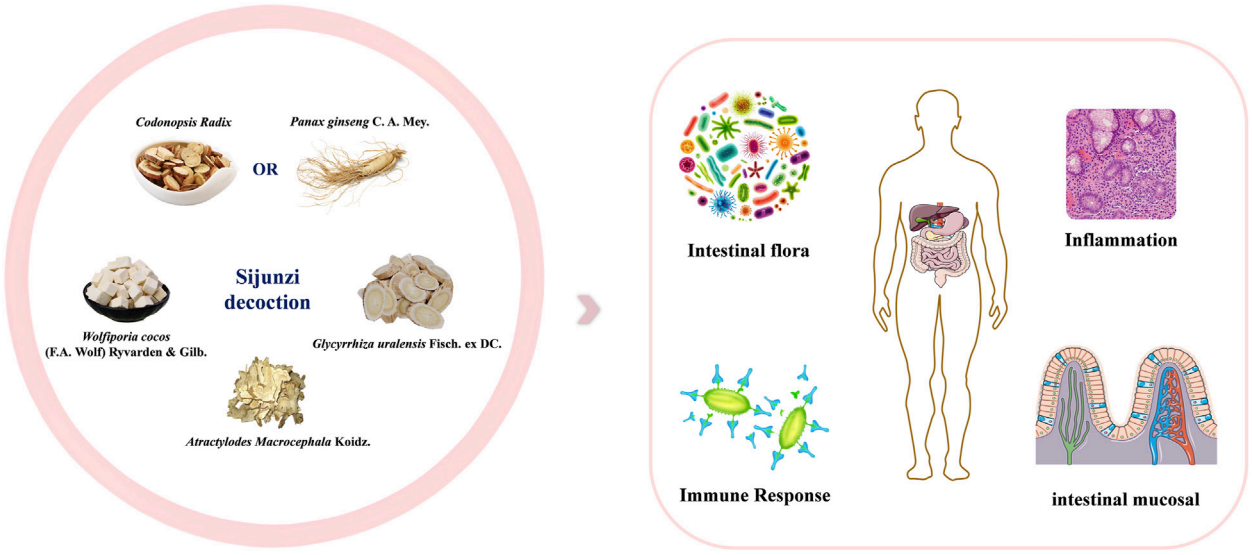


FIGURE 1
The mechanism of action of SJZD in the treatment of gastrointestinal disorders.

investigate its potential anti-diarrheal effects in cases of antibiotic-associated diarrhea. The results revealed that SJZD effectively promoted the growth of beneficial flora by increasing the populations of *Lactobacillus* and *Bifidobacterium*, while simultaneously reducing the abundance of pathogenic *Colibacillus* in the intestines. These actions contributed to alleviating diarrhea symptoms, regulating the gut flora, and maintaining the integrity of intestinal villi (Guo et al., 2022). UC is associated with microbial dysregulation, making the modulation of intestinal flora a critical approach for its treatment. Research has shown that SJZD can reduce inflammation, restore colorectal barrier function, and enhance intestinal permeability in mice with UC by regulating the levels of gut microbiota, specifically *Alistipes*, *Akkermansia*, and Lachnospiraceae_NK4A136_group (Li et al., 2024). The non-polysaccharide components of SJZD, including flavonoids, saponins, and terpenoids, serve as the pharmacodynamic basis for its efficacy. These non-polysaccharides can alleviate gastrointestinal-nervous system dysfunction by modulating the microbiota-gut-metabolites axis, resulting in an increased relative abundance of beneficial probiotics such as *Lactobacillus johnsonii* and *Lactobacillus_taiwanensis* (Dong et al., 2024). These findings suggest that the regulation of intestinal flora is a vital mechanism through which SJZD exerts its therapeutic effects on gastrointestinal diseases.

Anti-inflammation

Inflammation plays a crucial role in the body's immune defense, aiding in the eradication of pathogens, tissue repair, and regeneration (Grivennikov et al., 2010). However, chronic inflammation has been associated with the development and progression of several gastrointestinal disorders, including reflux esophagitis, gastritis, gastrointestinal cancer, inflammatory bowel disease, and diarrhea, among others. Consequently, anti-inflammatory interventions are essential in the treatment of these conditions.

Persistent inflammation of the gastric mucosa is recognized as a crucial contributor to the development and progression of gastritis. Research has demonstrated that SJZD effectively alleviates local gastric inflammation and reduces inflammation in peripheral blood leukocytes, thereby providing relief for chronic gastritis (Wang et al., 2020). Inflammation plays a pivotal role in the development of UC, which is characterized as a form of localized recurrence of intestinal inflammatory disease. A study has revealed that SJZD effectively enhances the inflammatory response induced by DSS in rats with UC. This beneficial effect is achieved through improvements in secretory IgA levels and the IL-2 level in the intestinal tissue (Yu et al., 2016). Subsequent investigations have provided additional evidence that SJZD effectively reduces the expression of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and NO, thereby exerting its anti-inflammatory effect and alleviating ulcerative colitis. Among its components, R1, Rg2, and Rb3 demonstrate the most pronounced impact on the anti-inflammatory action (Kan et al., 2023). Neutrophil extracellular traps contain various inflammatory factors and proteins that play a crucial role in the intestinal immune imbalance associated with UC. These factors act as triggers for inflammatory signaling pathways, contributing to intestinal mucosal inflammation in UC (Drury et al., 2021). SJZD treats UC by reducing the levels of intestinal neutrophil extracellular traps, primarily targeting IL1B and TNF (Zhang et al., 2023). In conclusion, SJZD exhibits promising anti-inflammatory activity, making it a potential candidate for future exploration and discovery of novel anti-inflammatory agents.

Regulating the immune response

Gastrointestinal immune homeostasis plays a vital role in human physiology, as it maintains a delicate equilibrium between immune responses against invading pathogens and tolerance towards symbiotic bacteria. Intestinal diseases, including

TABLE 1 Mechanism of action of SJZD in treatment of gastrointestinal disorders.

Disease	*Codonopsis radix* or *radix ginseng*	Mechanism	Apply styles	References
None	*Codonopsis radix*	increase the abundance of *Bifidobacterium_pseudolongum*	*in vivo*	Zhang et al. (2022b)
Dysbacteriotic diarrhea	*Codonopsis radix*	increase the populations of *Lactobacillus* and *Bifidobacterium*, reduce the abundance of pathogenic *Colibacillus* in the intestines	*in vivo*	Guo et al. (2022)
UC	*Panax ginseng* C. A. Mey	inhibit inflammation, remodel the intestinal barrier, reduce intestinal epithelial permeability by increasing the abundance of *Akkermansia* and Lachnospiraceae_NK4A136_group while decreasing *Bacteroides* and *Helicobacter*	*in vivo*	Li et al. (2024)
Spleen deficiency syndrome	*Panax ginseng* C.A.Mey	increase the abundance of *Lactobacillus johnsonii* and *Lactobacillus_taiwanensis*	*in vivo*	Dong et al. (2024)
Chronic gastritis	*Codonopsis radix*	suppress the local gastric inflammation and inflammations in peripheral blood leukocytes	*in silico*	Wang et al. (2020)
UC	*Panax ginseng* C.A.Mey	ameliorate inflammation by improving the content of sIgA in intestinal mucosa and the IL-2 level in the intestinal tissue	*in vivo*	Yu et al. (2016)
UC	*Panax ginseng* C.A.Mey	exert anti-inflammatory effect by reducing the expression of TNF-α, IL-1β, IL-6, and NO	*in vivo*	Kan et al. (2023)
UC	*Panax ginseng* C.A.Mey	reduce the production of Neutrophil Extracellular Traps through IL1B and TNF	*in vivo*	Zhang et al. (2023)
None	*Panax ginseng* C.A.Mey	improve the TNF-α production and NO production of macrophages	*in vitro*	Gao et al. (2019)
Spleen deficiency syndrome	Not mention	improve immune function of the rat through influencing the JAK-STAT signal pathway	*in vivo*	Xiong and Qian (2013)
Intestinal obstruction	*Panax ginseng* C.A.Mey	improve the immunity system by improving the immunoglobulins, complement components, $CD4^+$ and $CD4^+/CD8^+$ and reducing the TNF-α and $CD8^+$	*in vivo*	Li et al. (2017)
None	*Panax ginseng* C.A.Mey	enhance the phagocytosis and increase the NO production and TNF-α level	*in vitro*	Ji et al. (2017)
Spleen deficiency syndrome	*Panax ginseng* C.A.Mey	increase the expression of T lymphocyte cells and repair the intestinal barrier	*in vivo*	Ma et al. (2021)
Intestinal obstruction	*Panax ginseng* C.A.Mey	regulate the adaptive immune response by reducing the number of $CD3^+$T cells and $CD8^+$T cells while increasing the number of $CD4^+$T cells. promote the recovery of the integrity of the small intestine	*in vivo*	Yu et al. (2014)
Spleen deficiency syndrome	*Panax ginseng* C.A.Mey	repair intestinal epithelium injury through modulation of the FAK/PI3K/Akt signaling pathway	*in vivo* and *in vitro*	Ma et al. (2023)
Wounded intestinal epithelial cells	*Codonopsis radix*	promote intestinal restitution by increasing expression of genes coding for ion channels and transporters	*in vitro*	Liu et al. (2005)
Wounded intestinal epithelial cells	*Panax ginseng* C.A.Mey	promote intestinal epithelial restitution by regulating cellular levels of STIM1 and STIM2	vivo and *in vitro*	Shi et al. (2019)
Intestinal epithelial barrier dysfunction	*Panax ginseng* C.A.Mey and *Codonopsis radix*	attenuates the intestinal barrier dysfunction by inhibiting NF-κB p65-mediated phosphorylation of myosin light chain and myosin light chain kinase	*in vitro*	Lu et al. (2018)

inflammatory bowel disease (Hu et al., 2023), gastrointestinal neoplasms (Mima et al., 2021), and gastritis (Yang and Hu, 2022), are closely associated with immune dysfunction. This association leads to various alterations in immune responses, impaired barrier function, and abnormal activation of immune cells within the gastrointestinal tract.

Spleen deficiency is frequently associated with immune dysfunction. Research indicates that one of the mechanisms by which SJZD exerts its spleen-tonifying and Qi-replenishing effects is through the modulation of immune function, specifically by enhancing the production of TNF-α and NO in macrophages (Gao et al., 2019). To investigate the role of SJZD

in immune regulation, rats were administered SJZD. The results elucidated that SJZD enhances rat immune function by modulating the genetic expression of the JAK-STAT signaling pathway (Xiong and Qian, 2013). In other studies, SJZD was applied for treatment of post-operative ileus. Results demonstrated that SJZD has a moderating effect on immune function by improving the immunoglobulins, complement components, $CD4^+$ and $CD4^+$/$CD8^+$, and reducing the TNF-α and $CD8^+$ (Li et al., 2017). Furthermore, several studies investigated the immunomodulatory effects of polysaccharides present in SJZD. The findings revealed that these polysaccharides significantly enhanced macrophage phagocytic activity, stimulated the production of NO, and elevated the level of TNF-α (Ji et al., 2017). Similarly, another study demonstrated that a specific active polysaccharide known as S-3, found in SJZD, augmented intestinal immunity by upregulating the expression of T lymphocyte cells (Ma et al., 2021). These findings indicate that SJZD can target the immune system among its multiple targets.

Promoting intestinal mucosal restitution

The mucosal barrier assumes a pivotal role as the gastrointestinal tract's primary defense mechanism, safeguarding against an array of dysfunctions (Sánchez de Medina et al., 2014). Impairment to this barrier constitutes a noteworthy driver of gastrointestinal disturbances, encompassing infections, gastrointestinal inflammatory diseases, and tumors (Dahiya and Nigam, 2023; Song et al., 2023). It is essential to preserve the integrity of the mucosal barrier to ensure optimal gastrointestinal function and impede the onset or progression of these disorders.

In a study examining the impact of SJZD on the restoration of intestinal function in a rabbit model following obstruction relief, the application of SJZD yielded significant outcomes. The findings demonstrated that SJZD effectively facilitated the restoration of intestinal function through multiple mechanisms, including reducing intestinal mucosal permeability, enhancing the secretion of intestinal mucins, and promoting the recovery of small intestine integrity (Yu et al., 2014). Furthermore, network pharmacology and experimental studies have demonstrated that the active components of SJZD possess the ability to repair intestinal epithelium injury induced by spleen deficiency syndrome through modulation of the FAK/PI3K/Akt signaling pathway (Ma et al., 2023). Polysaccharides are abundantly present in Chinese herbs and play a significant role in various physiological functions. As the principal component of SJZD, studies have revealed that these polysaccharides can promote intestinal restitution and provide protection against indomethacin-induced damage to intestinal epithelial cells by enhancing the expression of genes coding for ion channels and transporters (Liu et al., 2005). Intestinal epithelial cell migration is a crucial mechanism in the healing process of mucosal wounds. Experimental studies have demonstrated that the polysaccharides present in SJZD exert a positive effect on intestinal epithelial restitution by differentially regulating the cellular levels of STIM1 and STIM2. Specifically, these polysaccharides stimulate the translocation of STIM1, facilitate the association between STIM1 and TRPC1, and decrease the levels of both STIM1 and STIM2 (Shi et al., 2019). Additionally, another study has demonstrated that the polysaccharides in SJZD can alleviate the impairment of the intestinal epithelial cell barrier function induced by TNF-α by inhibiting NF-κB p65-mediated phosphorylation of myosin light chain and myosin light chain kinase (Lu et al., 2018). In summary, SJZD enhances intestinal mucosal restitution through various mechanisms, highlighting its therapeutic potential in gastrointestinal disorders.

Conclusion

Digestive diseases encompass a variety of conditions that have the potential to disrupt the normal digestive process, resulting in a spectrum of health effects that can range from mild to severe. It is crucial to emphasize the significance of early diagnosis, appropriate management, and regular monitoring in order to maintain optimal digestive health. Evidence has shown that TCM provides effective therapeutic outcomes for digestive diseases, with its multi-component, multi-target, and multi-pathway approach to overall regulation. As a classical prescription in TCM, SJZD has achieved good curative effects in gastrointestinal disorders in clinical practice due to its multicomponent and multitarget characteristics. In this review, we provide a comprehensive summary of the clinical research advancements surrounding the utilization of SJZD in the treatment of prevalent digestive system diseases. The therapeutic mechanism of SJZD in addressing these disorders is also analyzed, highlighting its significant role in regulating intestinal flora, mitigating inflammation, modulating the immune response, and facilitating mucosal repair.

Although significant progress has been made in exploring the mechanisms by which SJZD prevents and treats digestive diseases, several shortcomings persist. There are only a limited number of prospective, multi-center, and large-sample controlled studies available. Furthermore, as a compound prescription, current research on SJZD primarily examines its mechanisms of action from the perspective of the entire formula or analyzes the effects of individual Chinese herbs and herbal monomers. However, there is a notable lack of experimental studies investigating the interactions among the various components. Given the importance of SJZD in managing gastrointestinal disorders, further investigation into its clinical effects and underlying mechanisms is crucial. Future research should prioritize the clinical translation of SJZD, providing new insights and references for its application in the prevention and treatment of gastrointestinal inflammatory diseases, functional disorders, and tumors. Such efforts will enhance our understanding and optimize the therapeutic use of SJZD in gastrointestinal diseases.

Author contributions

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Formal Analysis, Investigation, Writing–review and editing. FL: Conceptualization, Funding acquisition, Investigation, Visualization, Writing–review and editing.

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Conflict of interest

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

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EDITED BY
Daniele Maria-Ferreira,
Instituto de Pesquisa Pelé Pequeno Príncipe, Brazil

REVIEWED BY
Hanan Salah El-Abhar,
Cairo University, Egypt
Marwan Almoiliqy,
University of Texas MD Anderson Cancer Center, United States

*CORRESPONDENCE
Tongqing Xue,
vh6376@163.com
Zhongzhi Jia,
jiazhongzhi.1998@163.com

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Paricalcitol alleviates intestinal ischemia-reperfusion injury via inhibition of the ATF4-CHOP pathway

Jiawei Zhang[1], Tingting Liu[2], Tongqing Xue[3]* and Zhongzhi Jia[1]*

[1]Department of Interventional and Vascular Surgery, The Third Affiliated Hospital of Nanjing Medical University (Changzhou Second People's Hospital), Changzhou, China, [2]Graduate College of Dalian Medical University, Dalian, China, [3]Department of Interventional Radiology, Huaian Hospital of Huai'an City (Huaian Cancer Hospital), Huai'an, China

Introduction: Intestinal ischemia reperfusion (I/R) injury is a severe condition characterized by inflammation, oxidative stress, and compromised intestinal barrier function, which can lead to death. This study investigated the effects of paricalcitol, a synthetic vitamin D receptor (VDR) agonist, on intestinal I/R injury, focusing on the activating transcription factor 4 (ATF4)-C/EBP homologous protein (CHOP) signaling pathway and the modulation of endoplasmic reticulum stress (ERS).

Methods: This study consists of both *in vivo* and *in vitro* experiments. *In vivo* experiment, a mouse model of intestinal I/R injury was established by clamping the superior mesenteric artery, and followed by 24 or 72 h of reperfusion. 6-week-old male C57BL/6 J mice were randomly assigned to six groups: sham, I/R 24h, I/R 72 h, and their respective paricalcitol-treated counterparts. VDR knockout mice and wild-type mice were assigned to WT, VDR-KO, WT + I/R and VDR-KO + I/R groups. The paricalcitol-treated groups received oral gavage of paricalcitol (0.3 μg/kg) once daily for 5 days before I/R. *In vitro*, IEC-6 cells were incubated in a microaerophilic system (5% CO_2, 1% O_2, 94% N_2) for 6 h to induce hypoxia. The cells were then transferred to complete medium with or without paricalcitol (200 nM) and cultured under normoxic conditions for 24 h to establish the hypoxia/re-oxygenation (H/R) model and investigate the protective effects of paricalcitol on H/R-induced injury in cells. We further utilized VDR- and ATF4-silenced cells to examine how paricalcitol regulates the expression of VDR, ATF4, and CHOP.

