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Emerging horizons of metformin: exploring recent advances and addressing challenges in research and clinical utilization

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Emerging horizons of metformin: exploring recent advances and addressing challenges in research and clinical utilization

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Editorial: Emerging horizons of metformin: exploring recent advances and addressing challenges in research and clinical utilization

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Editorial on the Research Topic
Emerging horizons of metformin: exploring recent advances and addressing challenges in research and clinical utilization

Drug repurposing, or drug repositioning, is a popular strategy in drug development that aims to accelerate the process and reduce costs associated with discovering new drugs. (Tanoli et al., 2025). Metformin is an FDA-approved medication for type 2 diabetes that is also being researched for other potential uses (Saengboonmee et al., 2021; Foretz et al., 2023). Metformin has been shown to have multiple beneficial effects on various non-communicable diseases, particularly those related to metabolic dysregulation, such as obesity (Abbasi et al., 2022; 2024a; Abbasi et al., 2024b), neurodegenerative diseases (Kruczkowska et al., 2025), and cancers (Saengboonmee et al., 2017; Panaampon et al., 2023). Metformin's mechanisms are not fully understood, but it is known to enhance insulin sensitivity, activate AMP-activated protein kinase (AMPK), inhibit mitochondrial respiratory complex I, and modulate gut microbiota. Additionally, it impacts lipid metabolism and inflammatory signaling, promotes autophagy, improves DNA repair, and reduces adipogenesis and adipokine secretion. Currently, metformin is a major focus of research beyond diabetes treatment, with many studies showing promising results already applied in clinical practice (Teede et al., 2023). This Research Topic compiles articles and reviews on the potential repurposing of metformin, aiming to deepen our understanding of its effects and benefits in various diseases.

This Research Topic presents seven original articles, including a systematic review, meta-analysis, and a brief research report. The studies cover preclinical models to randomized controlled trials, featuring a review and a perspective on metformin's advancements. Metformin has shown real-world therapeutic efficacy in four clinical studies, including two randomized controlled trials. A pilot study by AlRasheed et al. found that Parkinson's disease patients receiving levodopa/carbidopa along with metformin experienced significant improvements on the Unified Parkinson's Disease Rating Scale

(UPDRS) compared to their baseline scores. In contrast, those receiving only levodopa/carbidopa showed no significant changes. While final UPDRS scores were not significantly different between the groups, biomarkers in the metformin group indicated a trend toward positive outcomes. A double-blind, randomized, controlled trial by Binsaleh et al. found that adding metformin to standard treatment for mild to moderate ulcerative colitis may slow disease progression, as shown by a reduced disease activity index. A group of patients receiving metformin alongside mesalamine, a standard treatment for inflammatory bowel diseases, showed significantly decreased inflammation biomarkers and disease severity. This suggests metformin may have anti-inflammatory effects that could benefit other inflammatory conditions. Additionally, a clinical study reported that metformin concentrations affected plasma galectin-3 levels, a biomarker linked to polycystic ovary syndrome (PCOS), in patients preparing for *in vitro* fertilization, as noted by Nikolic et al. Metformin plasma concentration showed a weak correlation with galectin-3 levels, but its dosage was linked to plasma galectin-3 levels. This suggests that personalized metformin dosing is important for managing PCOS. Furthermore, another study indicated that metformin improved symptoms, knee joint function, and quality of life in individuals with impaired glucose tolerance and knee osteoarthritis (Halabitska et al.). Metformin is recommended for diabetes prevention in individuals with impaired glucose tolerance, and this study suggests that its early use may also benefit those without hyperglycemia. Patients with diabetes on metformin may not need to stop their medication before medical imaging with contrast agents. A systematic review and meta-analysis by Xu et al. found no significant impact of metformin on the risk of contrast-induced acute kidney injury, impaired renal function, or elevated lactate levels.

In addition to clinical studies, the present issue also delves into mechanistic studies using preclinical models, both *in vitro* and *in vivo* experiments. The anti-inflammatory effects of metformin were emphasized in a mouse model of neovascular age-related macular degeneration (AMD). The insight into the intracellular signaling of inflammation-mediated AMD was also reported in the same study by Wang et al. Mice with AMD that were treated with metformin showed significantly reduced retinal vascular leakage and neovascularization, as well as lower levels of inflammatory markers and phosphorylated STAT3, a key transcription factor involved in inflammation. However, it was noted that retinal fibrosis increased in mice receiving metformin, indicating that while metformin might be beneficial for specific stages of AMD, further studies are needed to explore this more thoroughly. The perspective of metformin's roles, as well as other anti-diabetic drugs, on the prevention of AMD was also reported by Zhou and Xue in the present issue.

The AMPK-dependent effects of metformin are not only involved in anti-inflammation, but they also protect osteoblast cells from ferroptotic cell death under diabetic conditions. Using metformin treatment for patients with diabetes may help prevent diabetic osteoporosis by protecting osteoblasts, as shown by *in vitro* and *in vivo* experiments conducted by Liu et al. The results from this study are consistent with the clinical findings in the aforementioned article of Halabitska et al. Finally, the anti-cancer effects of metformin remain a hot issue in the present decade, as shown in the growing evidence in several cancers. The updated overview of metformin's benefit in treating gynecological disorders, including cancers, is also included in this issue (Nie et al.).

This Research Topic enhances our understanding of metformin and its potential applications beyond diabetes treatment, suggesting it remains a strong candidate for drug repurposing. Already integrated into some clinical practices, metformin is the focus of extensive research from preclinical studies to clinical trials. Despite its long history and benefits for various conditions, the underlying molecular mechanisms of its effects have not been fully clarified (Foretz et al., 2023). Caution is advised in using metformin for age-related macular degeneration (AMD) treatment. A recent study suggests that while metformin may be beneficial, it could also increase the risk of retinal fibrosis in animal models. Furthermore, the pharmacokinetics of metformin in patients with diverse conditions is essential for its effective use. Future research on metformin's molecular mechanisms and clinical benefits is expected to be important over the next decade, potentially broadening its medical applications (Zhou and Xue, 2025). This Research Topic issue could highlight the potential of repurposing drugs in drug discovery to improve human health and inspire further research into metformin and other drugs for treating various diseases.

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Revolutionary drug repositioning: the preventive and therapeutic potential of metformin and other antidiabetic drugs in age-related macular degeneration

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Age-related macular degeneration (AMD) is a leading cause of blindness among the elderly worldwide. Anti-vascular endothelial growth factor (anti-VEGF) injections remain the first-line therapy for AMD. However, their high cost and the need for frequent administration pose challenges to long-term adherence, highlighting the need for accessible and cost-effective preventive strategies. Emerging evidence suggests that traditional antidiabetic drugs, such as metformin, sulfonylureas, and thiazolidinediones, may offer neuroprotective benefits, opening new avenues for AMD prevention. Among these, metformin has emerged as the most promising candidate, demonstrating significant potential in reducing AMD risk, even at low cumulative doses, primarily through AMP-activated protein kinase (AMPK) activation. Sulfonylureas, although effective in stimulating insulin secretion, carry risks such as hypoglycemia, hyperinsulinemia, and a possible association with increased cancer risk. Similarly, thiazolidinediones, while improving insulin sensitivity, are associated with adverse effects, including cardiovascular risks and macular edema, limiting their broader application in AMD prevention. This paper explores the preventive potential and underlying mechanisms of these antidiabetic drugs in AMD and discusses the role of artificial intelligence in optimizing individualized prevention strategies. By advancing precision medicine, these approaches may improve public health outcomes and reduce the burden of aging-related vision loss.

KEYWORDS

age-related macular degeneration (AMD), antidiabetic drugs, metformin, AMPK activation, precision medicine

1 Introduction

1.1 The global challenge of AMD: an unresolved issue

Age-related macular degeneration (AMD) is a degenerative eye disease primarily affecting individuals aged 55 years and older, and it is a leading cause of irreversible vision loss in developed countries (Li et al., 2020). Globally, approximately 8.7% of the population is affected by AMD, with an estimated 196 million patients in 2020, projected to increase to 288 million by 2040 (Wong et al., 2014). Late-stage AMD includes neovascular (wet) and geographic atrophy (late dry, GA) forms, both of which

are closely associated with significant vision loss. Major risk factors include smoking, poor nutrition, cardiovascular disease, and genetic predisposition (Lim et al., 2012; de Jong et al., 2020). Early symptoms of AMD include blurry vision, central vision loss, and distorted lines, which may ultimately lead to complete central vision loss. These impairments severely impact daily life and increase the risk of mental health issues such as anxiety, depression, and social isolation (Gheorghe et al., 2015; Hwang et al., 2023a).

From an economic perspective, the treatment costs of AMD impose a significant burden on individuals, families, and society. Neovascular AMD is a primary cause of irreversible vision loss, with patients incurring an average cost of €17,265 in the first year post-diagnosis, primarily attributed to direct medical expenses (Abraldes et al., 2024). Although anti-vascular endothelial growth factor (anti-VEGF) therapy is the current mainstay of treatment, its high cost and the need for frequent injections make it difficult for many patients, particularly those with lower incomes, to maintain long-term treatment adherence (Brown et al., 2021; Spooner et al., 2018). Therefore, there is an urgent need to develop new, cost-effective, and broadly applicable treatment and prevention strategies, especially given the growing patient population and increasing aging demographic.

1.2 The unexpected potential of antidiabetic drugs: a possible game changer

In recent years, traditional antidiabetic drugs have shown potential in treating a variety of diseases, prompting renewed attention from the academic community. Diabetes, especially type 2 diabetes, is considered a potential risk factor for AMD (Hwang et al., 2023b; Chen et al., 2014). Diabetes-induced oxidative stress, chronic inflammation, and the accumulation of advanced glycation end products may contribute to the development of AMD by damaging the retinal pigment epithelium (RPE) and endothelial cells (Jadeja and Martin, 2021; Tian et al., 2005; Dionysopoulou et al., 2023; Amato et al., 2021).

In this context, antidiabetic drugs, particularly metformin, have garnered increasing attention for their potential in preventing AMD progression. Research indicates that metformin may offer neuroprotection by improving metabolic status and reducing inflammation (Romdhoniyyah et al., 2021; Francisco and Rowan, 2023; Du et al., 2022; Brown et al., 2019), especially in high-risk elderly populations, thereby delaying disease progression and enhancing quality of life (Holtz et al., 2023; Amin et al., 2022; Khanna et al., 2022; Kaufmann et al., 2023). Other antidiabetic drugs, such as sulfonylureas and thiazolidinediones, have also shown potential for AMD prevention in early studies (Francisco and Rowan, 2023; Picard et al., 2024). These findings suggest that drug repurposing may provide new preventive pathways and offer a more cost-effective solution for AMD patients. Additionally, novel delivery systems, such as lipid-based nanoparticles, may enhance ocular bioavailability and support the application of antidiabetic drugs in targeting the posterior segment of the eye (Puglia et al., 2021). Therefore, this paper will further explore the potential and practical applications of these antidiabetic drugs in AMD prevention and treatment.

2 Multifunctional mechanisms of diabetes drugs: from glucose lowering to retinal protection

2.1 Retinal protective effects of metformin: an affordable star drug

Metformin, a traditional antidiabetic drug, has garnered increasing attention in recent years for its potential protective effects against AMD. These protective effects involve several interrelated mechanisms. Firstly, metformin activates the AMP-activated protein kinase (AMPK) pathway, which plays a key role in protecting RPE cells by inhibiting oxidative stress and inflammation (Datta et al., 2017; Xu et al., 2018). AMPK activation inhibits Mechanistic target of rapamycin (mTOR) and activates the Unc-51-like kinase (ULK) complex, initiating autophagy to clear damaged organelles and decrease reactive oxygen species (ROS) This leads to the inhibition of NOD, LRR and pyrin domain-containing protein 3 (NLRP3) inflammasome activation, thereby protecting RPE cells (Meyer et al., 2019; Zhao et al., 2020; Yang et al., 2019). Furthermore, metformin activates mitophagy, reducing mitochondrial ROS (mtROS) and chronic inflammation (Lu et al., 2021; Toppila et al., 2024). Additionally, AMPK activation also enhances mitochondrial function by improving oxidative phosphorylation, restoring ATP levels, and meeting the high metabolic demands of RPE cells, thus maintaining mitochondrial homeostasis (Xu et al., 2018; Dieguez et al., 2024). Through these combined mechanisms, metformin protects RPE cells from oxidative damage and is hypothesized to slow the progression of AMD.

One of the pathological features of advanced AMD is vascular endothelial growth factor (VEGF)-driven neovascularization (Ohno-Matsui et al., 2001). Studies have shown that metformin inhibits pathological neovascularization by downregulating the VEGF receptor Flk1, which has potential benefits for preventing or treating nAMD (Joe et al., 2015; Han et al., 2018). However, the effect of metformin on angiogenesis is inconsistent across studies, potentially due to tissue-specific differences, necessitating further research to clarify these mechanisms (Dallaglio et al., 2014). Recent studies have also shown that metformin indirectly inhibits neovascularization by modulating the gut microbiome (Zhang et al., 2023). Metformin increases the abundance of Bifidobacterium and Akkermansia and promotes the production of short-chain fatty acids, thereby reducing pathological retinal neovascularization through the "gut-retina axis." In addition to neovascularization, epithelial-mesenchymal transition (EMT) is another critical pathological process in late-stage AMD that is associated with subretinal fibrosis (Wu et al., 2022; Shu et al., 2020). Metformin inhibits EMT by upregulating microRNA-140-3p, suppressing Lin-28 Homolog B activity, and consequently downregulating the JNK/STAT3 pathway, reducing fibrosis (Hua et al., 2023; Mitra et al., 2024; Wang et al., 2021). The detailed mechanisms are illustrated in Figure 1.

In addition to the support from mechanistic studies, recent epidemiological research has also provided evidence for the preventive role of metformin in reducing the risk of AMD. Multiple studies, as summarized in Table 1, have demonstrated a significant association between metformin use and reduced AMD