Results: We demonstrated that protective paricalcitol treatment reduces ERS and apoptosis by activating VDR and inhibiting the ATF4-CHOP pathway, thereby alleviating intestinal I/R injury *in vivo* and H/R injury *in vitro*. Furthermore, experiments with VDR knockout mice demonstrated that the absence of VDR exacerbated I/R injury, underscoring the protective role of VDR in intestinal epithelial cells.

Discussion: These findings suggest that the protective effects of paricalcitol may offer a promising therapeutic strategy for managing intestinal I/R injury.

KEYWORDS

intestinal, ischemia reperfusion injury, paricalcitol, ATF4, CHOP, VDR (vitamin D receptor)

Introduction

Intestinal ischemia reperfusion (I/R) injury is a life-threatening condition that is typically caused by ischemic or septic shock in patients with acute mesenteric ischemia (Reintam et al., 2024). Intestinal I/R injury is a complex condition marked by the excessive release of inflammatory cytokines and oxidative stress, leading to epithelial cell death and compromised intestinal barrier function. This disruption results in increased intestinal permeability and reduced nutrient absorption, which can facilitate the passage of macromolecules (Wang et al., 2024). Currently, there is no established clinical treatment for intestinal I/R injury.

Recent research has focused on the role of endoplasmic reticulum stress (ERS) and the activating transcription factor 4 (ATF4)-C/EBP homologous protein (CHOP) pathway in the pathogenesis of I/R injury (Tang et al., 2023; Zhao et al., 2024). ERS occurs when there is an excessive accumulation of unfolded or misfolded proteins in the endoplasmic reticulum. Excessive ERS can trigger inflammation and eventually lead to programmed cell death (Stengel et al., 2020). ATF4, a member of the activating transcription factor family, plays a crucial role in gene regulation (Chen et al., 2022). Under normal conditions, its expression is low but significantly upregulated upon stimulation (Gachon et al., 2001). Previous studies have shown that ATF4 plays a key role in apoptosis and regulates various physiological processes, including amino acid metabolism, redox homeostasis, and mitochondrial function (Wei et al., 2021; Ohoka et al., 2005). As a key effector of ERS, ATF4 regulates downstream genes involved in apoptosis, inflammation, and oxidative stress (Steiger et al., 2004). CHOP, another critical mediator of ERS-induced cell death, is upregulated by ATF4. Their overexpression enhances oxidative stress and cell death (Han et al., 2013). Notably, inhibition of ATF4-CHOP signaling has been shown to reduce mitophagy, ERS, apoptosis and ischemia-reperfusion injury (Tang et al., 2023; Chen et al., 2022; Chen et al., 2024).

The vitamin D receptor (VDR) is a nuclear transcription factor that is widely present in cells of various tissues and is highly expressed in the intestines (Hamza et al., 2023). Recent research has shown that VDR has immunoregulatory effects, promotes the differentiation and proliferation of intestinal tissues, and plays an important role in maintaining the normal barrier function of intestinal epithelial cells (Sun and Zhang, 2022). Additionally, experimental evidence has suggested a close association between VDR and I/R injury in organs such as the heart, liver, brain, and kidneys (Qian et al., 2019; Wu et al., 2023; Kim et al., 2017). Numerous studies have demonstrated that activating VDR can inhibit ERS (Zhou et al., 2020; Haas et al., 2016; Ahmad et al., 2022), and activation of VDR has been found to alleviate I/R-induced renal injury by suppressing ERS, partly through transcriptional regulation of the ATF4/CHOP pathway (Tang et al., 2023). However, no studies to date have demonstrated whether paricalcitol can mitigate intestinal I/R injury or whether the ATF4-CHOP pathway plays a role in the progression of this injury.

The aim of this study was to evaluate the effects of paricalcitol, a synthetic VDR agonist, in an intestinal I/R model, focusing on the ATF4-CHOP signaling pathway.

Materials and methods

Animals

All animals in this study were treated in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. And all procedures were approved by the animal ethics committee of Changzhou Second People's Hospital (Permit Number: 2024KY206-01).

C57BL/6 mice (6 weeks old, 16–21 g) (Huachuang Sino Tech Ltd., Nanjing, China) and VDR knockout (VDR-KO) mice (6 weeks old, 16–21 g) (Gem Pharma Tech Ltd., Nanjing, China) were used for the *in vivo* studies. Because of the salutary effect of estrogen, female mice are more resistant to intestinal I/R injury than male mice (Chai et al., 2019; Ricardo-da-Silva et al., 2017); therefore, only male mice were used. Animals were housed in ventilated cages at a temperature of 20°C–24°C, with relative humidity (40%–70%) and a 12-h light/dark cycle. The mice had free access to food and water. All of the mice were allowed to adapt to this environment for 1 week before any experiments were conducted.

Intestinal I/R injury model and experimental groups

The intestinal I/R injury models were established as described previously (Wen et al., 2019; Jia et al., 2017). Briefly, the mice were anesthetized with 2% pentobarbital sodium (0.3 mg/10 g) via intraperitoneal injection. A midline incision was made, and the superior mesenteric artery (SMA) was clamped with a microvascular clip for 45 min to induce ischemia. The clip was then removed and the incision was sutured, and this was followed by 24 or 72 h of reperfusion. Paricalcitol (HY-50919, MCE, Princeton, NJ, United States) and corn oil were administered once daily for five consecutive days before surgery, according to the specific treatment groups.

The C57BL/6 mice were randomly assigned to one of six groups (n = 6 per group): sham or paricalcitol groups, in which mice underwent laparotomy without SMA occlusion and received oral gavages of corn oil (0.5 mL/10 g) or paricalcitol (0.3 μg/kg) once daily for 5 days before the procedure; I/R 24 h or I/R 72 h groups, in which mice underwent 45 min of ischemia followed by 24 or 72 h of reperfusion, and were given corn oil (0.5 mL/10 g) before the procedure; or paricalcitol + I/R 24 h or paricalcitol + I/R 72 h groups, in which mice underwent ischemia and 24 or 72 h of reperfusion, with paricalcitol (0.3 μg/kg) administered once daily for 5 days before the procedure based on data from previous studies (Hong et al., 2017).

The VDR-KO mice were randomly divided into two groups (n = 6 per group): an VDR-KO + I/R group, in which the VDR-KO mice underwent 45 min of ischemia followed by 72 h of reperfusion; and an VDR-KO group, which did not undergo ischemia or reperfusion. Similarly, wild-type (WT) mice were divided into corresponding groups including a WT group and a WT + I/R group, following the same protocol as the VDR-KO mice.

After reperfusion, the mice were euthanized via exsanguination under isoflurane anesthesia, followed by cervical dislocation. Two 0.5-cm segments of the ileum were collected 10 cm proximal to the

terminal ileum. One segment was fixed in 10% formalin and embedded in paraffin for histopathological evaluation, while the other was preserved in electron microscope fixative (glutaraldehyde, 4%) for subsequent transmission electron microscopy analysis. Additionally, a 10-cm segment was washed with PBS, dried, and stored at −80°C for further biochemical analysis.

Biochemical analysis

The intestinal tissues were homogenized in ice-cold normal saline. The homogenates were then centrifuged at 3500 *g* at 4°C for 20 min. The supernatant fraction of the intestinal homogenates was collected, and the levels of superoxide dismutase (SOD) and glutathione (GSH) were determined using ELISA kits (A001-three to two, A006-one to one, Jianchen, Nanjing, China).

Histological and immunohistochemistry (IHC) analysis

The isolated ileum segments were fixed in 4% paraformaldehyde for at least 24 h and then embedded in paraffin and sectioned into 4-μm-thick slices. The segments were stained with hematoxylin and eosin (H&E) for histological analysis. The H&E-stained images were assessed using a digital pathology scanner (KFBIO, Zhejiang, China) and evaluated at ×5 magnification. The images were scored by three pathologists blinded to this research according to the methods of Chiu et al. (1970). The Chiu scoring criteria are as follows: 0 – normal intestinal mucosal villi morphology; one – expansion of the subepithelial Gruenhagen's space in the villous core, capillary congestion, and epithelial damage; 2 – further expansion of the subepithelial space with significant separation between the epithelial layer and the lamina propria; three – increased subepithelial space with occasional denuded villous tips; 4 – severe villous damage and denudation, accompanied by capillary dilation in the lamina propria; 5 – ulceration and hemorrhage of the lamina propria.

For IHC analysis, the ileum segments were deparaffinised with xylene and were then hydrated in serial dilutions of alcohol. The sections were immersed in 3% hydrogen peroxide solution to inhibit endogenous peroxidase activity and were then incubated with antibodies against the tight junction protein zonula occludens-1 (ZO-1) (82870-7-RR, Proteintech, Wuhan, China; 1:100) or VDR (12550S, Cell Signaling Technology, Danvers, MA, United States; 1:100). This was followed by the addition of anti-rabbit immunoglobulin and streptavidin conjugated to horseradish peroxidase. The ileum segments were then stained with 3,3′-diaminobenzidine (DAB) and hematoxylin for counter staining. A digital pathology scanner was used to assess the segments. The protein expression levels and histological changes were evaluated at ×10 magnification.

Transmission electron microscopy

The intestinal tissues were placed in electron microscope fixative (glutaraldehyde, 4%) at 4°C. The tissue was then embedded and cut into ultrathin sections of 60–80 nm. This was followed by uranium-lead double staining. The morphology of the endoplasmic reticulum in the intestinal epithelial cells was observed using transmission electron microscopy.

Cell culture and hypoxia/re-oxygenation (H/R) model

IEC-6 cells (Pricella, Wuhan, China) were cultured in DMEM with 10% fetal bovine serum. Paricalcitol treatment concentration (200 nM) was determined based on a previous study by Tang et al. (2023). The experiment consisted of three phases. In the first phase, cells were divided into control, paricalcitol, H/R, and H/R + paricalcitol groups. Cells of the HR group were incubated in a microaerophilic system (Thermo Fisher Scientific, Waltham, MA, United States) with 5% CO_2 and 1% O_2 and balanced with 94% N_2 for 6 h, followed by 24 h of reoxygenation, while the H/R + paricalcitol group was reoxygenated in complete medium containing paricalcitol (200 nM) for 24 h. In the second phase, cells were assigned to control, siVDR, siVDR + H/R, and siVDR + H/R + paricalcitol groups. In the siVDR group, cells were transfected with siVDR using Lipofectamine 3000 (L3000015, Invitrogen, Carlsbad, CA, United States), incubated with 50 nM siRNA for 6 h, and then cultured in complete medium with or without paricalcitol (200 nM) for 24 h. Hypoxia and reoxygenation conditions were identical to those in the first phase. In the third phase, siATF4 replaced siVDR, forming control, siATF4, siATF4 + H/R, and siATF4 + H/R + paricalcitol groups, with experimental conditions identical to those in the second phase.

Cell counting Kit-8 assays

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) (CK04, Dojindo, Japan) following the manufacturer's instructions. IEC-6 cells were seeded in 96-well plates (5,000 cells/well), and 10 μL of CCK-8 reagent was added to each well. After 1 h of incubation at 37°C, absorbance was measured at 450 nm. Data were analyzed using GraphPad Prism 5.0 (GraphPad Prism Software, San Diego, CA, United States).

TUNEL assay

Apoptotic cells were detected using the TUNEL assay kit (E-CK-A320, Elabscience, Wuham, China), following the manufacturer's instructions. IEC-6 cells were seeded in 24-well plates. After treated, cells were fixed with 4% paraformaldehyde at room temperature for 30 min, then incubated with a terminal deoxynucleotidyl transferase reaction mixture at 37°C for 1 h. After washing with PBS, the cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and observed under a fluorescence microscope.

Western blotting

Total protein was extracted from the intestinal mouse tissues and from the IEC-6 cells. Proteins were separated on 10% SDS-

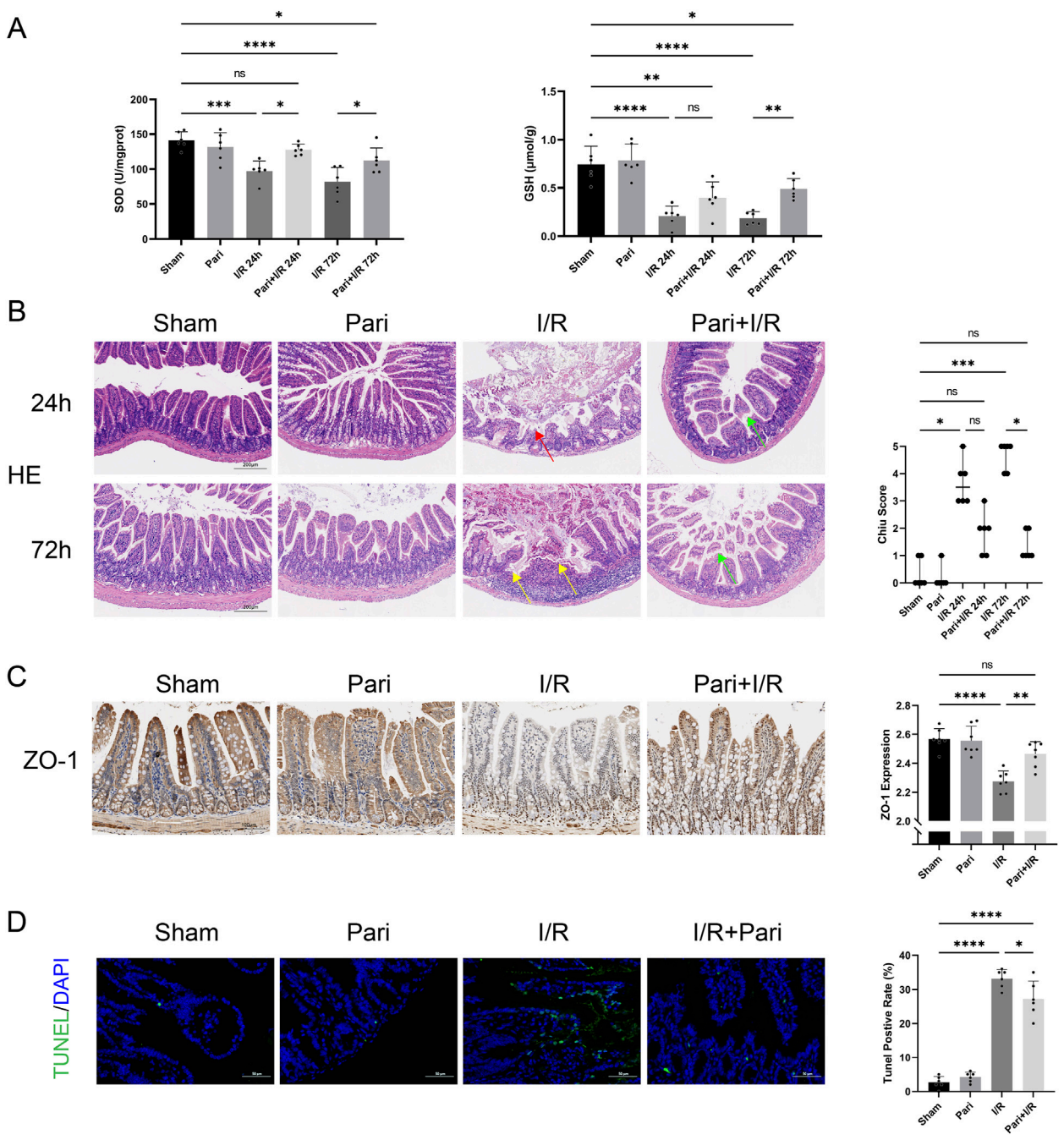

FIGURE 1
Paricalcitol alleviates intestinal injury and apoptosis caused by ischemia reperfusion (I/R) **(A)** Levels of superoxide dismutase (SOD) and glutathione (GSH) in intestinal tissues across various groups (n = 6 in each group). **(B)** Representative images of intestinal hematoxylin and eosin (H&E) staining results and Chiu scores. Red arrow represents severe villous denudation, yellow arrow represents severe villous denudation and destruction to the lamina propria, green arrow represents mild denudation of the villous tips. (scale bar = 200 μm; n = 6 in each group). **(C)** Representative immunohistochemistry results and expression analysis zonula occludens-1 (ZO-1) in intestinal tissues after 72 h reperfusion (scale bar = 100 μm; n = 6 in each group). **(D)** Representative TUNEL staining (green) and nuclear staining (blue) results and apoptosis analysis of intestinal tissues after 72 h reperfusion (scale bar = 50 μm; n = 6 in each group). Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test for parametric data and the Kruskal–Wallis test followed by Dunn's *post hoc* test for non-parametric data. Parametric data are presented as mean ± SD, while non-parametric data are presented as median (min-max). *, P values <0.05; **, *P* values <0.01; ***, *P* values <0.001; ****, *P* values <0.0001.