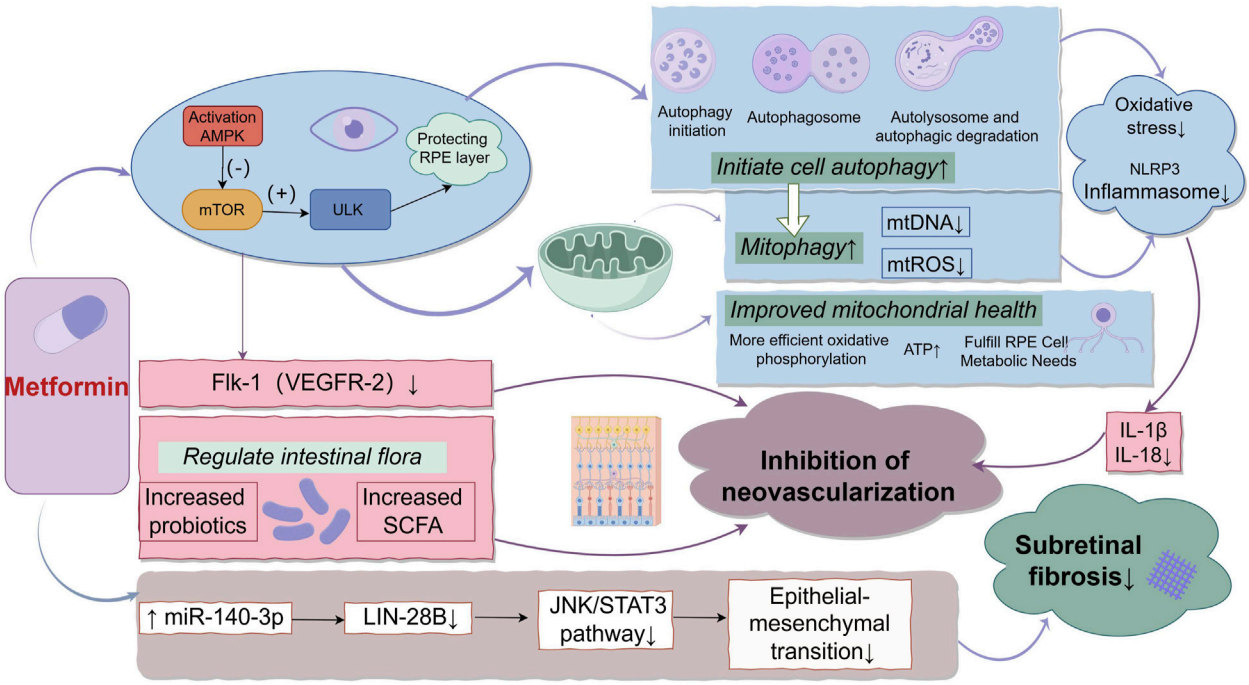

FIGURE 1
The Multifaceted Mechanisms of Metformin in AMD. Illustrates the multifaceted mechanisms of metformin in the treatment of AMD. Metformin activates the AMPK pathway, which inhibits oxidative stress and inflammation. The activation of AMPK initiates both cellular and mitochondrial autophagy, reducing the release of mitochondrial DNA and ROS, thereby inhibiting NLRP3 inflammasome activation and protecting RPE cells. In addition, AMPK improves mitochondrial function, restores ATP levels, and meets the high metabolic demands of RPE cells. Metformin also inhibits pathological neovascularization by downregulating the VEGF receptor (Flk-1/VEGFR-2). Furthermore, it modulates the gut microbiome, increasing SCFA production, which further suppresses retinal neovascularization. Lastly, metformin upregulates miR-140-3p, suppresses LIN28B activity, and inhibits the JNK/STAT3 pathway, thereby reducing EMT and inhibiting subretinal fibrosis.

risk, with some studies highlighting a dose-response relationship (Khanna et al., 2022; Kaufmann et al., 2023; Moir et al., 2024; Aggarwal et al., 2024; Jiang et al., 2022; Tseng, 2023). Furthermore, a recent meta-analysis integrating multiple studies has further supported this protective trend (Holtz et al., 2023). However, some studies have not found a significant effect of metformin on AMD risk, indicating that, while the overall evidence leans positive, the heterogeneity among study results warrants attention (Shen et al., 2024; Eton et al., 2022; Huang et al., 2023). Recent epidemiological research has predominantly supported the preventive role of metformin in AMD, while its potential therapeutic effects remain under investigation. Therefore, larger-scale, well-designed prospective studies are needed to clarify the actual efficacy of metformin in AMD prevention and treatment.

2.2 Neuroprotective effects of sulfonylureas: untapped potential

Glibenclamide, a traditional sulfonylurea used to control blood glucose levels in type 2 diabetes, has recently attracted attention for its neuroprotective effects in the retina. By targeting sulfonylurea receptor 1 (SUR1) and co-localizing with potassium channels (Kir6.2) and cation channels (TRPM4), glibenclamide modulates ion flow across the cell membrane, reducing cell depolarization and effectively mitigating oxidative stress-induced damage to RPE cells. Additionally, it inhibits the activation of the NLRP3 inflammasome, thereby reducing chronic inflammatory responses (Berdugo et al., 2021; Zhang et al., 2017; He et al., 2022), improving retinal cell function, balancing growth factors, and reducing retinal damage (Wong et al., 2014) as well as extending the lifespan of retinal ganglion cells (Conti et al., 2022; Chou et al., 2018). Recent *in vitro* experiments and case-control studies have demonstrated that glibenclamide protects cone cells from oxidative stress and apoptosis, thereby lowering the risk of developing late-stage dry AMD (Picard et al., 2024). However, large-scale clinical trials are currently lacking, and further research is needed to determine the practical application and feasibility of glibenclamide in AMD prevention and treatment.

2.3 Thiazolidinediones: a double-edged sword for retinal protection

Thiazolidinediones (TZDs), such as rosiglitazone and pioglitazone, are Peroxisome proliferator-activated receptor gamma (PPARγ) agonists initially used to control blood glucose levels in type 2 diabetes but have shown complex effects in AMD. TZDs can inhibit VEGF gene promoter activity, reducing VEGF expression and suppressing neovascularization (Peeters et al., 2006). However, they may also increase VEGF levels, leading to vascular leakage and new vessel formation (Ku et al., 2017). Additionally, TZDs reduce chronic inflammation by inhibiting Tumor necrosis

TABLE 1 Metformin use and risk of AMD: Summary of evidence.

Author(s)/ Country/ Region	Study type	Database	Age	Diagnostic criteria	Sample size	AMD risk (OR/HR)	Conclusion
Moir et al. (2024), United States	Observational Cohort	Merative MarketScan Database	≥60	ICD-11	Total: 21,007 (Non-diabetic: 15,219)	OR = 0.88 (95% CI: 0.79–0.99)	Positive—Metformin reduces GA risk
Khanna et al. (2022), United States	Case-Control Study	Merative MarketScan Database	≥55	ICD-9, ICD-10	Total: 173,848	OR = 0.95 (95% CI: 0.91–0.98)	Positive—Metformin reduces AMD risk
Aggarwal et al. (2024), United States	Case-Control Study	Merative MarketScan Database	≥55	ICD-10	Total: 464,021 participants (Non-diabetic)	OR = 0.83 (95% CI: 0.74–0.87)	Positive—Reduced AMD risk in non-diabetic
Kaufmann et al. (2023), United States	Case-Control Study	Merative MarketScan Database	≥55	ICD-9, ICD-10	Total: 388,125 (Diabetic: 99,448)	OR = 0.97 (95% CI: 0.95–0.99)	Positive—Reduced dry AMD risk
Jiang et al. (2022), China	Retrospective Study	Hospital Records	≥50	ICD-10	Total: 324	OR = 0.23 (95% CI: 0.13–0.38)	Positive—Significant reduction in AMD risk
Tseng (2023), Taiwan	Retrospective Cohort	Taiwan National Health Insurance	50–79	ICD-9-CM	Total: 26,606 (Ever Users: 13,303, Never Users: 13,303)	HR = 0.756 (95% CI: 0.673–0.850)	Positive—Significant reduction in AMD risk
Shen et al. (2024), United States	Randomized Phase II	Multi-center Study	≥55	Image-based Diagnosis of GA	Total: 66 participants (Non-diabetic)	Rate Difference = 0.07 mm/year (95% CI: −0.05 to 0.18, p = 0.26)	Neutral—No significant effect on GA progression
Elhalag et al. (2024), United States	Meta-analysis	PubMed, Scopus, Web of Science	Unlimited	Various	Total: 1,447,470 patients (Diabetic)	OR = 0.37 (95% CI: 0.14–1.02), p = 0.05	Neutral—No significant difference
Huang et al. (2023a), Taiwan	Cohort Study	Taiwan National Health Insurance	≥50	ICD-9, ICD-10	Total: 728,703 new (Diabetic)	OR = 0.93 (<5 defined daily dose/month); OR = 1.39 (>25 defined daily dose/month)	Neutral—Dose-dependent association with AMD risk
Eton et al. (2022), United States	Retrospective Cohort	Clinformatics™ Database	≥55	ICD-9, ICD-10	Total: 1,007,226 (Diabetic)	Current Users: HR = 1.08 (95% CI: 1.04–1.12); Prior Users: HR = 0.95 (95% CI: 0.92–0.98)	Neutral—Conflicting associations observed

factor-alpha (TNF-α) (Carta et al., 2011). Due to tissue-specific effects, the impact of TZDs can vary across different pathological conditions. A 2-year study found that patients using TZDs experienced a reduction in subretinal fluid after anti-VEGF treatment, but with an associated increased risk of intraretinal fluid (IRF), indicating the need for further investigation into their long-term effects (Core et al., 2023).

3 The prospects of drug repurposing: overcoming the barriers of indications

3.1 Drug repurposing: bridging endocrinology and ophthalmology

Drug repurposing, the application of approved drugs to new indications, has gained significant attention in recent years. Its advantages include shortening drug development timelines, reducing costs and risks, and accelerating clinical translation to benefit more patients (Pushpakom et al., 2019). Classic examples include Viagra (originally developed for cardiovascular conditions but later used for erectile dysfunction and pulmonary hypertension) (Ghofrani et al., 2006), and Thalidomide (repurposed from a morning sickness treatment to a therapy for leprosy and multiple myeloma) (Wang et al., 2016). The repurposing of diabetes drugs, such as metformin, holds the potential to offer a more affordable and accessible treatment option for patients. This interdisciplinary approach shows promise in providing safe and effective strategies for managing retinal degenerative diseases, including AMD.

3.2 Exploring applications beyond diabetes: a revolutionary approach

A bold but worth-exploring question is whether metformin could be integrated into health management plans for high-risk

elderly populations to prevent AMD. Similar to the widespread use of aspirin in cardiovascular prevention, metformin's potential preventive effects in non-diabetic populations are gaining attention. A recent study in JAMA Ophthalmology found that metformin use was associated with a reduced risk of AMD, even in patients without diabetes (Aggarwal et al., 2024). However, it is important to interpret this finding with caution. Most evidence supporting metformin's role in AMD prevention, including this study, comes from observational data, which may not entirely exclude the possibility of undiagnosed diabetes among registered metformin users. Since metformin is primarily prescribed for type 2 diabetes, it is likely that some of these users were in prediabetic or early diabetic stages. This limitation highlights the necessity of future prospective studies to validate metformin's independent preventive effects in strictly non-diabetic populations and to elucidate its underlying mechanisms.

In comparison, the potential of sulfonylureas and TZDs for AMD prevention is more limited. Sulfonylureas, though effective in stimulating insulin secretion, are associated with higher risks of hypoglycemia, hyperinsulinemia (Tseng and Tai, 1992), and possibly cancer (Hsieh et al., 2012). TZDs, while improving insulin resistance, carry risks such as cardiovascular events with rosiglitazone and bladder cancer with pioglitazone (Tseng, 2012). Their association with macular edema further restricts their potential use in retinal disease prevention (Nien and Tseng, 2014).

Metformin's anti-inflammatory and antioxidant properties not only support AMD prevention but also suggest broader benefits, including anti-aging, cardiovascular protection, cancer prevention, and depression management (Kulkarni et al., 2020; Dihoum et al., 2023; Yao et al., 2024; Ríos et al., 2024; Syed et al., 2022). Additionally, epidemiological studies indicate metformin may reduce risks of dementia (Chin-Hsiao, 2019), hypertension (Tseng, 2018), atrial fibrillation (Tseng, 2021a), heart failure (Tseng, 2019), and inflammatory bowel disease (Tseng, 2021b). These findings highlight metformin's unique value across multiple fields, supporting its potential as a widely applicable preventive medication.

4 Safety, controversies, and risks

4.1 Long-term use in non-diabetic populations: side effects and solutions

While metformin shows potential in preventing AMD in non-diabetic populations, its long-term use raises some safety concerns. The most common side effects are gastrointestinal issues, including diarrhea, nausea, and abdominal discomfort, particularly during the early stages of treatment (Bonnet and Scheen, 2017). Another key concern is vitamin B12 deficiency, which may lead to anemia and neurological symptoms, especially in elderly patients (Infante et al., 2021). Regular monitoring of vitamin B12 levels and supplementation when necessary is recommended for patients on long-term metformin therapy (Shahjahan et al., 2024). Although rare, there is a risk of lactic acidosis, particularly in patients with impaired liver or kidney function (Visconti et al., 2016). Notably, recent studies have highlighted that type 2 diabetes patients hospitalized for heart failure and/or acute coronary syndrome may face an elevated risk of metformin-related lactic acidosis, which, though infrequent, can be fatal (Tseng, 2024). This underscores the importance of careful patient selection and monitoring when prescribing metformin in populations with comorbidities.

Genetic testing can identify individuals most likely to benefit from metformin while minimizing side effect risks. Variations in organic cation transporter 1 (OCT1), encoded by the SLC22A1 gene, significantly influence metformin absorption and efficacy (Chan et al., 2018). For instance, the rs72552763 (Met420del) variant reduces drug uptake, increasing gastrointestinal side effects, while rs628031 (Met408Val) may lower OCT1 expression, affecting absorption and efficacy differently across populations (Aladhab et al., 2023; Mato et al., 2018). Incorporating genetic testing into clinical practice enables targeted therapy by identifying high-risk individuals, optimizing metformin use, and supporting personalized strategies, particularly for AMD prevention.

4.2 Should metformin be combined with anti-VEGF therapy?

Current research on metformin primarily focuses on its preventive effects against AMD; however, a few studies have begun to explore its potential in treating AMD (Ebeling et al., 2022; Luo et al., 2021). Anti-VEGF therapy is the current standard treatment for wet AMD, significantly improving vision by inhibiting the growth of pathological neovascularization (Amoaku et al., 2015). While there is no direct evidence supporting metformin as a standalone treatment for AMD, findings from diabetic macular edema (DME) research provide valuable insights. For instance, one study reported that combining metformin with anti-VEGF therapy significantly improved visual acuity and central macular thickness in DME patients, while reducing the frequency of anti-VEGF injections (Shao et al., 2022). Another study suggested that metformin may enhance vision recovery, reduce retinal thickness, and mitigate anti-VEGF resistance (Uwimana et al., 2022). Although these findings hint at a potential auxiliary role for metformin in AMD treatment, they are primarily derived from DME studies. High-quality clinical trials are needed to determine whether similar benefits can be observed in AMD patients. Future research should also clarify the underlying mechanisms and assess the safety and efficacy of metformin when used in combination with anti-VEGF therapy.

5 Future directions: establishing a new framework for AMD prevention

5.1 Personalized treatment and AI optimization

Future research should focus on identifying which patients are most likely to benefit from metformin for AMD prevention. Additionally, its potential role in treatment for certain AMD subtypes could also be explored. By integrating patient genetic profiles, inflammation levels, and other biomarkers with the chemical properties of the drug, AI (Artificial Intelligence) can combine genetic or proteomic data with chemical structures to score treatment effectiveness, helping to select patients who are

most likely to respond favorably (Romm and Tsigelny, 2020). Additionally, AI can predict drug interactions based on structural and target similarities, optimizing dosage regimens to maximize efficacy (Chen et al., 2023). These AI-driven approaches will contribute to building personalized treatment models, shifting AMD management from "one-size-fits-all" to precision medicine.

5.2 The necessity of clinical trials and multidisciplinary collaboration

The successful implementation of drug repurposing requires close interdisciplinary collaboration. Experts in ophthalmology, endocrinology, and public health should work together to design clinical trials that assess the efficacy of metformin for AMD prevention in diverse populations. Multicenter collaborations can ensure that the treatment is applicable to a broad patient base, particularly in resource-limited regions. Additionally, the involvement of social scientists can effectively evaluate the societal acceptance and cost-effectiveness of repurposed drugs, facilitating global adoption.