PAGE gels and were then transferred to PVDF membranes (Immobilon, Darmstadt, Germany). The membranes were blocked with fast blocking buffer (Servicebio, Wuhan, China) and incubated overnight with primary antibodies, including VDR (12550S, Cell Signaling Technology, Danvers, MA, United States), ATF4 (28657-1-AP, Proteintech, Wuhan, China), CHOP (15204-1-AP, Proteintech, Wuhan, China) and β-actin (HRP-81115, Proteintech, Wuhan, China). After three washes, they were

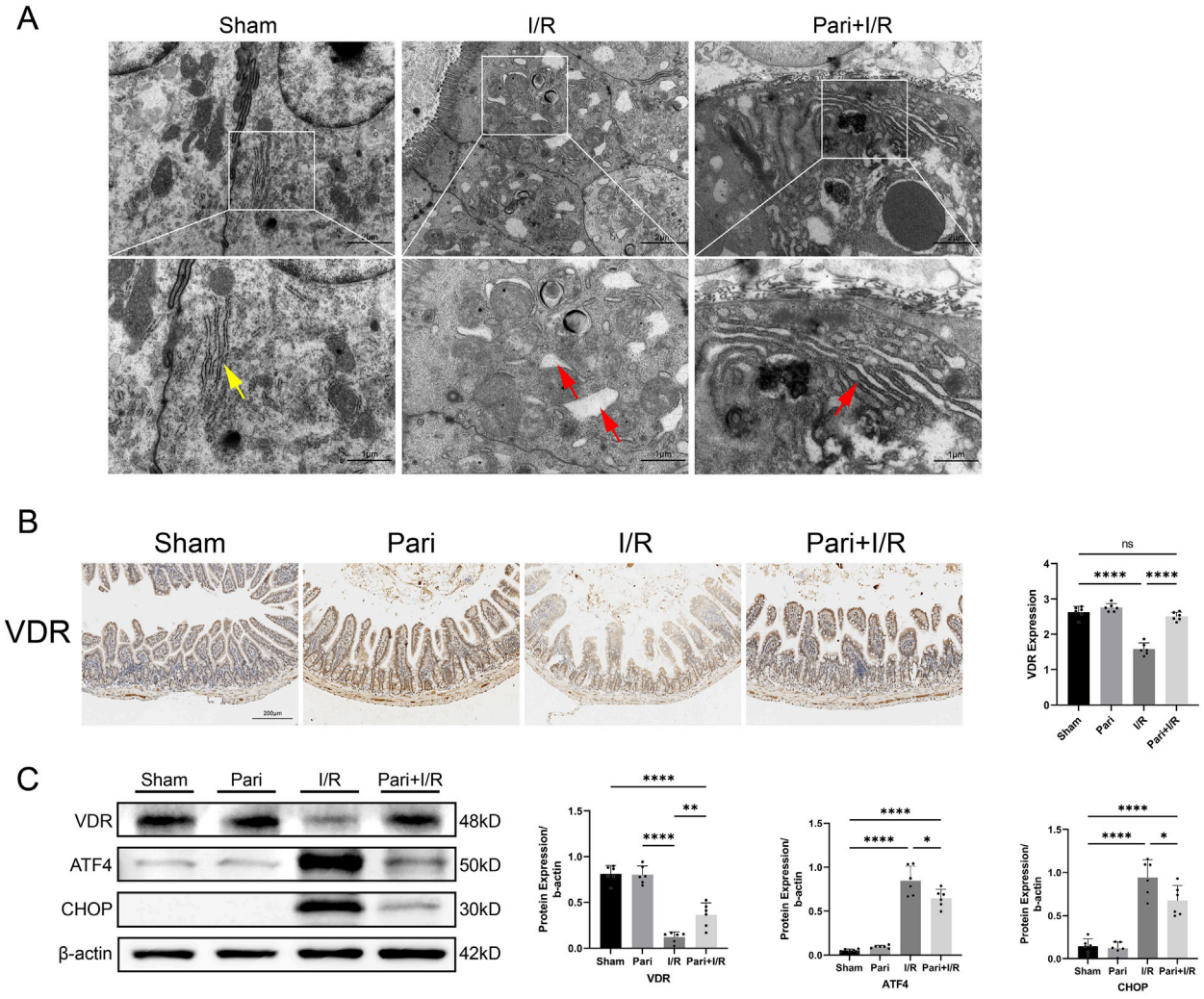

FIGURE 2
Paricalcitol alleviates endoplasmic reticulum stress induced by intestinal ischemia reperfusion (I/R) injury through the activation of vitamin D receptor (VDR) **(A)** Representative transmission electron microscopy images showing endoplasmic reticulum damage in intestinal epithelial cells. Yellow arrows represent normal endoplasmic reticulum, red arrows represent endoplasmic reticulum changes (scale bar = 2 or 1 μm; n = 3 in each group). **(B)** Representative immunohistochemistry results for vitamin D receptor (VDR) expression in intestinal tissues and corresponding expression level analysis (scale bar = 200 μm; n = 6 in each group). **(C)** Western blot analysis and densitometric quantification of VDR, activating transcription factor 4 (ATF4), and C/EBP homologous protein (CHOP) expression levels (n = 6 in each group). Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. All data are presented as mean ± SD. *, P values <0.05; **, *P* values <0.01; ****, *P* values <0.0001.

incubated with a secondary antibody (SA00001-2, Proteintech, Wuhan, China) for 1 h. Protein detection was performed using an enhanced chemiluminescence (ECL) system, and quantification was performed using ImageJ, normalized to β-actin.

Statistical analysis

Parametric data with normal distributions were expressed as mean ± standard deviation (SD) and analyzed using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls (SNK) test. Non-parametric data were analyzed using the Kruskal–Wallis test, followed by Dunn's *post hoc* test, and presented as median ± range (minimum–maximum). All experimental results were obtained from at least three independent experiments. Statistical analysis was performed using GraphPad Prism 5.0. Statistical significance was inferred at *P* values <0.05.

Results

Paricalcitol alleviated intestinal I/R injury

In the intestinal I/R injury model, reperfusion for 24h and 72 h reduced average SOD activity to 69% and 57% of the sham group, respectively. However, with paricalcitol pretreatment, SOD activity decreased only to 90% and 79% of the original levels, showing a significant increase compared to untreated mice ($P < 0.05$). Similarly, paricalcitol pretreatment significantly opposed the

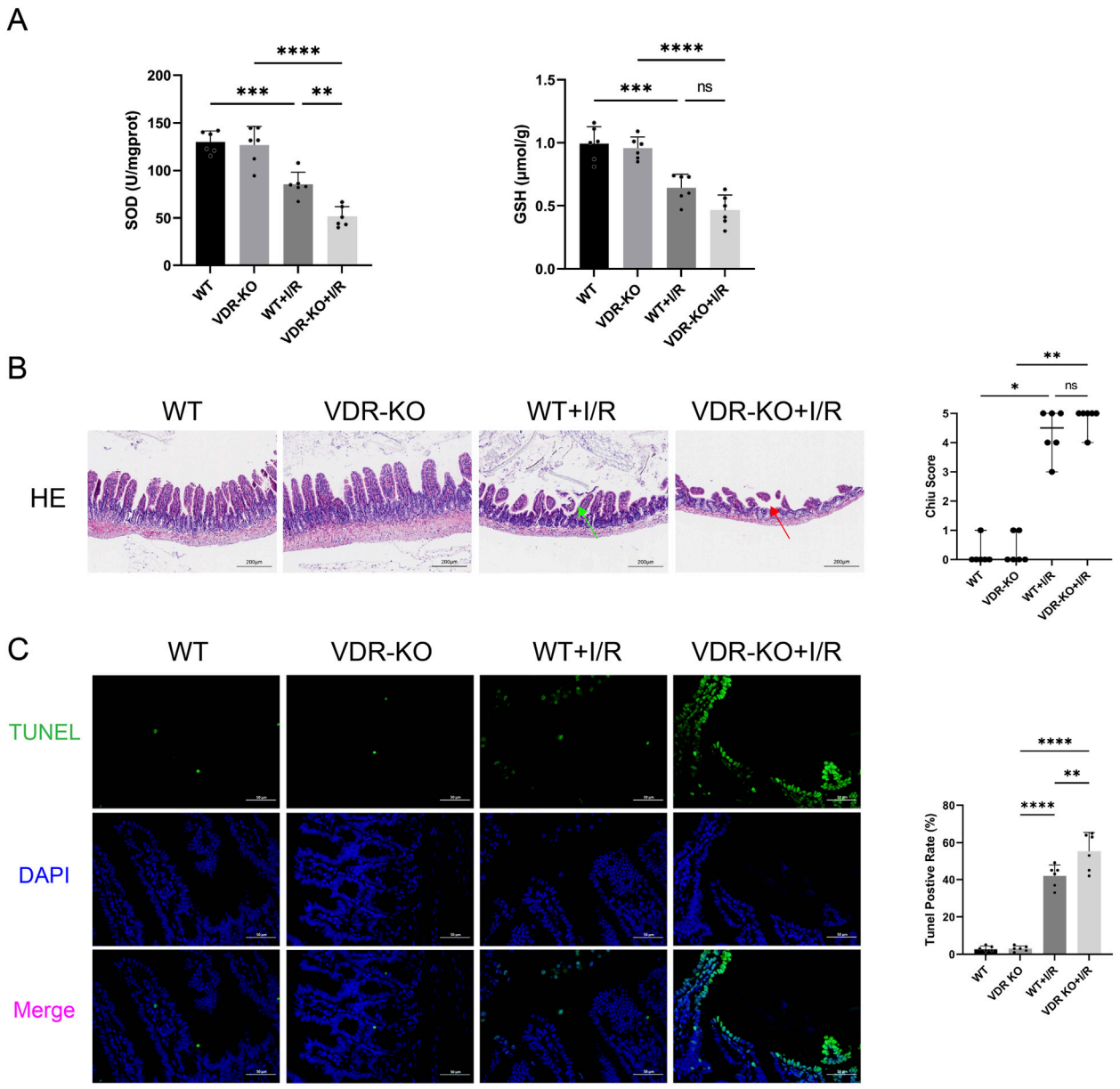

FIGURE 3
Vitamin D receptor (VDR) knockout (KO) exacerbates intestinal ischemia reperfusion (I/R) injury and cell apoptosis **(A)** Levels of superoxide dismutase (SOD) and glutathione (GSH) in intestinal tissues of VDR-KO mice (n = 6 in each group). **(B)** Representative images of intestinal hematoxylin and eosin , (H&E) staining results and Chiu scores for VDR-KO mice. Red arrow represents severe villous denudation, green arrow represents mild denudation of the villous tips (scale bar = 200 μm; n = 6 in each group). **(C)** Representative TUNEL staining (green) and nuclear staining (blue) results and apoptosis analysis in intestinal tissues of VDR-KO mice (scale bar = 50 μm; n = 6 in each group). Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test for parametric data and the Kruskal–Wallis test followed by Dunn's *post hoc* test for non-parametric data. Parametric data are presented as mean ± SD, while non-parametric data are presented as median (min-max). *, P values <0.05; **, *P* values <0.01; ***, *P* values <0.001; ****, *P* values <0.0001.

I/R-induced reduction in GSH levels, increasing GSH levels by 2.7-fold after 72 h of reperfusion compared to the I/R 72 h group ($P < 0.01$). Similar results were observed in the assessments of H&E-stained images and Chiu scores. In the I/R 24 h group, severe mucosal epithelial detachment was observed, while in the I/R 72 h group, more severe epithelial necrosis and hemorrhage were noted, leading to an increase in the Chiu scores ($P < 0.01$). However, pretreatment with paricalcitol significantly alleviated intestinal epithelial injury, with only mild mucosal epithelial detachment. Furthermore, there was no significant difference in the Chiu scores compared to the sham group (Figure 1B). IHC analysis demonstrated that paricalcitol significantly mitigated the loss of ZO-1 caused by I/R injury in intestinal tissue and restored ZO-1 expression to normal levels (Figure 1C). Additionally, TUNEL assay indicated that paricalcitol significantly reduced I/R-induced intestinal cell apoptosis. Compared to the I/R group, paricalcitol pretreatment reduced the apoptosis rate of intestinal epithelial cells by 18% ($P < 0.05$) (Figure 1D).

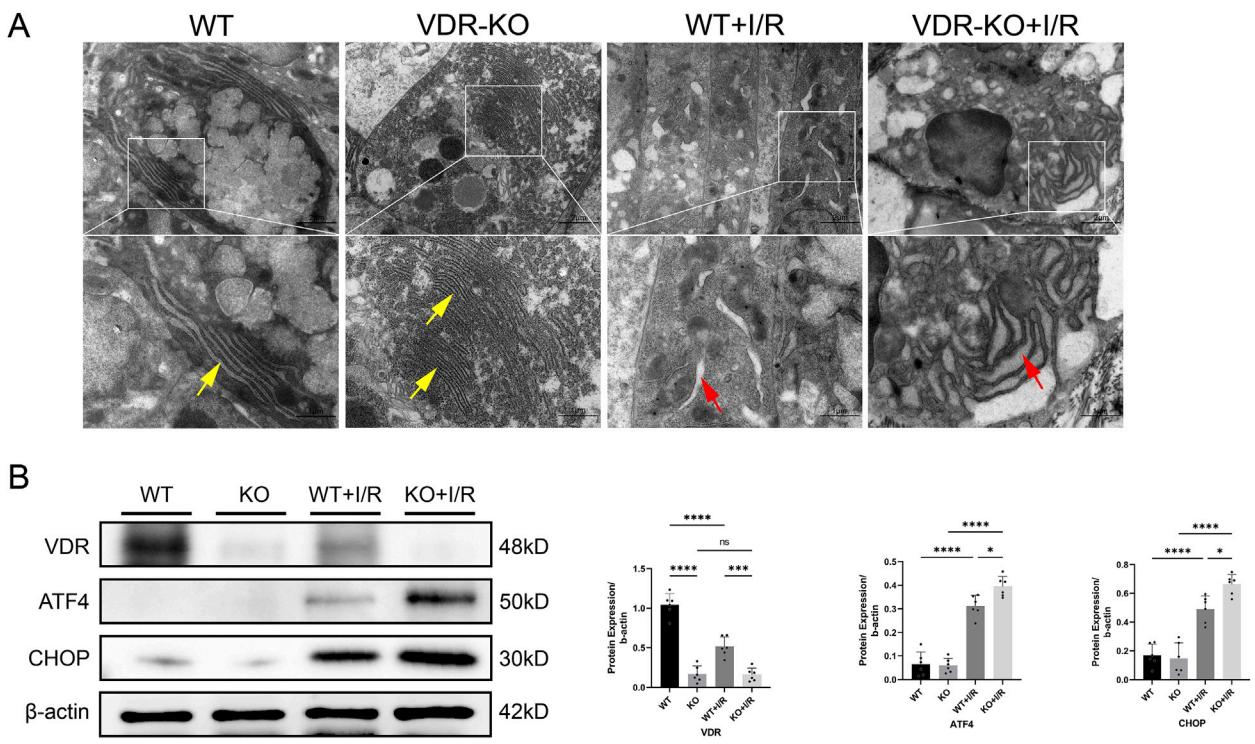

FIGURE 4
Vitamin D receptor (VDR) knockout (KO) exacerbates intestinal ischemia-reperfusion (I/R) injury by inducing endoplasmic reticulum stress (ERS) **(A)** Representative transmission electron microscopy images showing endoplasmic reticulum damage in intestinal epithelial cells of VDR-KO mice. Yellow arrows represent normal endoplasmic reticulum, red arrows represent endoplasmic reticulum changes (scale bar = 2 or 1 μm; n = 3 in each group). **(B)** Western blot analysis and densitometric quantification of VDR, activating transcription factor 4 (ATF4), and C/EBP homologous protein (CHOP) expression levels (n = 6 in each group). Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. All data are presented as mean ± SD. *, P values <0.05; **, *P* values <0.01; ***, *P* values <0.001; ****, *P* values <0.0001.

Paricalcitol inhibited I/R-induced ERS in intestinal mucosal epithelial cells through the activation of VDR

On transmission electron microscopy, I/R was seen to cause swelling and rupture of the endoplasmic reticulum in intestinal mucosal epithelial cells, which was alleviated by pretreatment with paricalcitol. After pretreatment, only mild swelling of the endoplasmic reticulum was observed (Figure 2A). IHC analysis demonstrated that paricalcitol pretreatment restored the intestinal VDR protein expression level, which was reduced by I/R injury, to normal levels (Figure 2B). Western blotting similarly demonstrated that paricalcitol mitigated the I/R-induced downregulation of VDR expression and inhibited the upregulation of ATF4 and CHOP (Figure 2C).

VDR-KO aggravated intestinal I/R injury and ERS

We found that in VDR-KO mice, I/R injury reduced intestinal SOD activity to only 40% of the original level, while in WT mice, the reduction was to 65%. The absence of VDR significantly exacerbated the decrease in SOD activity caused by I/R injury ($P < 0.01$) (Figure 3A). H&E staining indicated that intestinal damage caused by I/R could be more severe in VDR-KO mice than in WT mice, although the difference was not statistically significant (Figure 3B). TUNEL analysis revealed that the apoptosis rate of intestinal epithelial cells in the VDR-KO + I/R group was elevated by 31% compared to the WT + I/R group ($P < 0.01$) (Figure 3C).

Electron microscopy analysis showed that, compared to the WT + I/R group, the VDR-KO + I/R group exhibited more pronounced endoplasmic reticulum swelling, rupture, and loss of normal structure (Figure 4A). This suggests that VDR knockout may exacerbate intestinal I/R injury by intensifying I/R-induced ERS. Western blotting results indicated that VDR deficiency increased the accumulation of ATF4 and CHOP proteins (Figure 4B).

Paricalcitol mitigated H/R injury in IEC-6 cells

TUNEL assay demonstrated that the rate of apoptotic cells in the paricalcitol-treated H/R group was reduced by 28% compared to the H/R group without paricalcitol treatment ($P < 0.05$) (Figure 5A). CCK-8 assay demonstrated that H/R injury reduced the viability of IEC-6 cells to 35% of the control group ($P < 0.0001$). However, with paricalcitol treatment, cell viability was maintained at 54% of the control level, indicating that paricalcitol significantly mitigated the H/R-induced decline in cell viability ($P < 0.05$) (Figure 5B), and Western blotting indicated that H/R led to downregulation of VDR expression and upregulation of ATF4 and CHOP in IEC-6 cells, which were reversed by paricalcitol pretreatment (Figure 5C).