5.3 Integrating metformin into elderly health management

Incorporating metformin into elderly health management as a preventive medication could be a promising future strategy. For elderly individuals with high AMD risk factors (e.g., family history, smoking, malnutrition), metformin may play a critical role in reducing AMD risk and slowing disease progression. Early intervention with metformin could not only lower AMD incidence but also reduce healthcare costs. Governments and health organizations should support related research and develop guidelines to implement this preventive strategy, achieving true "prevention before disease" in public health.

6 Conclusion

The application of metformin has extended beyond diabetes management, with recent studies highlighting its unique advantages in AMD prevention and treatment. This paper explores the strategy of drug repurposing, positioning this "cost-effective" medication as a solution to the challenge of vision loss in AMD. Compared to existing high-cost treatments, the cross-application of metformin, especially in resource-limited areas, may offer a more affordable alternative. While research on sulfonylureas and TZDs in AMD prevention remains preliminary, their neuroprotective effects provide important directions for future study. Additionally, integrating AI to predict drug selection and individual responses could help advance precision medicine in ophthalmology. Overall, metformin not only offers new hope for AMD patients but also presents a novel opportunity for health management in an aging society.

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

YTZ: Conceptualization, Visualization, Writing–original draft, Writing–review and editing. FX: Conceptualization, Supervision, Writing–original draft, Writing–review and editing.

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Conflict of interest

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

Generative AI statement

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

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Metformin dosage and galectin-3 levels: insights from PCOS patients preparing for IVF

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This study explores the impact of metformin dosage and hyperprolactinemia on galectin-3 levels in women with Polycystic Ovary Syndrome (PCOS), providing novel insights into their roles in the metabolic and hormonal management of the condition. A cohort of 53 women, diagnosed using the Rotterdam criteria and undergoing *in vitro* fertilization (IVF) preparation, was analyzed to determine how these factors influence galectin-3, a biomarker in PCOS. Using high-performance liquid chromatography to measure metformin concentrations and ELISA for galectin-3, our results revealed that both metformin dosage and hyperprolactinemia significantly statistically associated with galectin-3 levels, while body mass index (BMI) showed no significant association. These findings challenge prior assumptions and suggest that galectin-3 may be regulated via pathways independent of metformin pharmacokinetics. Notably, the correlation between galectin-3 levels and metformin concentration was either absent or weak after adjusting for the daily dose, indicating that treatment duration and dosage, rather than absolute drug levels, may more critically influence galectin-3. This study offers deeper insights into the role of personalized metformin dosing in managing PCOS, enhancing the understanding of metabolic and hormonal regulation in this condition, and laying the groundwork for future targeted therapies.

KEYWORDS

polycystic ovary syndrome (PCOS), galectin-3, metformin, hyperprolactinemia, body mass index, metabolic pathways, insulin resistance

1 Introduction

Polycystic Ovary Syndrome (PCOS) affects up to 10% of women of reproductive age and is characterized by hyperandrogenism and chronic anovulation (Escobar-Morreale, 2018; Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group, 2004). This condition is intricately linked with significant metabolic disturbances, including insulin resistance and dyslipidemia, which elevate the risk of developing type 2 diabetes and cardiovascular diseases. These metabolic alterations, exacerbated by chronic low-grade inflammation, make insulin resistance particularly central to the pathology of PCOS, guiding therapeutic interventions such as metformin (Rudnicka et al., 2021). While recent studies have illuminated the role of metformin in enhancing endothelial function in women

with PCOS (Agarwal et al., 2010), the broader impacts of this medication on additional biomarkers remain less understood. This gap in knowledge suggests the need for further investigation into how treatments like metformin affect various aspects of PCOS beyond the commonly studied parameters. Within this context, galectin-3 emerges as a biomarker of interest, offering insights into the complex interactions between insulin resistance, chronic inflammation, and PCOS (Kruszewska et al., 2022).

Galectin-3 plays a critical role in modulating trophoblast functions such as invasion and vascular formation in normal pregnancies, which are essential for successful implantation and placental development (Chen M. et al., 2022). Research indicates that galectin-3 levels increase as a normal pregnancy progresses, suggesting its protective role in maintaining gestation (Chen M. et al., 2022; Freitag et al., 2020). Conversely, in pathological conditions like gestational diabetes mellitus, altered galectin-3 levels are observed, correlating with poor pregnancy outcomes, thus marking its significance as a potential biomarker and therapeutic target (Talmor-Barkan et al., 2020; Zhang et al., 2021). Additionally, the modulation of galectin-3 is also critical in the context of PCOS, particularly for patients undergoing *in vitro* fertilization, where its regulatory roles can impact both the implantation success and the overall health of the pregnancy (Chen M. et al., 2022; Freitag et al., 2020).

Recent studies suggest that hyperprolactinemia associated with PCOS may arise from various independent etiologies rather than a direct pathophysiological link (Delcour et al., 2019). While prolactin-secreting pituitary adenomas, a common manifestation among PCOS patients, pose concerns, their presence is not universal (Delcour et al., 2019). Nonetheless, galectin-3 has been identified as a key marker in assessing the aggressiveness of these adenomas, shedding light on their potential implications within the context of PCOS (Dai et al., 2014). This connection not only underscores the significance of galectin-3 in understanding the metabolic aspects of PCOS but also highlights its potential role in managing reproductive health challenges linked to pituitary abnormalities.

Recent studies have begun to explore the interaction of metformin with galectin-3 levels. However, significant gaps remain in our understanding, particularly concerning reproductive treatments like IVF in women with PCOS. This study aims to address these gaps by investigating how both metformin dosage and hyperprolactinemia influence galectin-3 levels, potentially offering new insights into personalized treatment protocols. To ensure the relevance and applicability of our findings, our study was conducted on a real-life cohort of women preparing for *in vitro* fertilization (IVF), with minimal exclusion criteria. This approach captures a wide range of clinical scenarios, reflecting the complexities encountered in everyday management of PCOS. By doing so, we aimed to provide insights that are directly translatable to routine clinical practice.

2 Materials and methods

2.1 Design of the study and data collection

2.1.1 Objective and design

Our study aimed to identify factors influencing galectin-3 levels in patients with Polycystic Ovary Syndrome (PCOS). We designed a comprehensive protocol to collect clinical, demographic, and lifestyle data, ensuring a robust analysis of potential influences on galectin-3 levels.

2.1.2 Participant selection

We enrolled 53 women aged 23 to 43, diagnosed with PCOS based on the Rotterdam criteria. All participants were preparing for *in vitro* fertilization and had been undergoing metformin therapy for at least 1 month. The short protocol using recombinant gonadotropins, combined with GnRH antagonists, is a commonly recommended method for ovarian stimulation in women with PCOS undergoing IVF (Kotlyar and Seifer, 2023), as it minimizes the risk of ovarian hyperstimulation syndrome (OHSS) while offering flexibility and efficiency in ovarian response management. Data were collected in standard clinical settings as part of routine IVF preparation, without additional interventions that could skew the results. By including patients with hyperprolactinemia, we aimed to reflect the true diversity and complexity of the PCOS population undergoing IVF treatments. This inclusion helps in understanding the broader implications of metabolic and hormonal interactions in PCOS, enhancing the relevance and applicability of our findings across a wider spectrum of the PCOS community.

2.1.3 Data collection

Clinical and demographic data were systematically extracted from medical records. Lifestyle habits were assessed through structured interviews with each participant, providing insights into non-clinical factors that might influence galectin-3 levels.

2.1.4 Sample collection and analysis

Blood samples were collected from each participant using two separate procedures. The first blood sample was collected for routine laboratory analyses, while the second sample was specifically designated for measuring metformin concentrations and galectin-3 levels. For the latter, blood was collected in tubes containing EDTA to prevent coagulation and ensure plasma preservation. Plasma was then separated from the blood cells through centrifugation and stored at the recommended temperature until analysis. Samples were drawn just before the next scheduled dose of metformin, after participants had been on therapy for at least 1 month, to ensure that steady-state concentrations were reached and to allow for a comprehensive evaluation of the impact of metformin therapy on galectin-3 levels. This approach was crucial for accurately measuring serum galectin-3 levels and understanding the interaction between metformin and galectin-3 in the context of PCOS.

2.2 Measurement of plasma Galectin-3 levels

Plasma levels of galectin-3 were quantified using an ELISA (Enzyme-Linked Immunosorbent Assay) kit provided by Elabscience Corp. (Model E-EL-H1470). This method is widely used for its high specificity and sensitivity in detecting proteins. The detection range of the galectin-3 assay was 0.16–10 ng/mL, with a sensitivity of 0.1 ng/mL, suitable for the expected concentration range in clinical samples. All procedures were performed strictly following the manufacturer's guidelines to ensure consistency and

reliability of the results. This kit was selected based on its proven accuracy and compatibility with our plasma samples, which is crucial for the validity of our findings.

2.3 Determination of metformin concentration

Chromatographic analysis was performed using Agilent Technologies 1,200 Series HPLC system with a DAD detector. The HPLC column was a Zorbax NH2 column (4.6 × 150 mm, 5.0 μm) and data were managed by the Agilent ChemStation program. Metformin hydrochloride certified reference material was purchased from Sigma-Aldrich (St. Louis, MO, United States). LC-MS grade acetonitrile and methanol were obtained from Honeywell Riedel-de Haen (Germany). A slightly modified method established by Mary Rebecca et al. was used for metformin analysis in plasma samples (Rebecca et al., 2019). The mobile phase was 100% acetonitrile and the flow rate was 0.725 mL/min. The column temperature was maintained at 30°C and the UV detector was set at 232 nm. The partial validation of analytical method was conducted according to ICH guidelines. The linearity was found to be over the metformin concentration range of 0.5-5.0 μg/mL with the linear regression coefficient of R2 = 0.988 for a 95% confidence level. The experimentally estimated values of limit of detection and limit of quantification were 0.15 μg/mL and 0.40 μg/mL, respectively. The within-run accuracy of the method was found to be 87%, while within-run precision expressed as the relative standard deviation was 9.0%.

The plasma samples were prepared by mixing with acetonitrile in the volume ratio 1:1 (v/v). After centrifugation and filtration through a 0.45 μm syringe filter, 20 μL of the sample was injected into the column. The metformin concentrations were calculated by the calibration curve method by using the external standard method (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, 2022). To account for differences in metformin dosing among participants, the dose-corrected metformin plasma concentration was calculated by dividing the measured plasma concentration by the corresponding daily dose (mg). This adjustment allows for the standardization of metformin levels, enabling a more accurate exploration of its pharmacodynamic effects, such as its impact on galectin-3 concentrations.

2.4 Ethical statement

The study protocol was reviewed and approved by the Institutional Review Boards of the University Clinical Center Nis (approval number 38212). The study adhered to the guidelines outlined in the Helsinki Declaration, emphasizing ethical principles for medical research involving human subjects. Each subject enrolled in the study received detailed information about the study protocol upon which written informed consent was obtained.

2.5 Statistical analysis

Data is shown as mean, standard deviation, and, for categorical variables, number and percentage. To compare data across groups, the Student's t-test or Mann-Whitney *U*-test was used, depending on data distribution. Correlation between continuous variables was assessed according to Pearson's correlation coefficient. Additionally, multivariate regression analysis assessed factors affecting galectin-3 and metformin concentrations. Analysis was conducted using SPSS v27.0, with statistical significance established at 0.05.

3 Results

3.1 Descriptive statistics and correlation analysis

Our study examined various parameters in women diagnosed with PCOS, summarized in Table 1. We noted several key relationships:

- Metformin Dosage and Galectin-3 Levels: There was a weak positive correlation between daily metformin dose and galectin-3 concentrations ($r = 0.366$, $p < 0.05$), suggesting that higher doses of metformin may modulate galectin-3 levels. Additionally, a weak negative correlation between galectin-3 concentrations and metformin concentration adjusted by the daily dose ($p < 0.05$) indicates complex pharmacokinetic interactions.
- Hyperprolactinemia Effects: Multivariate regression analysis, as summarized in Table 2, showed that hyperprolactinemia (Beta = 0.484, $p = 0.036$) and metformin dose (Beta = 0.461, $p = 0.050$) were significantly associated with galectin-3 levels, highlighting the influence of hormonal environment and treatment regimen. Figure 1 illustrates these findings, showing the average galectin-3 concentration and metformin dosage in PCOS patients, categorized by the presence of hyperprolactinemia. The bar heights represent the average galectin-3 levels, while the overlaid lines indicate the average metformin dosages.
- BMI Independence: Despite known influences of BMI on pharmacokinetics, BMI did not significantly affect galectin-3 levels ($p = 0.991$), suggesting an independent regulatory mechanism separate from typical metformin pharmacokinetics.

3.2 Implications of findings

The complexity of galectin-3 regulation in PCOS emphasizes the need for further investigation into the metabolic and hormonal pathways influenced by these interactions. The lack of a significant direct correlation between absolute metformin concentrations and galectin-3 levels underscores the importance of metformin dosage and administration timing, suggesting the potential for personalized treatment strategies based on individual metabolic responses. Our findings are directly applicable to clinical practice as they are based on real-world data collected from patients during routine IVF preparation. The real-life approach of our study enhances the relevance of the results and provides a better understanding of how different metformin dosages and the presence of hyperprolactinemia influence galectin-3 levels, potentially leading to more personalized therapeutic strategies.

TABLE 1 Descriptive statistics of the examined parameters and laboratory findings.

Parameter	Mean ± SD (Min-Max)	Parameter	Mean ± SD (Min-Mx)
Age	32.08 ± 4.41 (23–43)	HbA1c	31.41 ± 2.82 (26.8–38.0)
Body Mass Index	23.03 ± 2.96 (19.1–30.8)	Leukocytes (109/L)	7.54 ± 2.12 (4.5–12.7)
Hyperprolactinemia (%)	11 (20.8%)	Lymphocytes (%)	31.82 ± 6.97 (16.8–47.2)
Number of patients with hypothyroidism	12 (22.6%)	Monocytes (%)	5.37 ± 1.87 (1.9–10.6)
Number of patients with hyperthyroidism	3 (5.7%)	Granulocytes (%)	65.53 ± 8.15 (53.5–80.4)
Duration of therapy (months)	12.19 ± 12.59 (1–72)	Neutrophils (109/L)	4.24 ± 1.57 (2.27–7.99)
Metformin dosage (mg)	1,166.67 ± 577.35 (500–2,500)	Erythrocytes (1012/L)	4.55 ± 0.9 (3.91–6.08)
Glucose (mmol/L)	4.93 ± 0.69 (3.9–7.3)	Hematocrit	0.40 ± 0.03 (0.33–0.47)
Urea (mmol/L)	3.80 ± 1.13 (1.5–6.4)	MCV (fL)	88.63 ± 5.16 (70.5–96.5)
Creatinine (mmol/L)	68.38 ± 10.10 (44.8–88.2)	MCH (pg)	29.03 ± 2.52 (20.1–32.8)
Albumin (g/dL)	43.50 ± 0.71 (43.0–44.0)	MCHC (g/L)	328.92 ± 12.55 (298–351)
Cholesterol (total) (mmol/L)	5.25 ± 0.95 (3.49–7.97)	RDW (%)	12.13 ± 0.94 (10.4–15.4)
HDL-C (mmol/L)	1.47 ± 0.36 (0.63–2.52)	Trombocytes (109/L)	261.06 ± 1.52 (162–485)
LDL-C (mmol/L)	3.18 ± 0.90 (1.31–5.50)	MPV (fL)	8.34 ± 1.14 (6.4–12.3)
Triglycerides (mmol/L)	1.34 ± 0.74 (0.52–4.16)	PDW (%)	18.9 ± 2.12 (15.4–21.8)
AST (U/L)	17.15 ± 4.37 (11–28)	PCT (ng/mL)	0.02 ± 0.005 (0.01–0.04)
ALT (U/L)	18.72 ± 13.08 (7–81)	Metformin (mg/L)	1.88 ± 0.28 (1.24–2.38)
CRP (mg/L)	4.20 ± 3.88 (0.3–20.5)	Galectin-3 (pg/mL)	3.16 ± 1.17 (1.55–6.12)

Abbreviations: HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; AST, aspartate aminotransferase; ALT, alanine amiotransferase; CRP, C-reactive protein; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; MPV, mean platelet volume; PDW, platelet distribution width; PCT, procalcitonin.