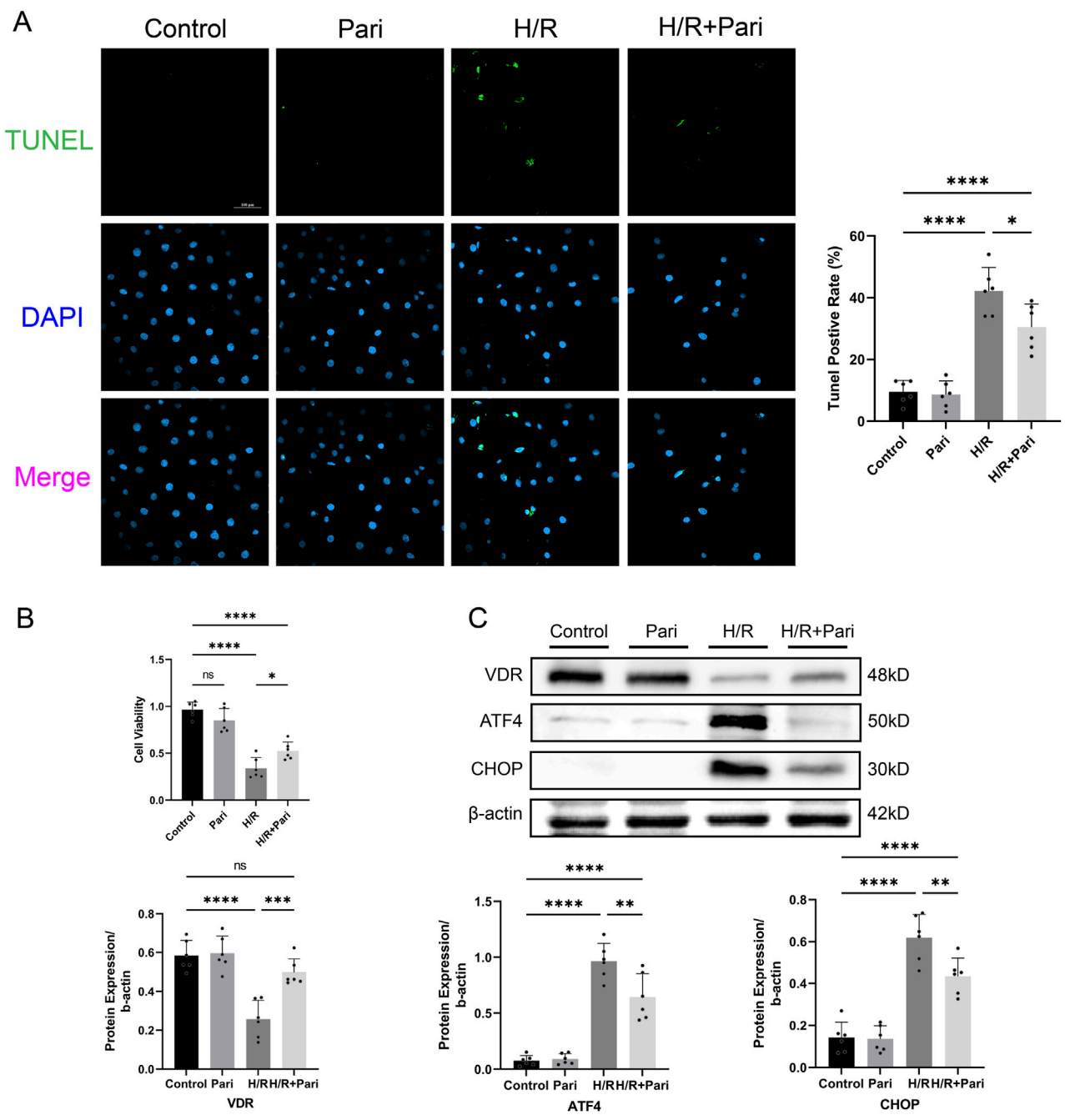

FIGURE 5
Paricalcitol mitigates hypoxia-reoxygenation (H/R) injury in IEC-6 cells **(A)** Representative TUNEL staining (green) and nuclear staining (blue) results and apoptosis analysis of IEC-6 cells (scale bar = 100 μm; n = 6 in each group). **(B)** Differences in IEC-6 cell viability among groups assessed using the cell counting kit-8 assay (n = 6 in each group). **(C)** Western blot analysis and densitometric quantification of vitamin D receptor (VDR), activating transcription factor 4 (ATF4), and C/EBP homologous protein (CHOP) expression levels in IEC-6 cells (n = 6 in each group). Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. All data are presented as mean ± SD. *, P values <0.05; **, *P* values <0.01; ***, *P* values <0.001; ****, *P* values <0.0001.

Silencing VDR or ATF4 abolished the protective effect of paricalcitol in IEC-6 cells

CCK-8 assay results indicated that after H/R injury, cell viability in the VDR-silenced and non-silenced groups decreased by 2.3-fold and 4.8-fold, respectively, compared to the control group ($P < 0.0001$). However, there was no significant difference between the H/R group and siVDR + H/R groups. In the siVDR + H/R group, subsequent treatment with paricalcitol did not restore the H/R-induced decrease of cell viability (Figure 6A). This contrasts with the protective effect observed with paricalcitol treatment in Figure 5B. Western blotting results showed that after siVDR treatment, ATF4 and CHOP expression remained similar regardless of paricalcitol treatment, indicating that silencing VDR abolished the effect of paricalcitol

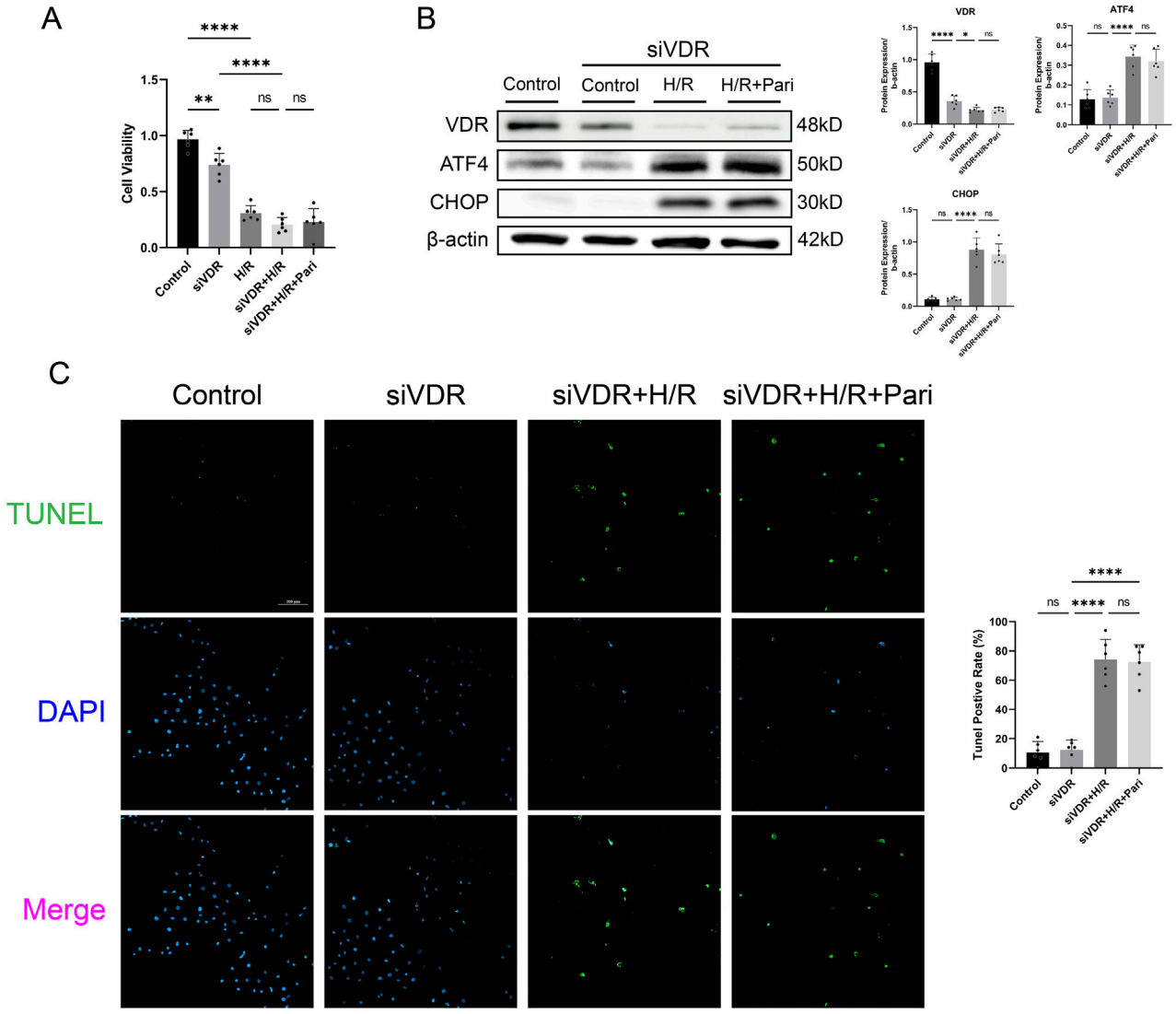

FIGURE 6
Silencing vitamin D receptor (VDR) abolished the protective effect of paricalcitol in IEC-6 cells **(A)**The effect of siVDR treatment on cell viability assessed using cell counting kit-8 assay in IEC-6 cells (n = 6 in each group). **(B)** Western blot analysis and densitometric quantification of VDR, ATF4, and CHOP expression levels in IEC-6 cells after siVDR treatment (n = 6 in each group). **(C)** Representative TUNEL staining (green) and nuclear staining (blue) results for IEC-6 cells after siVDR treatment (scale bar = 200 μm; n = 6 in each group). Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. All data are presented as mean ± SD. *, P values <0.05; **, P values <0.01; ***, P values <0.001; ****, P values <0.0001.

(Figure 6B). TUNEL staining yielded similar results, showing that silence VDR abolished the protective effect of paricalcitol against cell apoptosis (Figure 6C).

CCK-8 assay showed that H/R treatment significantly reduced cell viability in both ATF4-silenced and non-silenced cells, by 3-fold and 1.6-fold, respectively, compared to the control group ($P < 0.0001$). Compared to the H/R group, cell viability in the siATF4 + H/R group increased by 1.8-fold, indicating that silencing ATF4 alleviated the reduction in cell viability caused by H/R injury ($P < 0.001$). However, paricalcitol treatment was unable to further enhance cell viability (Figure 7A). Western blotting results showed that after ATF4 silencing, H/R increased CHOP expression, while paricalcitol treatment did not significantly alter CHOP expression (Figure 7B). This suggests that the protective effect of paricalcitol was suppressed after ATF4 silencing. Likewise, TUNEL staining showed that after ATF4 silencing, paricalcitol treatment did not significantly affect cell apoptosis levels (Figure 7C).

Discussion

In this study, we found that paricalcitol alleviates intestinal I/R injury by activating VDR signaling. Paricalcitol markedly downregulated ATF4 and CHOP expression, thereby mitigating ERS, apoptosis, and intestinal barrier damage caused by I/R injury (Figure 8). Conversely, VDR knockout worsened intestinal I/R injury and increased ATF4 and CHOP levels. *In vivo* experimental results demonstrated that paricalcitol significantly alleviated H/R-induced injury in IEC-6 cells, activated VDR, and reduced ATF4 and CHOP protein expression. Notably, we observed that VDR silencing led to a marked decrease in cell viability and an

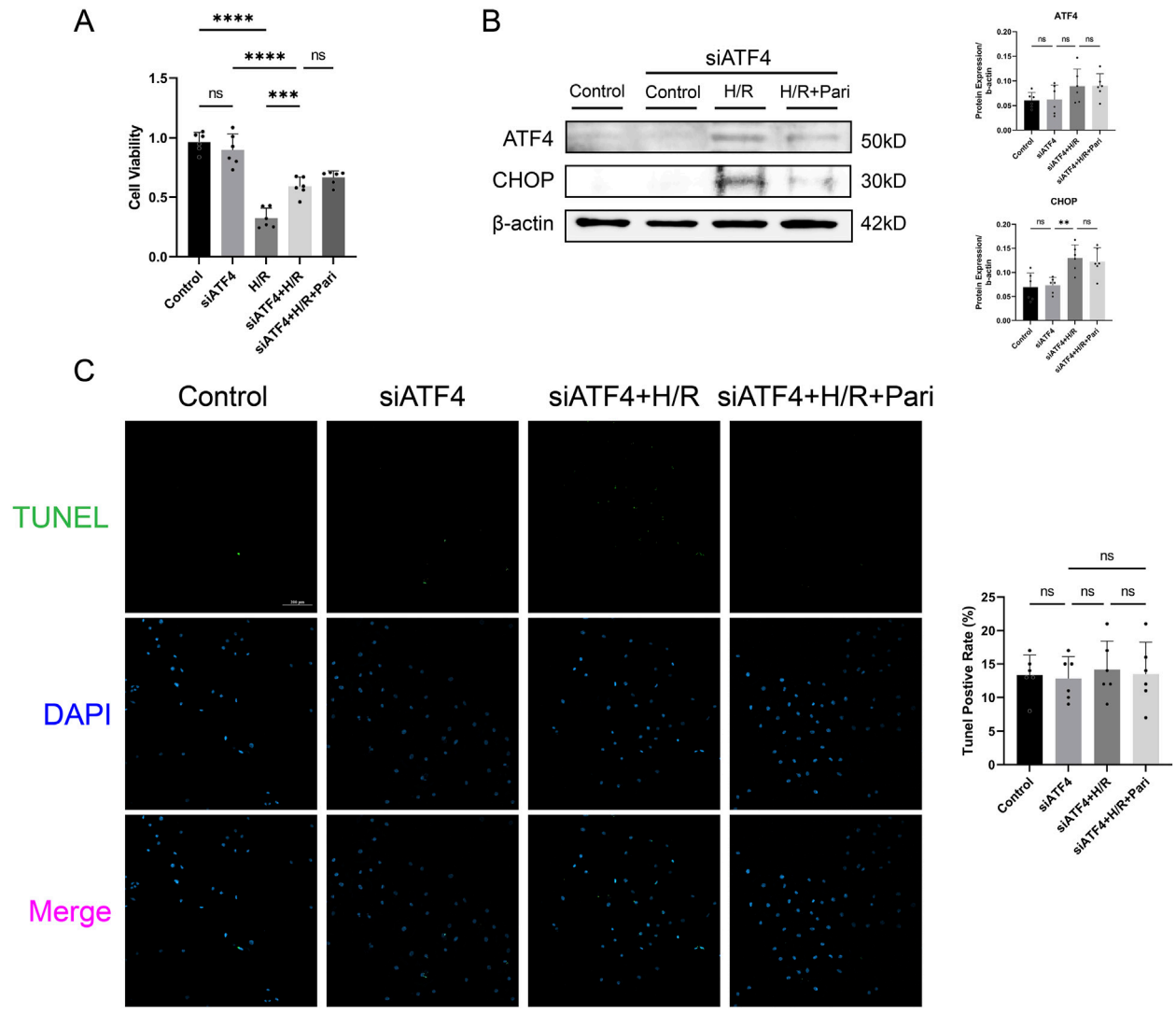

FIGURE 7
Silencing activating transcription factor 4 (ATF4) abolished the protective effect of paricalcitol in IEC-6 cells **(A)** The effect of siATF4 treatment using cell counting kit-8 assay on cell viability in IEC-6 cells (n = 6 in each group). **(B)** Western blot analysis and densitometric quantification of ATF4 and CHOP expression levels in IEC-6 cells after siATF4 treatment (n = 6 in each group). **(C)** Representative TUNEL staining (green) and nuclear staining (blue) results for IEC-6 cells after siATF4 treatment assay (scale bar = 200 μm; n = 6 in each group). Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. All data are presented as mean ± SD. *, P values <0.05; **, *P* values <0.01; ***, *P* values <0.001; ****, *P* values <0.0001.

increase in apoptosis, regardless of paricalcitol treatment. This suggests that the protective effect of paricalcitol against H/R injury is mediated through VDR activation. Furthermore, ATF4 silencing significantly improved cell viability following H/R injury, similar to the effect of paricalcitol. However, paricalcitol treatment did not further enhance cell viability in ATF4-silenced cells, indicating that ATF4 suppression alone is sufficient to mitigate H/R injury. Despite this, ATF4 silencing did not result in significant changes in apoptosis levels or CHOP protein expression after H/R injury. Given that ATF4 is not the sole upstream regulator of CHOP, their interaction may be more complex, warranting further investigation.

Previous studies have shown that VDR, as a transcription factor, can directly bind to the ATF4 promoter, inhibiting its protein expression (He et al., 2024). This aligns with our findings, as we confirmed the regulatory effect of VDR on downstream ATF4 using VDR-KO mice and *in vitro* transfection of siVDR. ATF4, as a key mediator of ERS, plays a crucial role in ERS-associated apoptosis (Ren et al., 2021). Research has shown that activation of the ATF4-CHOP pathway promotes apoptosis in porcine intestinal epithelial cells (Li et al., 2024). Conversely, blocking this pathway has been shown to reduce ERS and mitigate dextran sulfate sodium–induced colitis (Fan et al., 2024), and silencing ATF4 *in vitro* has been found to substantially opposed H/R-induced ERS and protected cells from H/R damage (Liu et al., 2024). Our study further expands on the role of the ATF4-CHOP pathway in intestinal I/R injury and suggests a potential therapeutic approach for this condition.