TABLE 2 Multivariate regression analysis outcomes of galectin-3 levels in PCOS patients.

	Regression analysis			
	B	Se	Beta	*p*-value
Constant	4.197	2.845		0.156
Age	−0.080	0.074	−0.230	0.290
Smoking	0.185	0.603	0.077	0.763
BMI	0.002	0.138	0.004	0.991
Creatinine clearance	−0.001	0.019	−0.021	0.960
Hyperprolactinemia	1.306	0.580	0.484	0.036
Dose of metmorfin	0.001	0.001	0.461	0.050

4 Discussion

We found a significant correlation between metformin dosage and galectin-3 levels, but no association with BMI or dose-corrected metformin plasma concentration. This suggests that the total metformin dosage plays a more critical role in modulating galectin-3 levels, highlighting its pharmacodynamic effects. Our previous study Nikolić et al. (2024) demonstrated that BMI significantly influences dose-corrected plasma concentration, underscoring the pharmacokinetic impact of BMI. However, in the current study, pharmacokinetic factors such as BMI and plasma concentration were not linked to galectin-3 modulation, suggesting that the therapeutic impact of metformin may operate through pharmacodynamic mechanisms, particularly in modulating inflammatory and metabolic pathways.

In the context of emerging research on PCOS and galectin-3 levels, our study presents a unique insight into the influence of metformin dosage and hyperprolactinemia. While previous studies indicated no significant difference in galectin-3 levels between PCOS patients and healthy controls, they did find associations with metabolic parameters like BMI, insulin levels, and HOMA-IR (Alves et al., 2020). In contrast Yilmaz et al. (2022) reported significantly higher galectin-3 levels in PCOS patients compared to controls, correlating with insulin resistance markers and hormone levels. Our findings expand on this by demonstrating a direct correlation between metformin dosage and galectin-3 levels, suggesting a dose-dependent modulation of galectin-3 in PCOS management, which has not been explicitly documented before. Moreover, we report that hyperprolactinemia significantly elevates galectin-3 levels, adding another layer to the multifaceted relationship between metabolic, hormonal, and therapeutic factors in PCOS.

Our study found that hyperprolactinemia, as indicated by bromocriptine use, was significantly associated with galectin-3 levels. However, since prolactin levels were not measured at the time of blood sampling for galectin-3 and metformin, the precise

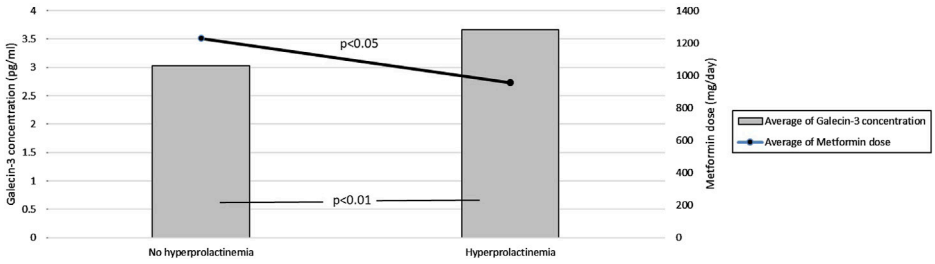

FIGURE 1
Effect of Metformin Dosage on Galectin-3 Levels in PCOS Patients with and without Hyperprolactinemia. This figure compares average galectin-3 concentrations and metformin dosages in PCOS patients with and without hyperprolactinemia. The bar heights represent mean galectin-3 levels, while the overlaid lines indicate mean metformin dosages.

role of prolactin in modulating galectin-3 levels remains unclear. Future studies should include prolactin measurements and explore the relationship between different prolactin levels (mild vs. severe) and galectin-3 regulation to better understand how prolactin influences galectin-3 in the context of PCOS.

The potential impact of gonadotropins, particularly human chorionic gonadotropin (hCG), on galectin-3 levels during ovarian stimulation protocols is an additional factor to consider in understanding our findings. Previous studies have indicated that hCG, along with hormones such as estrogen and progesterone, can regulate galectin-3 expression, particularly in endometrial cells and trophoblastic cells, which play a role in embryo-maternal interactions (Yang et al., 2013). However, since baseline galectin-3 measurements were not taken before gonadotropin therapy in our study, we cannot confirm or exclude their contribution to the observed galectin-3 levels. This limitation emphasizes the need for further research to explore the direct effects of gonadotropin therapy on galectin-3 and its role in the metabolic and hormonal imbalances associated with PCOS.

Recent studies have broadened our understanding of the pleiotropic effects of metformin beyond its glucose-lowering properties. Metformin may modulate galectin-3 levels through the mTOR signaling pathway, a critical regulator of both metabolic processes and inflammatory responses (Chen X. et al., 2022). Galectin-3 has been implicated in mTORC1 signaling, particularly in cellular metabolism and its compartmentalization in lysosomes, suggesting that the suppression of this pathway by metformin could reduce galectin-3 expression, thereby alleviating insulin resistance and chronic inflammation in PCOS (Rudnicka et al., 2021; Chen X. et al., 2022). This hypothesis provides a molecular framework for how metformin exerts its multifaceted therapeutic effects. Further research is warranted to explore this intricate molecular interaction, particularly in the context of the role of galectin-3 in the pathophysiology of PCOS and its potential as a therapeutic target.

Additionally, the effect of metformin on mitochondrial function, particularly its interaction with mitochondrial complex I, may represent another mechanism through which it alleviates metabolic disturbances in PCOS (Foretz et al., 2023). Mitochondrial dysfunction is a hallmark of PCOS, contributing to insulin resistance and inflammation, both of which are central to the condition's pathophysiology (Finsterer, 2023). By reducing oxidative stress and enhancing mitochondrial efficiency, metformin lowers inflammation and insulin resistance, key contributors to galectin-3 overexpression (Zhang et al., 2021; Zhu et al., 2023; Kingwell, 2016). Although the connection between mitochondrial modulation and galectin-3 appears indirect, reducing inflammation and improving insulin sensitivity may ultimately result in lower galectin-3 levels, further supporting the therapeutic effects of metformin in PCOS.

The dual action of metformin—on metabolic pathways and galectin-3 suppression—may underlie its overall therapeutic efficacy in managing PCOS. This is particularly relevant given that chronic low-grade inflammation is central to PCOS pathophysiology. By lowering galectin-3, which is associated with inflammatory signaling, metformin may improve clinical outcomes, including reproductive success (Zhu et al., 2023; Hirsch et al., 2012; Zhang et al., 2019).

Furthermore, the observation that galectin-3 levels were not significantly associated with metformin concentration in the studied population indicates that galectin-3 may be more sensitive to the dosage and administration pattern of metformin rather than its absolute concentration in the bloodstream. Additionally, our previous findings have demonstrated that BMI plays a significant role in influencing metformin plasma concentration, even when dosage is adjusted. Higher BMI was associated with lower dose-corrected metformin concentrations, potentially diminishing the drug's efficacy in PCOS management (Alves et al., 2020). This highlights the importance of personalized dosing strategies, particularly in individuals with elevated BMI, to optimize metformin's therapeutic effects in PCOS. This insight opens up new avenues for research into the pharmacodynamics of metformin in patients with PCOS and its interaction with galectin-3, a marker that has been increasingly recognized for its role in inflammatory processes and insulin resistance (Zhang et al., 2021; Zhu et al., 2023).