Studies on inflammatory bowel disease have shown that moderate ERS helps maintain intestinal homeostasis, protecting the normal function of the mucosal epithelium. However, excessive ERS activation can lead to inflammation, epithelial cell apoptosis, and disruption of the intestinal mucosal barrier (Kaur and Debnath, 2015; Qiao et al., 2021). Currently, ERS is understood to be initiated by three

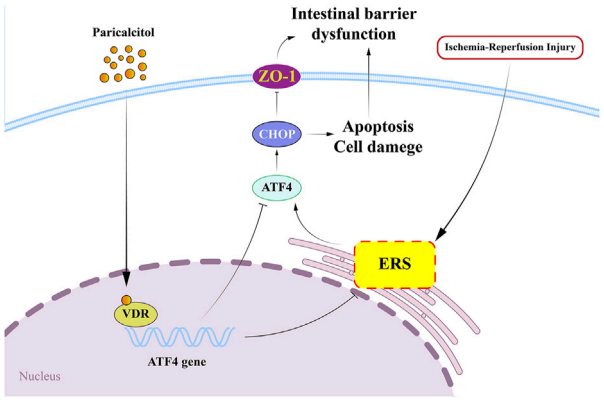

FIGURE 8
Paricalcitol alleviates intestinal ischemia-reperfusion injury by activating vitamin D receptor (VDR) and downregulated transcription factor 4 (ATF4) - C/EBP homologous protein (CHOP) expression VDR, vitamin D receptor; ERS, endoplasmic reticulum stress; ATF4, downregulated transcription factor 4; CHOP, C/EBP homologous protein; ZO-1, zonula occludens-1.

endoplasmic reticulum transmembrane sensors: inositol-requiring enzyme 1 (IRE1), protein kinase R-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6) (Wu et al., 2024). Binding immunoglobulin protein (BiP) interactions with nucleotides primarily mediate these processes (Pobre et al., 2019). Under ERS, BiP dissociates to activate IRE1, PERK, or ATF6, initiating a cascade of ERS and downstream signaling (Ma et al., 2017). In the PERK pathway, PERK oligomerization and phosphorylation activate eukaryotic initiation factor 2α (eIF2α), leading to ATF4 expression, which is induced by eIF2α phosphorylation (Vattem and Wek, 2004). This increases CHOP expression, ultimately inducing apoptosis (Rozpedek et al., 2016). Our study expands on this understanding, demonstrating that VDR activation can directly inhibit ATF4-CHOP expression. This suggests that the ATF4-CHOP pathway may be regulated by both PERK and VDR and that normal activation of VDR is essential in maintaining endoplasmic reticulum homeostasis.

This study had some limitations. First, we only investigated protein expression changes after paricalcitol intervention without measuring mRNA levels or examining transcriptional changes. Second, although previous research has shown that VDR can bind to the ATF4 promoter and repress its transcription, we did not confirm this in our study; further studies are needed to assess the molecular interactions between VDR and ATF4. Third, in our experiments silencing VDR and ATF4, we did not include an H/R group with non-silenced cells, therefore, we cannot determine the specific effects of VDR and ATF4 silencing on H/R injury. Fourth, we only investigated the protective effects of paricalcitol pre-treatment on I/R but did not examine its potential effects as a post-treatment. Although using paricalcitol after I/R would be more clinically relevant, we have not yet explored this aspect. Last, the mechanisms of intestinal I/R injury are complex and multisystemic. We restricted our focus to the effects of paricalcitol on the intestines and intestinal epithelial cells, overlooking potential interactions among different organs and cell types. Therefore, the full role of paricalcitol in intestinal I/R injury warrants further investigation.

In conclusion, our work demonstrates that the ability of paricalcitol to activate VDR offers protection against intestinal I/R injury by inhibiting ERS, primarily through the ATF4-CHOP pathway. The role of paricalcitol and the ATF4-CHOP pathway in intestinal I/R injury had not been previously reported. Our study highlights a potential link between these factors and ERS, providing new insights into the mechanisms of intestinal injury and potential therapeutic approaches.

Data availability statement

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

Ethics statement

The animal study was approved by The animal ethics committee of Changzhou Second People's Hospital (Permit Number: 2024KY206-01). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JZ: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft. TL: Conceptualization, Data curation, Investigation, Validation, Writing – review and editing. TX: Funding acquisition, Project administration, Supervision, Writing – review and editing. ZJ: Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review and editing.

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Conflict of interest

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

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

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EDITED BY
Daniele Maria-Ferreira,
Instituto de Pesquisa Pelé Pequeno Príncipe, Brazil

REVIEWED BY
Livio Muccillo,
AREA Science Park, Italy
Nadeem Bhat,
University of Miami, United States

*CORRESPONDENCE
Yufen Wang,
wzybmxbj@126.com
Yingde Wang,
albertwyd@dmu.edu.cn

†These authors contributed equally to this work and share first authorship

‡These authors are co-corresponding authors

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MiR-3613-5p targets AQP4 to promote the progression of chronic atrophic gastritis to gastric cancer

Jian Bi[1†], Yufen Wang[2*†‡] and Yingde Wang[1*‡]

[1]Department of Gastroenterology, First Affiliated Hospital of Dalian Medical University, Dalian, China, [2]Department of Digestive Endoscopy, First Affiliated Hospital of Dalian Medical University, Dalian, China

Introduction: Gastric cancer (GC) exhibits high invasiveness, delayed diagnosis, and poor prognosis. Chronic atrophic gastritis (CAG), an initial stage within the Correa cascade, induces gastric mucosal inflammation and atrophy, promoting genetic and epigenetic alterations. MicroRNAs (miRNAs) dysregulation has been implicated in gastric tumorigenesis, yet their specific roles in CAG progression to GC remain unclear.

Methods: Using clinical data from the GEO database, we identified miRNAs differentially expressed in gastric mucosa and serum samples from GC patients. Murine CAG models were established through administration of N-methyl-N-nitrosourea (MNU) and high-salt diet (HSD). In vitro functional assays evaluated proliferation and migration after miRNA modulation in gastric cancer cell lines. MiRNA target validation involved luciferase reporter assays.

Results: MiR-3613-5p expression was significantly elevated in gastric mucosal and serum samples of GC patients, mucosal tissues of CAG patients, tumor tissues, and human gastric cancer cell lines. Murine models demonstrated increased miR-3613-5p expression in gastric mucosa following MNU and HSD-induced CAG. Functionally, miR-3613-5p overexpression promoted gastric cancer cell proliferation and migration in vitro, whereas silencing miR-3613-5p alleviated pathological gastric mucosal alterations (atrophy, hyperplasia, inflammatory infiltration) in vivo. Mechanistically, miR-3613-5p inhibited Aquaporin 4 (AQP4) expression by directly targeting its 3′UTR.

Discussion: Our findings provide the first evidence that miR-3613-5p facilitates CAG progression toward GC via negative regulation of AQP4. These results highlight miR-3613-5p as a promising biomarker and therapeutic target, suggesting antagomiR-3613-5p as a potential novel strategy to prevent gastric carcinogenesis.

KEYWORDS

chronic atrophic gastritis, miR-3613-5p, gastric cancer, Aquaporin 4, intestinal metaplasia

Highlights

- miR-3613-5p is significantly overexpressed in gastric mucosal tissue samples from CAG patients, tumor samples from gastric cancer patients, human gastric cancer cell lines, and the gastric mucosa of CAG mice.

- Overexpression of miR-3613-5p promotes the proliferation and migration of gastric cancer cells, while silencing miR-3613-5p alleviates pathological changes, including atrophy, hyperplasia, and inflammatory accumulation, in the gastric mucosa of CAG mice.
- miR-3613-5p inhibits the expression of the AQP4 gene by binding to its 3′UTR, thereby promoting the progression from CAG to gastric cancer.

Introduction

Gastric cancer (GC) is a major global health issue, with the highest prevalence observed in East Asia (Sung et al., 2021). It has a high mortality rate due to its aggressive invasiveness, late-stage diagnosis, and poor prognosis (Zheng et al., 2004). Chronic atrophic gastritis (CAG) is a well-established precursor of gastric cancer (Hahn et al., 2024). CAG is characterized by the progressive loss of gastric glandular cells, leading to thinning of the gastric mucosa, with subsequent replacement by fibrous tissue and intestinal metaplasia (Jia et al., 2024). Clinically, CAG often presents with non-specific symptoms, including epigastric discomfort, bloating, and anemia due to impaired absorption of vitamin B12 and iron (Li et al., 2024). The Correa cascade model outlines the sequential progression of gastric carcinogenesis, starting with CAG and advancing through intestinal metaplasia, dysplasia, and ultimately gastric adenocarcinoma (He et al., 2022; Tong et al., 2024). The persistent inflammation and atrophy of the gastric mucosa caused by CAG promote both genetic and epigenetic changes (He et al., 2022). Existing studies identify key signaling pathways, including Wnt/β-catenin, NF-κB, RAS/RAF/MEK/ERK, and PI3K/AKT/mTOR, which promote cell proliferation, migration, and invasion, driving the transition to intestinal metaplasia and dysplasia (Akhavanfar et al., 2023; Guo et al., 2024; Lim et al., 2023). Investigating the factors and specific molecular mechanisms that induce the progression of CAG to gastric cancer is crucial for early diagnosis and intervention.

MicroRNAs (miRNAs) are a class of small, non-coding RNA molecules, typically 18–25 nucleotides in length, that play a critical role in regulating gene expression (Chong, 2024). They primarily function by binding to complementary sequences in the 3′untranslated regions (3′UTR) of target messenger RNAs (mRNAs), leading to mRNA degradation or translational repression (Sun et al., 2024). This post-transcriptional regulation allows miRNAs to fine-tune gene expression in various biological processes, including development, differentiation, apoptosis, and proliferation (Yu et al., 2024). Dysregulation of miRNAs has been implicated in the pathogenesis of numerous diseases, including cancer, cardiovascular diseases, and neurological disorders (Gan et al., 2024; Di Fiore et al., 2024). In gastric cancer (GC), miRNAs play a crucial role in regulating key cancer-related genes and signaling pathways, functioning as oncogenes or tumor suppressors (Alessandrini et al., 2018). For example, miRNA-21 is upregulated in GC and promotes cell proliferation and invasion by targeting PTEN in the PI3K pathway (Motoyama et al., 2010). In contrast, miRNA-375 is downregulated in GC and acts as a protective factor by targeting PDK1, thereby reducing cell viability (Tsukamoto et al., 2010). At least dozens of miRNAs have been shown to play key roles in GC initiation, metastasis, and drug resistance (Christodoulidis et al., 2024). However, the exact role of miRNAs in the transition from chronic atrophic gastritis (CAG) to GC remains unclear. We hypothesize that specific miRNAs play a pivotal role in the progression of CAG to GC by regulating key signaling pathways and gene expression. Identifying these miRNAs could provide novel therapeutic targets for preventing the development and progression of GC.

The ability of miRNAs to simultaneously regulate multiple genes and pathways makes them critical players in disease development and progression. As a result, miRNAs are being explored as potential biomarkers for diagnosis and prognosis, as well as therapeutic targets for novel treatments (Leng et al., 2024; Hu et al., 2024). The main strategies for miRNA-targeted therapy include the use of miRNA mimics and antagomirs. miRNA mimics are synthetic double-stranded RNA molecules designed to restore the function of miRNAs that are downregulated in diseases (Lee et al., 2024). Conversely, antagomirs are single-stranded oligonucleotides that bind to overexpressed miRNAs, preventing them from interacting with their target mRNAs and thereby reducing their pathogenic effects (Rupaimoole and Slack, 2017). miRNA-targeted therapies have shown significant potential in preclinical studies and are being evaluated in clinical trials for diseases such as cancer (Kara et al., 2022). In particular, different types or subtypes of cancer appear to have distinct miRNA expression profiles (Goh et al., 2016). When developing miRNA-targeted therapeutic approaches, it is crucial to identify specific miRNAs associated with tumor subtypes.

Through the analysis of clinical data, we found that miR-3613-5p is significantly upregulated in both the gastric mucosa and serum of gastric cancer patients. The expression of miR-3613-5p is significantly increased in both the gastric mucosa of CAG patients and the tumors of gastric cancer patients. High expression of miR-3613-5p was also observed in human gastric cancer cell lines and the gastric mucosa of CAG mice. Overexpression of miR-3613-5p promotes the proliferation and migration of gastric cancer cells and exacerbates atrophy, hyperplasia, and inflammatory accumulation in the gastric mucosa of CAG mice. We found that miR-3613-5p promotes the progression of CAG to gastric cancer by inhibiting the expression of AQP4. These results identify new potential targets for the early intervention of gastric cancer.

Materials and methods

Bioinformatics analysis

We searched the GEO database for non-coding RNA-seq profiles using the keywords “gastric cancer” and “gastritis” from the past 3 years. Raw gene expression profile data and clinical information were available from the GEO database. The following filtering criteria were used: The tissue were obtained from human gastric mucosal and serum, and the number of each group less than 10 were excluded. Finally, non-coding RNA-seq profiles GSE186595 and GSE211692 were obtained. The GSE186595 dataset was used on the GPL20795 platform. The dataset contained 13 gastric mucosa from gastric cancer patients and 20 from healthy controls. The GSE211692 dataset was used on

the GPL21263 platform. The dataset contained 1,418 serum microRNA profiles from gastric cancer patients and 5,643 from healthy controls. Furthermore, we also chose GSE130823 dataset to analysis the downregulated genes in gastric cancer carcinogenesis. This dataset used GPL17077 platform, and there are ninety-four biospy samples collected from gastric cancer carcinogenesis and paired controls. DeSeq2 and limma R packages were used to analyzed differentially expressed genes (DEGs) in datasets, and the standard p value <0.05 and logFC >2 was selected as the cut-off standard. For the correlation analysis between AQP4 and miR-3613-5p, we used the GSE224056 dataset from the GEO database, which includes mRNA and miRNA expression profiles from gastric cancer tissues and their corresponding non-tumorous adjacent tissues from five patients. A total of 439 gastric cancer cases with mRNA and miRNA expression profiles were obtained from The Cancer Genome Atlas (TCGA) database (https://www.cancer.gov/ccg/access-data). Pearson correlation analysis between AQP4 and miR-3613-5p was performed on the aforementioned data using R.

Human tissue specimens

This study included eight gastric mucosal from CAG patients and healthy individuals, as well as six human gastric cancer and adjacent non-tumor tissues. Samples were gathered from the First Affiliated Hospital of Dalian Medical University (Dalian, China) from December 2023 to January 2024. All patients provided informed consent. This study was approved by the First Affiliated Hospital of Dalian Medical University.

Animals

Mice were bred and maintained at Hubei Academy of Preventive Medicine, Hubei Provincial Center for Disease Control and Prevention. Animal studies were approved by the Laboratory Animal Care and Use Committee of Hubei Provincial Center for Disease Control and Prevention. The experimental procedures followed were in accordance with institutional guidelines. Experiments were performed using 4-week-old wild-type (WT) mice. All experiments on mice were conducted in a C57BL6/N background. Animals were housed under standard conditions and maintained on commercial mouse chow *ad libitum*. The environment was maintained at 22 °C with a 12-h light-12-h dark cycle. N-Nitroso-N-methylurea (MNU, HY-34758, MCE, United States) was added to the drinking water, which the mice were allowed to drink freely, and were gavaged with 1 mL of saturated NaCL solution every 3 days for 12 weeks.

Cell culture and transfections

The gastric carcinoma cell lines BGC-823, MKN-45, SGC-7901 and the normal gastric mucosal epithelial cell line GES-1 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). These cells were cultured in DMEM (Gibco, United States), supplemented with 10% fetal bovine serum (Gibco, United States) at 37 °C in a humidified incubator with 95% air and 5% CO_2. Cells were cultured in 6-well plates and transfected when they reached 60%–70% confluence. Agomirs, antagomirs, and their corresponding negative controls (20 nmol) were transfected into the cells using Lipofectamine 3,000 (Invitrogen, United States), following the manufacturer's instructions. The transfection reagent mixture was incubated with the cells at 37°C for 6 h. After incubation, the medium was replaced with DMEM supplemented with 10% FBS. Cells were collected for subsequent experiments 24 h after transfection. Agomirs and antagomirs and their negative controls were obtained from GenePharma (Suzhou, China). The AgomiR-3613-5p sequence was 5′- UGUUGUACU UUUUUUUUUGUUC-3′. The AgomiR-NC sequence was 5′- AUC UAUACUUUGUUUUUCUUCU-3′. The AntagomiR-3613-5p sequence was 5′- GAACAAUUUUUUUACACAA-3′. The Antagomir-NC sequence was 5′- CUUAGUUUUUAUCAUUCA A -3′.

Histology

Tissues were harvested from euthanized animals and immediately fixed in 4% paraformaldehyde fixative for 24 h at room temperature. After fixation, the samples were dehydrated through a graded ethanol series (70%, 80%, 95%, and 100%) and embedded in paraffin. Sections of 4 μm thickness were cut using a microtome and mounted on glass slides. To perform Hematoxylin and Eosin (H&E) staining, slides were deparaffinized with xylene and rehydrated through a descending ethanol series. The tissue sections were then stained with hematoxylin for 5 min, rinsed, and differentiated in 1% hydrochloric acid alcohol. Eosin staining was performed for 2 min, followed by dehydration, clearing, and mounting. Slides were observed under microscope (*Nikon*, Japan) for morphological analysis.

ELISA

The blood samples were left at room temperature for 2 h, and then centrifuged at 1,000°g at 4°C for 20 min. The supernatant was collected, and the levels of IL-6 (Mouse IL-6 ELISA Kit, 98027ES96, Yeasen Biotechnology, China), TNF-α (Mouse TNF-α ELISA Kit, 98029ES96, Yeasen Biotechnology, China) in the serum of mice were measured by ELISA using the corresponding commercial kits. The operation was conducted strictly according to the manufacturer's instructions. The standards were diluted according to the multiplicative dilution method, and a standard curve was prepared for each assay. Three replicate wells were set up for each sample, and each experiment was repeated three times.

Cell proliferation assay

Cells were cultured on 12-well slides for 24 h, fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and incubated with Ki-67 primary antibody (1:500, 27309-1-AP, Proteintech, China) at 4 °C overnight. After three washes with Tris Buffered Saline-Tween20, the sections were incubated with

miR-3613-5p	Forward primer	5′-UGUUGUACUUUUUUUUUGUUC-3′
	reverse primer	5′-GTGCAGGGTCCGAGGT -3’
PTGER3	forward primer	5′-CGCCTCAACCACTCCTACACA-3′
	reverse primer	5′-ATCCGCAATCCTCGCCAGAC-3’
HSPB7	forward primer	5′-CACCACCTCCAACAACCACATC-3′
	reverse primer	5′-TGGCACTTGTGAGCGAAGGT-3’
AQP4	forward primer	5′-AGCATCGCCAAGTCTGTCTTCT-3′
	reverse primer	5′-GAGACCATGACCAGCGGTAAGA-3’
ERBB4	forward primer	5′-GAACAGCAGTACCGAGCCTTG-3′
	reverse primer	5′-CGAACAGACCGCAGGAAGGA-3’
PAQR5	forward primer	5′-CCTGGACTATGGTGCCGTCAA-3′
	reverse primer	5′-GCCTGTGCTGAGGATGGTGTT-3’
U6 snRNA	forward primer	5′-CGCTTCGGCAGCACATATAC-3′
	reverse primer	5′-TTCACGAATTTGCGTGTCAT-3’

fluorescent secondary antibody (A23420, Abbkine, United States) for 1 h. Nuclei were stained with DAPI (P0131, Beyotime, China). Ki-67-positive cells and total cell counts were determined in 12 fields of view (×20 objective) from three replicate wells using the ImageJ analysis software. The Ki-67 index was calculated as the ratio of Ki-67-positive cells to the total number of cells.

Wound healing assay

At ~90% confluence, a scratch was made in the center of each well using the tip of a 200 μL pipette. Subsequently, the cells were cultured in serum-free medium. Images of the wounds were captured at 0 and 24 h by an Olympus CKX53 inverted microscope at ×4 magnification (Olympus Corporation) to record the scratch width marking the scratch location. The migration index was calculated to evaluate the cell migration capacity. Migration index = (A0h - A24h)/A0h. Scratch area (Area, A) was measured by ImageJ software (version 1.52a; National Institutes of Health). Every experiment was repeated 3-times.

Luciferase constructs and transfection

The 3′-UTR of AQP4 was amplified by PCR and subcloned into the dual-luciferase reporter vector pGL3 (Promega, United States) at Mlul and Xhol sites, termed Luc-AQP4-WT. The forward primer was 5′-CCGAGCTCTTACGCGCTAGTTGAG TCCTGGCTTT-3′. The reverse primer was 5′-GATCGCAGA TCTCGAGACAAGTAAGTGAGTCAGTAAC-3′. The mutant vectors with point mutations in miR-3613-5p binding sites were synthesized using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, United States), termed Luc-AQP4-MUT. HEK293T cells transfected with AgomiR-3613-5p and AntagomiR-3613-5p and their negative controls, with pGL3, Luc-AQP4-WT or Luc-AQP4-MUT plasmid. Renilla luciferase was co-transfected for the purposes of normalization. Cells were harvested 24 h after transfection and assayed for firefly luciferase and Renilla luciferase activities by Dual Luciferase Reporter Gene Assay Kit (KTA8010, Abbkine, United States).

RT-PCR

Total RNA was extracted using the RNAiso Plus (9,109, Takara, Japan) method according to the manufacturer’s protocol and reverse transcribed into cDNA using a cDNA synthesis kit (AT311, TransGen Biotech, China). For mRNA detection, gene expression was analyzed by the Tip Green qPCR SuperMix kit (AQ142, TransGen Biotech, China), and the results were normalized to GAPDH expression. For miRNA detection, cDNA was synthesized with the Mir-X™ miRNA First Strand Synthesis Kit (Takara), and subsequent qPCR analysis was performed using the Tip Green qPCR SuperMix kit (AQ142, TransGen Biotech, China). U6 snRNA was used as the endogenous control to normalize miRNA expression. Primers (synthesized by Sangon Biotech, China) for miR-3613-5p, PTGER3, HSPB7, AQP4, ERBB4, PAQR5, U6 snRNA and Gapdh were as follows.

Protein extraction and Western blot

Tissues and cells were lysed in RIPA Lysis buffer (Bioss, China) supplemented with cOmplete Protease Inhibitor EASYpacks (Roche, Switzerland) on ice for 30 min. Protein fractions were collected by centrifugation at 13,000 g at 4°C for 15 min. Protein samples were separated by 10% SDS–PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5%

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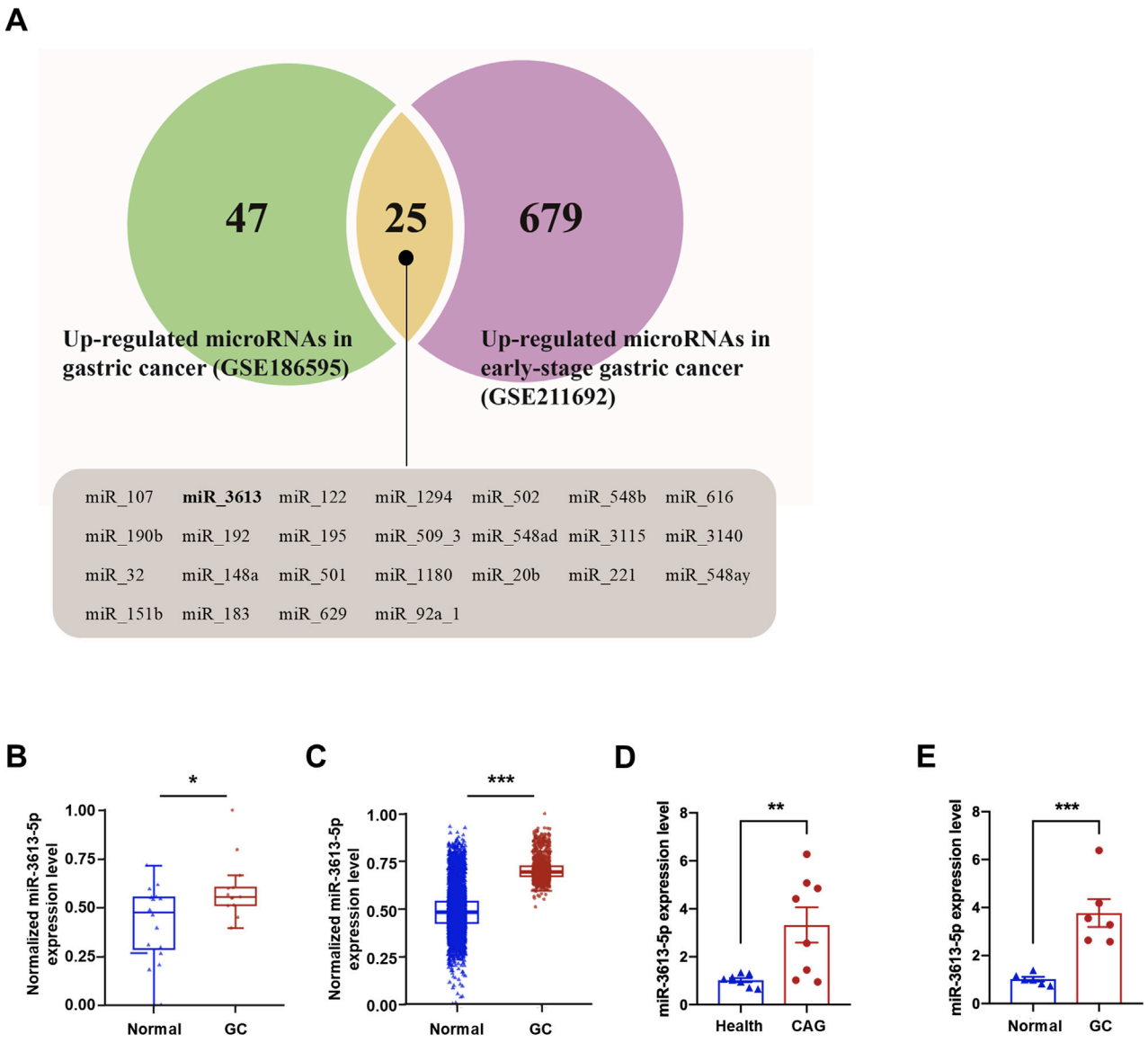

FIGURE 1
miR3613-5p is significantly overexpressed in human CAG and gastric cancer tissues. **(A)** Venn diagram of significantly upregulated miRNAs in the GSE186595 and GSE211692 datasets. A total of 72 differentially expressed genes (DEGs) for microRNAs were significantly upregulated in the gastric mucosal of gastric cancer patients compared to healthy controls in the GSE186595 dataset (green), while 704 DEGs were significantly upregulated in the serum of early gastric cancer patients compared to healthy controls in the GSE211692 dataset (purple). There are 25 microRNAs that are differentially expressed in both datasets (yellow), all of which are displayed below, including miR-3613. **(B, C)** Box plot of miR-3613-5p expression level in GSE186595 **(B)** and GSE211692 **(C)** dataset. **(D)** Relative expression levels of miR-3613-5p in gastric mucosal from CAG patients and healthy individuals (n = 8). **(E)** Relative expression levels of miR-3613-5p in gastric cancer and adjacent non-tumor tissues (n = 6). Data are presented as mean ± SEM. Statistical analysis is performed using a Student's t-test and statistical significance is shown as *p < 0.05, **p < 0.01, and ***p < 0.001.

skimmed milk and incubated with the primary antibodies overnight. Antibodies used were: Tubulin (1:5,000, ABL1030, Abbkine, China), AQP4 (1:1,000, 16473-1-AP, Proteintech, China).

Statistical analysis

For statistical analysis, all quantitative data are presented as the mean ± SEM. Statistical analysis for comparison of two groups was performed using two-tailed unpaired Student's t-test. Statistical differences among groups were analyzed by 1-way analysis of variance (ANOVA) or 2-way ANOVA (if there were two factor levels), followed by Bonferroni's *post hoc* test to determine group differences in the study parameters. Before ANOVA, we firstly test the homogeneity among variances. If it shows the variances are unequal, we then use Welch's ANOVA for 1-way analysis and the ordinary 2-way ANOVA for 2-way analysis after log-transformation. Pearson correlation coefficients was performed to assess the correlation between two variables. All statistical analyses were performed with GraphPad PrismV7 (GraphPad Prism, United States) and SPSS 23.0 software (SPSS, United States). Differences were considered significant at *P < 0.05, **P < 0.01, ***P < 0.001.

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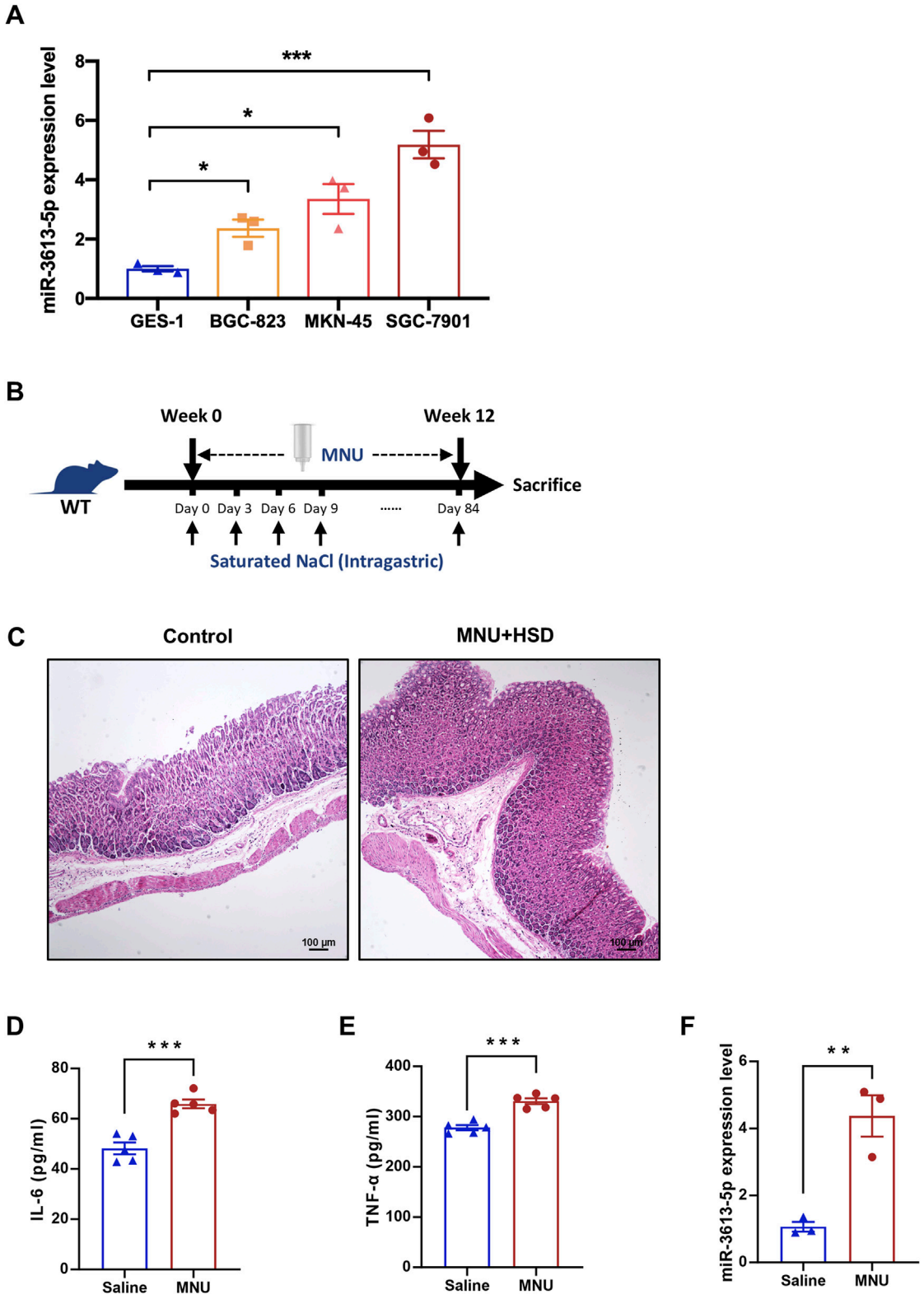

FIGURE 2
miR3613-5p is significantly overexpressed in gastric cancer cell lines and the gastric mucosa of CAG mice. **(A)** Relative expression levels of miR-3613-5p in human gastric cancer cell lines (BGC-823, MKN-45, SGC-7901) and the normal gastric mucosal epithelial cell line GES-1. **(B)** Schematic diagram of the modeling method for CAG mice. MNU was added to the drinking water for the mice to drink freely, and a 1mL saturated NaCl solution was administered by gavage every 3 days, continuing for 12 weeks. **(C)** Representative H&E staining images of gastric mucosal slices from control and MNU + HSD mice after 12 weeks of induction. **(D-E)** Levels of IL-6 and TNF-α in the serum of control and MNU + HSD mice detected by ELISA (n = 5). **(F)** Relative expression levels of miR-3613-5p in the gastric mucosa of control and MNU + HSD mice (n = 5). Data are presented as mean ± SEM. Statistical analysis is performed using a Student's t-test and statistical significance is shown as *p < 0.05, **p < 0.01, and ***p < 0.001.

Results

Result 1: miR3613-5p is significantly overexpressed in human CAG and gastric cancer tissues

We searched the GEO database for non-coding RNA-seq profiles from the past 3 years and obtained the GSE186595 and GSE211692 datasets. The GSE186595 dataset includes microRNA profiles from gastric mucosa tissues of 13 gastric cancer patients and 20 healthy controls. The GSE211692 dataset contains serum microRNA profiles from 1,418 early gastric cancer patients and 5,643 healthy controls. A total of 72 and 704 differentially expressed genes (DEGs) were identified in the GSE186595 (green) and GSE211692 (purple) datasets, respectively (Figure 1A). Among the two datasets, 25 miRNAs are commonly differentially expressed, including miR-3613. In both the GSE186595 and GSE211692 datasets, the expression of miR-3613-5p was significantly upregulated (Figures 1B,C). In recent years, miR-3613-5p has emerged as an important target in cancer research and has been shown to play a regulatory role in various tumor types (Luo et al., 2022; Zhan et al., 2021; Qin et al., 2019). Therefore, we aim to understand the role of miR-3613-5p in the progression of CAG to gastric cancer. We clinically collected gastric mucosa samples from eight patients with chronic atrophic gastritis (CAG) and healthy individuals to assess the expression of miR-3613-5p (ethics approval No. PJ-KS-KY-2023–528). Our findings revealed a significant upregulation of miR-3613-5p in the gastric mucosa of CAG patients (Figure 1D). Additionally, we examined miR-3613-5p expression in gastric cancer and adjacent non-tumor tissues. In 6 cases of gastric cancer, the expression level of miR-3613-5p was significantly higher than in the corresponding adjacent non-tumor tissues (Figure 1E). These results suggest that miR-3613-5p expression is increased in both CAG and gastric cancer tissues.

Result 2: miR3613-5p is significantly overexpressed in gastric cancer cell lines and the gastric mucosa of CAG mice

We examined the expression of miR-3613-5p in three human gastric cancer cell lines with varying degrees of differentiation, using the normal gastric mucosal epithelial cell line GES-1 as a control. RT-PCR results revealed a significant increase in miR-3613-5p expression across all three gastric cancer cell lines, with the highest expression observed in the moderately differentiated SGC-7901 cell line (Figure 2A). Subsequently, we induced a mouse model of CAG using N-Methyl-N-nitrosourea (MNU) combined with a high-salt diet (HSD) (Figure 2B). MNU is a potent chemical carcinogen that induces apoptosis or necrosis of gastric mucosal epithelial cells, triggering abnormal proliferation and atypical hyperplasia of gastric epithelial cells. A high-salt diet is considered one of the key risk factors for CAG and gastric cancer, as it increases the osmotic pressure of gastric contents, directly damaging gastric mucosal cells, leading to shedding, necrosis, and inflammatory responses in the mucosal epithelial cells. After 12 weeks of induction, the gastric mucosal morphology of the control mice remained normal, with gastric epithelial cells showing typical glandular structures, and the gastric pits comprising approximately one-third of the gland thickness. In contrast, the gastric mucosa of the induced mice exhibited severe atrophy, loss of intrinsic glands, hyperplasia-like changes, and the transformation of chief cells into mucous cells (Figure 2C). Significant infiltration of inflammatory cells was observed in the mucosal base and lamina propria. Consistently, the serum levels of inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were significantly increased (Figures 2D,E). Pathological and serological test results confirmed that the mice induced with MNU and a high-salt diet for 12 weeks developed the pathophysiological characteristics of CAG. Notably, the expression of miR-3613-5p was significantly increased in the gastric mucosa of the induced mice (Figure 2F).

Result 3: miR-3613-5p promotes the proliferation and migration of gastric cancer cells

Since the SGC-7901 cell line exhibited the highest expression of miR-3613-5p among the three gastric cancer cell lines tested, we selected it for small molecule delivery and functional assays. We used a synthetic small RNA fragment, agomiR-3613-5p, to mimic the function of endogenous miR-3613-5p, and antagomiR-3613-5p to specifically inhibit its function. RT-PCR results showed that agomiR-3613-5p could increase miR-3613-5p expression by more than tenfold in gastric cancer cells, while antagomiR-3613-5p significantly reduced its expression (Figures 3A,B). During the progression from CAG to gastric cancer, chronic inflammation progressively accumulates (Mustapha et al., 2014). The levels of cytokines, such as IL-6 and TNF-α, are elevated, stimulating cell cycle progression and preventing apoptosis, thereby allowing cancer cells to proliferate uncontrollably (Browning et al., 2018; Ji et al., 2014). Ki-67 is a cell proliferation marker that is specifically expressed during the cell cycle. Immunofluorescence staining revealed an increase in the number of Ki-67-positive nuclei after agomiR-3613-5p delivery, with statistical analysis showing a significantly higher percentage of Ki-67-positive cells compared to the negative control (NC) (Figures 3C,D). Conversely, the antagomiR-3613-5p inhibitor significantly reduced the percentage of Ki-67-positive cells (Figures 3E,F). These results suggest that miR-3613-5p promotes the proliferation of gastric cancer cells. The scratch assay results showed that overexpression of miR-3613-5p significantly accelerated the wound healing rate of gastric cancer cells (Figures 3G,H), while inhibition of miR-3613-5p expression markedly slowed the healing process (Figures 3I,J). These findings indicate that miR-3613-5p promotes the migration of gastric cancer cells.

Result 4: Overexpression of miR-3613-5p promotes the progression from CAG to gastric cancer in mice

During the final month of CAG model induction in mice, AgomiR-3613-5p was administered *via* tail vein injection to overexpress miR-3613-5p *in vivo* (Figure 4A). Pathological

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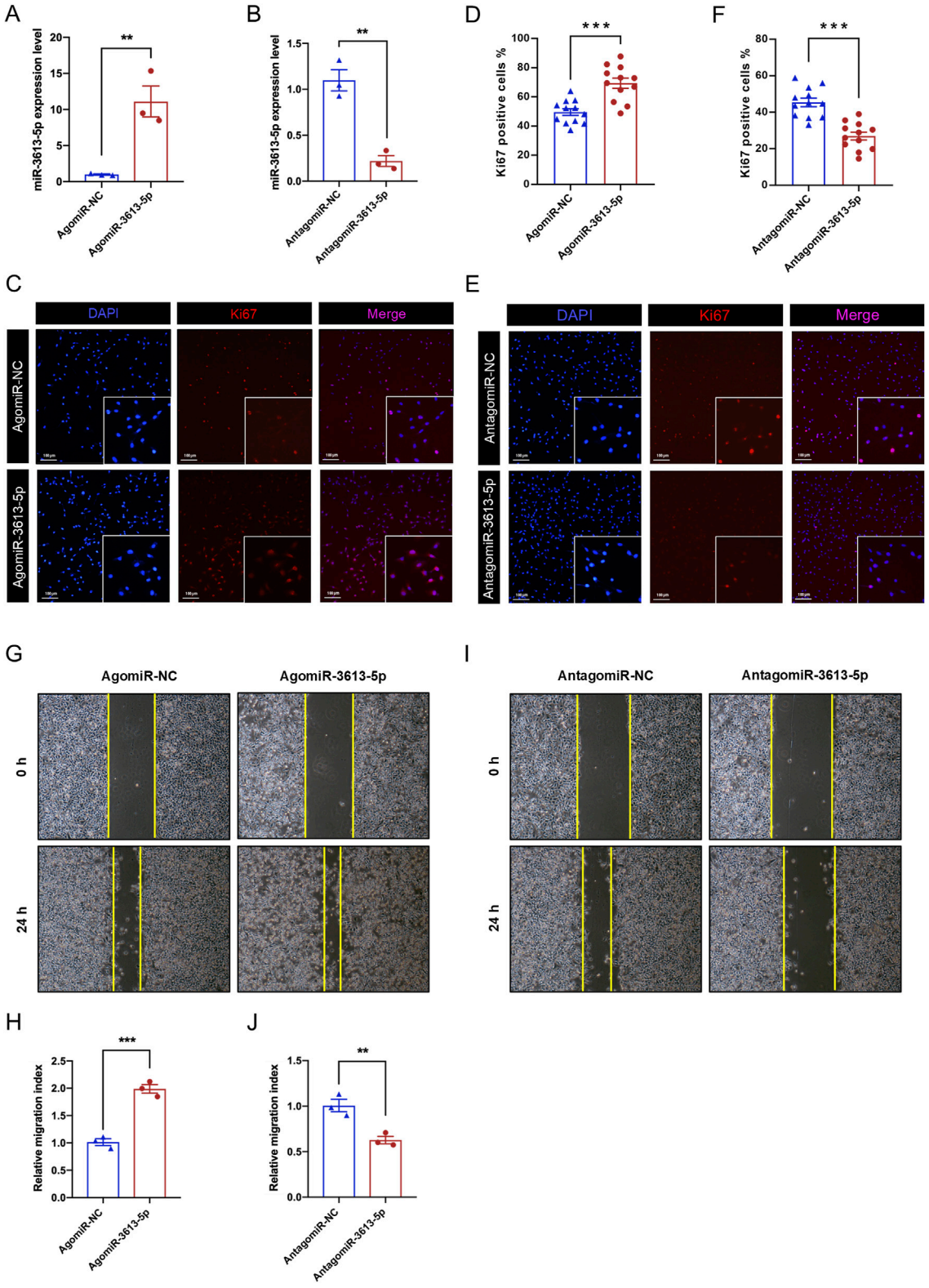

FIGURE 3
miR-3613-5p promotes the proliferation and migration of gastric cancer cells. **(A–B)** Relative expression levels of miR-3613-5p after transfection of AgomiR-3613-5p and AntagomiR-3613-5p in SGC-7901 cell line. **(C, E)** Representative images of Ki-67 immunofluorescence staining after transfection of AgomiR-3613-5p and AntagomiR-3613-5p in SGC-7901 cell line. **(D, F)** Statistical analysis of the percentage of Ki-67 positive cells after transfection of AgomiR-3613-5p and AntagomiR-3613-5p. **(G, I)**. Representative images from the cell wound healing assay after transfection of AgomiR-3613-5p and AntagomiR-3613-5p. **(H, J)** Statistical analysis of the wound healing assay. The migration index was calculated to evaluate the cell migration capacity. Migration index = (A0h - A24h)/A0h. Scratch area (Area, A) was measured by ImageJ software (version1.52a; National Institutes of Health). Every experiment was repeated 3 times. Data are presented as mean ± SEM. Statistical analysis is performed using a Student's t-test and statistical significance is shown as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

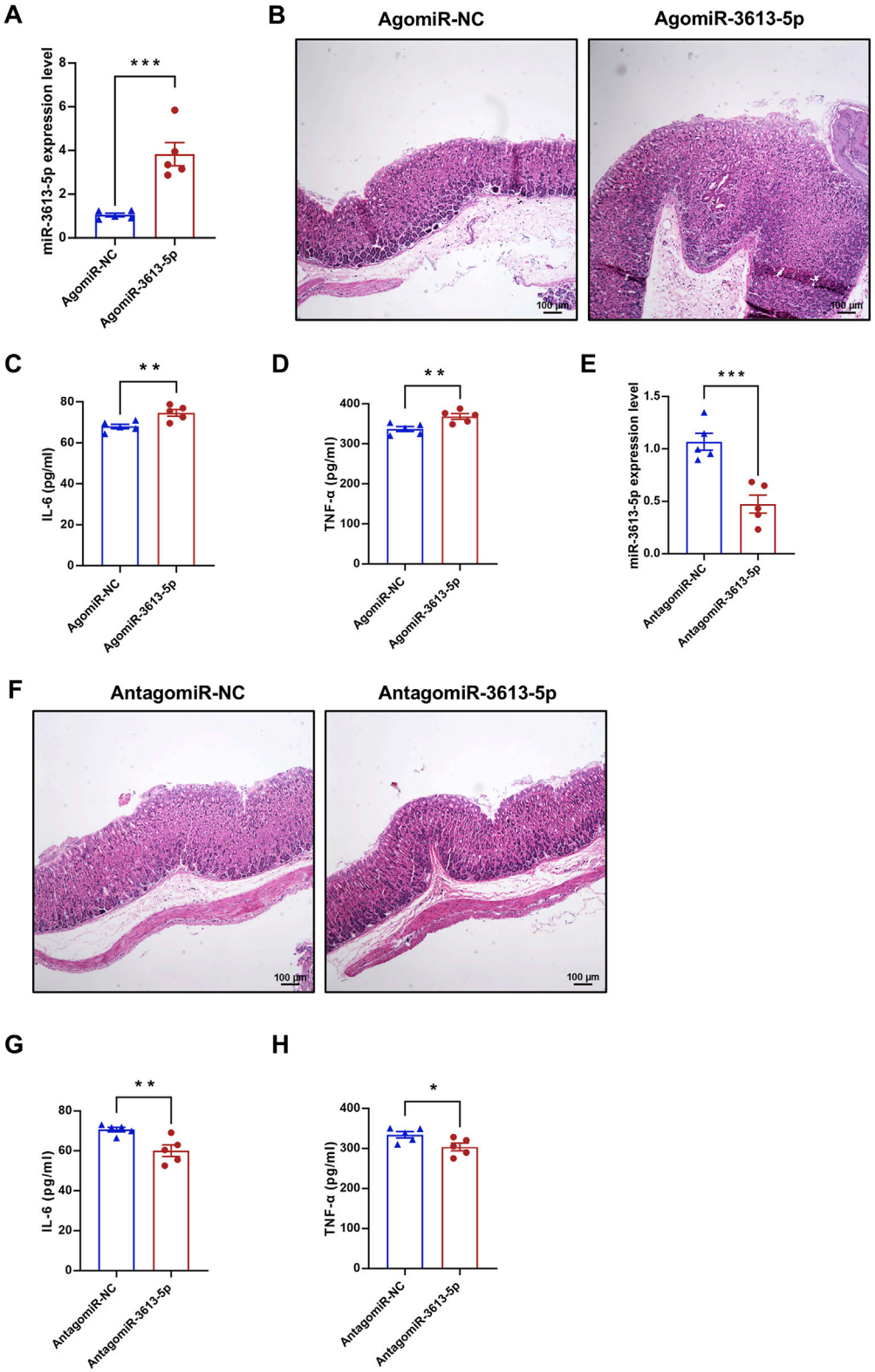

FIGURE 4
Overexpression of miR-3613-5p promotes the progression from CAG to gastric cancer in mice. **(A, E)** Relative expression levels of miR-3613-5p in gastric mucosa of CAG mice after tail vein injection of AgomiR-3613-5p or AntagomiR-3613-5p. **(B, F)** Representative images of H&E staining of mouse gastric mucosal slices. **(C, G)** Levels of IL-6 in mouse serum detected by ELISA (n = 5). **(D, H)** Levels of TNF-α in mouse serum detected by ELISA (n = 5). Data are presented as mean ± SEM. Statistical analysis is performed using a Student's t-test and statistical significance is shown as *p < 0.05, **p < 0.01, and ***p < 0.001.

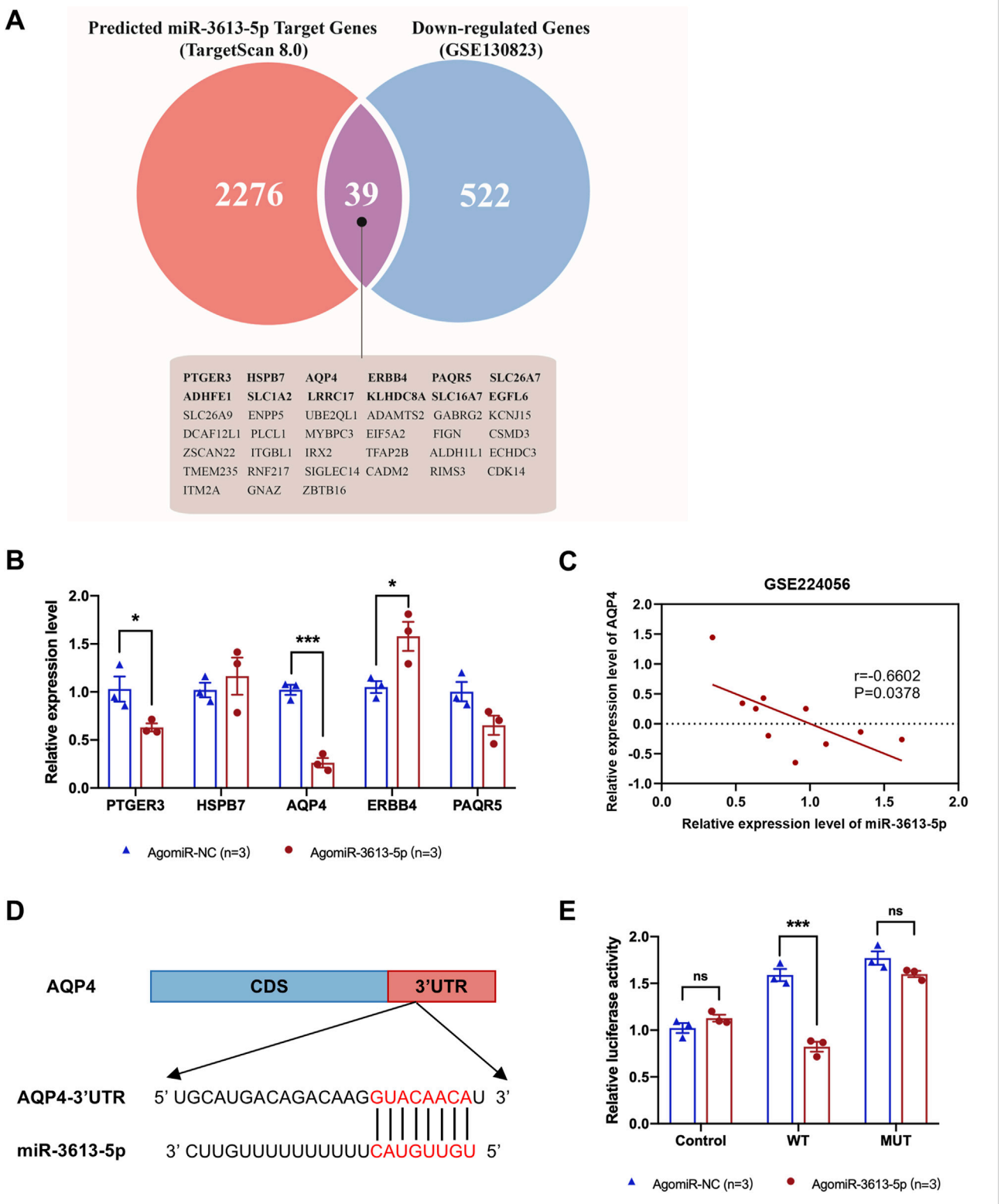

FIGURE 5
miR-3613-5p inhibits the expression of the AQP4 gene by binding to its 3′UTR. **(A)** The Venn diagram illustrates the overlap between the target genes predicted by TargetScan 8.0 (red) and the downregulated genes in the gastric mucosa of gastric cancer patients from the GSE130823 dataset (blue). The 39 presumed target genes in the intersection are listed below, including AQP4. **(B)** Relative mRNA expression levels of PTGER3, HSPB7, AQP4, ERBB4, and PAQR5 in SGC-7901 cell line after transfection with AgomiR-NC and AgomiR-3613-5p. **(C)** Pearson correlation coefficient between AQP4 and miR-3613-5p. Data from GSE224056. **(D)** Predicted binding sites of miR-3613-5p in the 3′ UTR of the AQP4 gene according to TargetScan 8.0. **(E)** Relative luciferase activity after co-transfection of SGC-7901 cells with pGL3, pGL3-AQP4-WT, pGL3-AQP4-MUT, as well as AgomiR-NC and AgomiR-3613-5p. Data are presented as mean ± SEM. Statistical analysis is performed using a Student's t-test and statistical significance is shown as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

analysis of the gastric mucosa revealed that, compared to AgomiR-NC, AgomiR-3613-5p exacerbated atrophy and hyperplasia of the gastric mucosal intrinsic glands, with intestinal metaplasia and the presence of goblet cells (Figure 4B). Consistently, serum levels of IL-6 and TNF-α significantly increased with miR-3613-5p overexpression, indicating that miR-3613-5p promotes the progression from CAG to gastric cancer in mice (Figures 4C,D). Subsequently, we suppressed miR-3613-5p expression in mice using AntagomiR-3613-5p (Figure 4E) and observed a significant reduction in glandular atrophy, with partial recovery in gland morphology and decreased hyperplasia (Figure 4F). Treatment with AntagomiR-3613-5p also significantly decreased serum levels of IL-6 and TNF-α (Figures 4G,H). This suggests that inhibiting miR-3613-5p expression can alleviate mucosal atrophy, hyperplasia, and inflammatory accumulation in CAG in mice.

Result 5: miR-3613-5p inhibits the expression of the AQP4 gene by binding to its 3′UTR

The GSE130823 dataset includes gene expression data from 94 gastric cancer tumor tissues and paired control biopsy samples. From this dataset, we identified genes significantly downregulated in gastric cancer and intersected them with miR-3613-5p predicted target genes from TargetScan 8.0, resulting in 39 potential target genes (Figure 5A). Based on differential expression significance, we measured the expression of the top five genes by RT-PCR in the SGC-7901 cell line and found that AQP4 mRNA was the most significantly downregulated (Figure 5B). For external validation, we used the GSE224056 dataset, which includes whole transcriptome sequencing data from gastric cancer tissues and their corresponding adjacent non-tumorous tissues from five patients. By integrating gene expression data with microRNA profiles, we found a negative correlation between miR-3613-5p and AQP4 expression (Figure 5C). A similar trend in AQP4 expression was observed in the Cancer Genome Atlas (TCGA) database (Supplementary Figure S1). We identified the predicted binding sites of miR-3613-5p in the 3′ UTR of the AQP4 gene and introduced mutations at these sites (Figure 5D). We verified the effect of miR-3613-5p on AQP4 gene expression using dual-luciferase reporter assays. Overexpression of miR-3613-5p significantly inhibited expression of the wild-type (WT) AQP4 gene, but had no effect on the AQP4 gene with mutated binding sites (MUT) (Figure 5E). This suggests that miR-3613-5p inhibits AQP4 gene expression by binding to its 3′ UTR.

Result 6: miR-3613-5p promotes the progression from CAG to gastric cancer by inhibiting the expression of AQP4

Aquaporins (AQPs) are integral membrane proteins that regulate the transport of water and small molecules across cell membranes. Thirteen types of AQPs (AQP0–AQP12) have been identified in humans, playing a crucial role in maintaining water homeostasis across various tissues, including the gastrointestinal tract (Nagaraju et al., 2016). In gastric cells, AQPs are involved in regulating gastric acid secretion and maintaining epithelial integrity. Among these, AQP4 is highly expressed in the chief and parietal cells of the gastric mucosa (Misaka et al., 1996). Parietal cells, which secrete HCl, rely on AQP4 to facilitate water entry into the glands, aiding in the dilution and secretion of hydrochloric acid. Dysregulation of AQP4 expression may lead to altered acid secretion and gastric mucosal damage, potentially contributing to ulcer formation (Demitrack et al., 2012). After overexpressing miR-3613-5p in the SGC-7901 cell line, we observed a significant decrease in AQP4 protein levels (Figures 6A,B). Similarly, in CAG mice, overexpression of miR-3613-5p led to a significant decrease in AQP4 protein levels as well (Figures 6C,D). These results confirm that AQP4 is a direct target gene of miR-3613-5p. AQP4 is responsible for water transport in gastric mucosal cells (Misaka et al., 1996). By inhibiting AQP4 expression, miR-3613-5p promotes the progression from CAG to gastric cancer.

Discussion

In recent years, miR-3613-5p has garnered attention as a potential regulatory factor in tumor biology, with studies suggesting its involvement in the development of various cancers. For example, Qin et al. identified miR-3613-5p as a negative prognostic indicator for hepatocellular carcinoma through a comprehensive analysis of miRNA expression profiles from The Cancer Genome Atlas (TCGA), and KEGG enrichment analysis indicated that it may contribute to tumorigenesis by regulating several signaling pathways, including PI3K/AKT and MAPK(28). Xu et al. found that miR-3613-5p is highly expressed in HCC and can regulate the expression of the tumor marker KMO (Xu et al., 2023). Mohsen et al. reported high expression of miR-3613-5p in renal clear cell carcinoma (RCC) tissues through bioinformatics analysis, where it was associated with clinical parameters such as pathological staging and histological grading (Ahmadi et al., 2023). In a clinical model of RCC constructed by Zhang et al., miR-3613-5p emerged as a prognostic indicator (Zhan et al., 2021). Luo et al. discovered that miR-3613-5p is upregulated in extracellular vesicles of breast cancer and regulates progression by targeting the tumor suppressor gene PTEN, enhancing resistance to doxorubicin (Luo et al., 2022). Fehmida et al. reported significantly elevated miR-3613-5p expression in tumor tissues of seven early-stage and 26 late-stage gastric cancer patients from a Saudi population (Bibi et al., 2016). However, the role of miR-3613-5p in gastric cancer has not been fully explored. Despite this, studies across various cancers confirm that miR-3613-5p is involved in regulating key processes such as cell proliferation, apoptosis, and migration, suggesting its potential role in the occurrence and progression of gastric cancer. Our research demonstrates that miR-3613-5p is significantly upregulated in gastric cancer tissue samples, and its overexpression promotes the proliferation and migration of gastric cancer cells.

Aquaporins (AQPs) are a family of small integral membrane proteins that facilitate the transport of water and small molecules across biological membranes. They play essential roles in maintaining water balance across various tissues, including the kidneys, brain, lungs, and gastrointestinal tract (Nagaraju et al., 2016). AQPs are also emerging as potential diagnostic and therapeutic targets for gastrointestinal cancers, influencing tumorigenesis by regulating cancer cell proliferation, migration, and angiogenesis (Xia et al., 2017). Among the AQPs, AQP4 is primarily expressed in the membranes of chief and

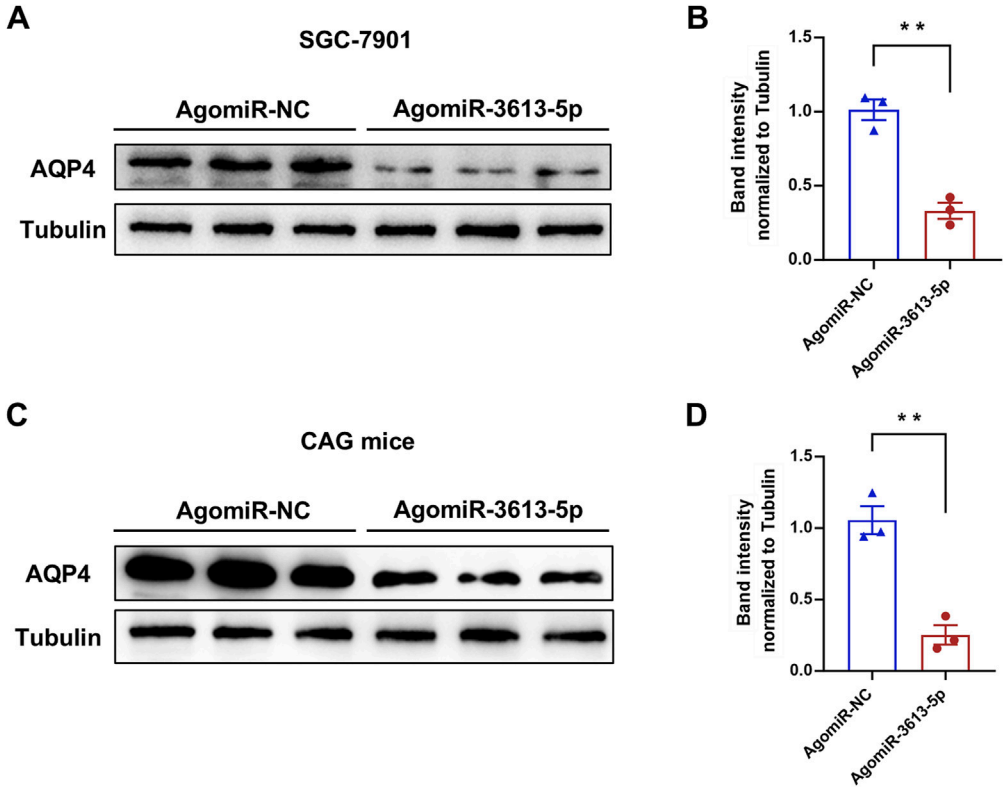

FIGURE 6
miR-3613-5p promotes the progression from CAG to gastric cancer by inhibiting the expression of AQP4. **(A)** Protein levels of AQP4 after transfection of AgomiR-NC or AgomiR-3613-5p in SGC-7901 cells. **(B)** Quantification of protein expression in **(A)**. **(C)** Protein levels of AQP4 in the gastric mucosa 1 month after tail vein injection of AgomiR-NC or AgomiR-3613-5p in MNU + HSD mice. **(D)** Quantification of protein expression in **(C)** Data are presented as mean ± SEM. Statistical analysis is performed using a Student's t-test and statistical significance is shown as *p < 0.05, **p < 0.01, and ***p < 0.001.

parietal cells in the gastric mucosa and is believed to contribute to gastric acid secretion. Parietal cells secrete HCl, and the transport of water is crucial for this process. AQP4 facilitates water entry into the gastric glands, essential for diluting and secreting hydrochloric acid. Dysregulation of AQP4 in the stomach may be linked to abnormal gastric acid secretion and the formation of ulcers (Misaka et al., 1996). In gastric cancer, AQP4 expression is typically decreased compared to normal gastric mucosa (Shen et al., 2010). This downregulation correlates with enhanced tumor invasiveness, poor prognosis, and increased cancer cell migration. Recent studies suggest that AQP4 downregulation may facilitate gastric cancer progression by promoting an inflammatory microenvironment conducive to tumor growth. For example, LINC00629 upregulates AQP4 expression by binding to miR-196b-5p, suppressing gastric cancer cell proliferation and migration (Li et al., 2020). Our findings further suggest that miR-3613-5p negatively regulates AQP4, exacerbating gastric mucosal inflammation and intestinal metaplasia, thus accelerating gastric cancer cell proliferation and migration. This implies that AQP4 may serve a protective role in gastric cancer development, highlighting its potential as a therapeutic target.

The gastric mucosa is regularly exposed to various inflammatory stimuli, such as *Helicobacter pylori* infection, oxidative stress, and gastric acid imbalance, all of which can lead to chronic inflammation (Yang et al., 2021; Butcher et al., 2017). AQP4 is involved in water transport and cellular homeostasis, contributing to the maintenance of mucosal integrity under normal conditions (Misaka et al., 1996). However, when AQP4 expression is downregulated, the gastric epithelium becomes more vulnerable to inflammatory damage, triggering cytokine signaling, particularly the activation of IL-6 and TNF-α. IL-6, a pro-inflammatory cytokine, plays a crucial role in tumorigenesis by promoting cell survival, proliferation, and immune evasion. Elevated levels of IL-6 are commonly observed in gastric cancer tissues and correlate with poor prognosis (Sánchez-Zauco et al., 2017). Our results show that overexpression of miR-3613-5p significantly increases IL-6 levels in the serum of CAG mice. The loss of AQP4 may amplify local IL-6 concentrations in CAG by promoting the activation of inflammatory cells, such as macrophages and neutrophils. IL-6 activates the JAK/STAT signaling pathway, which enhances cancer cell proliferation and resistance to apoptosis (Wu et al., 2025; Zhao et al., 2016). In gastric cancer, TNF-α contributes to angiogenesis, immune suppression, and cancer cell migration (Ji et al., 2014). In our study, serum levels of TNF-α were significantly elevated in mice with overexpression of miR-3613-5p. The downregulation of AQP4 may increase TNF-α expression in the gastric mucosa by activating the NF-κB pathway, further exacerbating inflammation and promoting cancer cell proliferation and migration (Kwon et al., 2012).

Chronic atrophic gastritis (CAG) is a precancerous lesion and is considered a critical step in the Correa cascade model of gastric

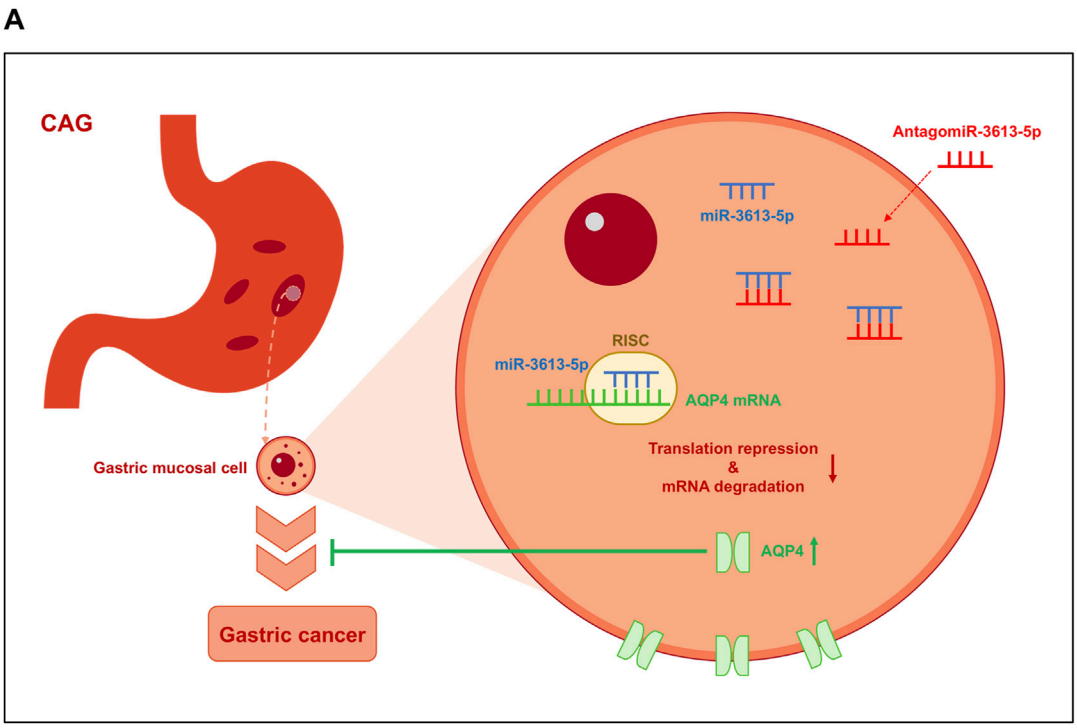

FIGURE 7
Model on how miR-3613-5p promotes the progression from CAG to gastric cancer. **(A)** miR-3613-5p inhibits the expression of the AQP4 gene by binding to its 3′ UTR, thereby promoting the progression from CAG to gastric cancer.

carcinogenesis. The progression from CAG to gastric cancer is influenced by multiple factors, including persistent inflammation, *H. pylori* infection, as well as genetic and epigenetic alterations (He et al., 2022). Intestinal metaplasia, the next stage in the Correa cascade, involves the replacement of gastric epithelial cells with intestinal-type cells. While this is considered an adaptive response to chronic inflammation, it significantly increases the risk of cancer. The underlying molecular mechanisms of this transformation involve key signaling pathways, including Wnt/β-catenin, EGFR, and Notch (Sugano et al., 2023). High-grade dysplasia is regarded as a precursor to invasive gastric cancer. At this stage, a considerable accumulation of genetic mutations and epigenetic changes occurs, driving the tissue toward malignant transformation. In our study, we found that overexpression of miR-3613-5p triggers immune responses by releasing pro-inflammatory cytokines such as IL-1β and TNF-α. Additionally, it regulates abnormal signaling pathways in gastric epithelial cells, leading to glandular atrophy and intestinal metaplasia, which in turn promote the proliferation and migration of gastric cancer cells. MiR-21 and miR-155 have been widely applied as biomarkers for early gastric cancer detection, and miR-3613-5p may also emerge as a promising new diagnostic target for assessing gastric cancer risk (Farasati Far et al., 2023).

While our study offers valuable insights into the role of miR-3613-5p in the progression from CAG to gastric cancer, there are several limitations to consider. First, the use of mouse models, though informative, may not fully capture the complexity of human gastric cancer. Additionally, the lack of long-term clinical validation means that the therapeutic potential of targeting miR-3613-5p remains to be fully explored. Future studies should aim to validate these findings in larger human cohorts and investigate combination therapies targeting miR-3613-5p to enhance treatment efficacy and prevent the progression of CAG to gastric cancer. Such approaches could pave the way for novel clinical interventions and preventive strategies for gastric cancer.

Conclusion

miR-3613-5p is significantly overexpressed in gastric mucosal tissue samples from CAG patients and in tumor samples from gastric cancer patients. High expression of miR-3613-5p was also observed in human gastric cancer cell lines and in the gastric mucosal of CAG mice. Overexpression of miR-3613-5p in gastric cancer cell lines promotes the proliferation and migration of gastric cancer cells, while silencing miR-3613-5p in CAG mice alleviates symptoms such as atrophy, hyperplasia, and inflammatory accumulation in the gastric mucosa. miR-3613-5p inhibits the expression of the AQP4 gene by binding to its 3′UTR, thereby promoting the progression from CAG to gastric cancer (Figure 7A).

Data availability statement

Experimental raw data are available at Figshare (DOI: 10.6084/m9.figshare.28632146). Bioinformatics analyses were performed using publicly available datasets from the GEO database. The processed data and scripts have been deposited in GitHub (https://github.com/YanXiu0105/AQP4-miR3613).

Ethics statement

The studies involving humans were approved by Ethics Committee of the First Affiliated Hospital of Dalian Medical University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by Laboratory Animal Care and Use Committee of Hubei Provincial Center for Disease Control and Prevention. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JB: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. YuW: Data curation, Resources, Software, Validation, Writing–original draft, Writing–review and editing. YiW: Conceptualization, Methodology, Resources, Writing–original draft, Writing–review and editing.

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Conflict of interest

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

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2025.1523689/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Pearson correlation coefficient between AQP4 and miR-3613-5p. Data from The Cancer Genome Atlas (TCGA) database (https://www.cancer.gov/ccg/access-data).

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