The role of hyperprolactinemia as a comorbidity in our real-life cohort offers important insights into the modulation of galectin-3 levels. Studies, such as those by Righi et al. (2010), have shown that galectin-3 is significantly upregulated in prolactin-secreting tumors, likely mediated by estrogen-related signaling pathways that influence both cellular growth and inflammation. While our cohort did not include confirmed pituitary tumors, the shared hormonal imbalances in PCOS and hyperprolactinemia suggest that similar mechanisms may contribute to elevated galectin-3 levels in these patients. The inclusion of hyperprolactinemic patients in our study strengthens the real-world applicability of our findings, but it also introduces complexities. Since galectin-3 levels were not directly correlated with metformin concentration, this may indicate a nuanced interaction between dosage, administration patterns, and the broader hormonal environment in hyperprolactinemia. Further research is warranted to

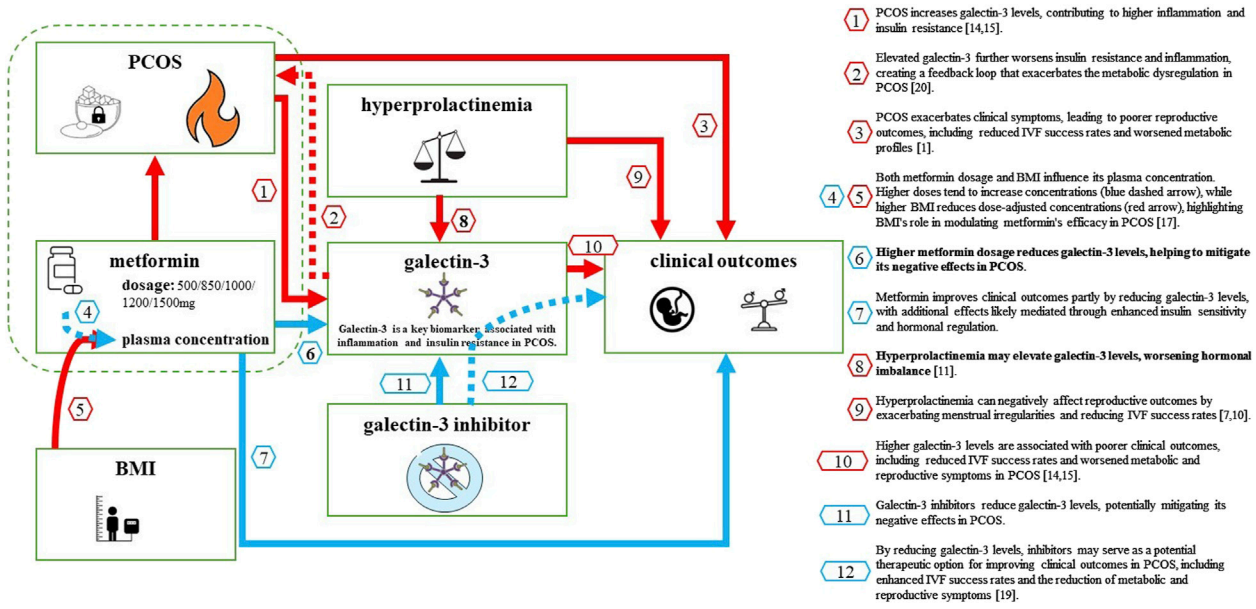

FIGURE 2
Key Factors Influencing Galectin-3 Levels in Women with PCOS and Their Impact on Clinical Outcomes. This diagram illustrates the complex relationships between factors that influence galectin-3 levels in women with PCOS and their impact on clinical outcomes. PCOS increases galectin-3 levels by elevating inflammation and insulin resistance (red arrow 1) and exacerbates clinical symptoms, leading to poorer reproductive outcomes (red arrow 2). Metformin, at higher dosages, reduces galectin-3 levels (blue arrow 3) and improves clinical outcomes both by lowering galectin-3 and through additional pathways, including enhanced insulin sensitivity (blue arrow 4). BMI also plays a significant role by reducing dose-corrected metformin plasma concentrations (red section of arrow 6), while higher metformin doses increase plasma concentrations (blue section of arrow 5), illustrating the need for personalized dosing in PCOS treatment. Hyperprolactinemia further elevates galectin-3 levels (red arrow 7) and negatively impacts reproductive outcomes (red arrow 8). Finally, potential Galectin-3 inhibitors could reduce galectin-3 levels (red arrow 10), indirectly improving clinical outcomes (dashed blue arrow 11). Fire icon: Represents inflammation, a key factor driving increased galectin-3 levels in PCOS; Sugar cube with a keyhole: Symbolizes insulin resistance, another major contributor to elevated galectin-3 levels; Unbalanced scales: Illustrates hormonal imbalance associated with hyperprolactinemia, affecting galectin-3 levels in certain patients; Crossed molecular structure icon: Represents the potential therapeutic galectin-3 inhibitor, a molecule that specifically blocks or reduces galectin-3 levels, which could help alleviate inflammation, improve insulin sensitivity, and enhance reproductive outcomes in women with PCOS. Arrow colours: Blue arrows indicate positive effects, where interventions like metformin or galectin-3 inhibitors contribute to better clinical outcomes or reduce galectin-3 levels; Red arrows indicate negative impacts, such as factors like PCOS and BMI increasing galectin-3 levels or reducing metformin efficacy.

disentangle these variables and better understand how hyperprolactinemia influences galectin-3 modulation in PCOS.

These findings emphasize the complexity of metabolic and hormonal regulation in PCOS and point to the importance of personalized treatment strategies. This real-life approach allowed for the inclusion of a broad spectrum of clinical scenarios, providing a realistic assessment of the impact of metformin and hyperprolactinemia on galectin-3 levels in women with PCOS. The inclusion of 11 participants receiving bromocriptine, while not confirming specific adenomas, contributes to understanding the association between hyperprolactinemia and galectin-3 levels in the context of IVF preparation. These findings emphasize the relevance of studying heterogeneous patient populations to uncover the complex interplay of metabolic and hormonal factors influencing galectin-3.

Additionally, galectin-3 inhibitors represent a promising area of exploration for future therapeutic interventions in PCOS. Recent advances in the development of galectin-3 inhibitors for other conditions, such as idiopathic pulmonary fibrosis and cardiovascular diseases, provide a rationale for investigating their application in PCOS management (Ahmed et al., 2023). Given the role of galectin-3 in driving both insulin resistance and inflammatory processes, targeting this pathway may offer new strategies for improving both metabolic and reproductive outcomes in women with PCOS (Kingwell, 2016; Yan et al., 2020).

Furthermore, an in-depth exploration of metformin's pharmacodynamics, particularly its interaction with galectin-3 in the presence of hyperprolactinemia, is critical. By advancing our understanding of these mechanisms, we could pave the way for more targeted, personalized treatment approaches that address the complex hormonal and metabolic dysregulation in PCOS.

4.1 Clinical implications

Our findings suggest that metformin dosage, rather than plasma concentration or BMI, plays a key role in modulating galectin-3 levels in women with PCOS. This insight highlights the importance of personalized metformin dosing strategies, particularly in patients with elevated BMI, where pharmacokinetic factors may reduce the drug's efficacy. Given the potential for galectin-3 to serve as a marker of both metabolic dysfunction and inflammation, targeting galectin-3 with inhibitors—alongside optimized metformin therapy—could provide a promising approach for improving clinical outcomes in PCOS, including metabolic and reproductive health. Large-scale clinical trials are essential to validate the use of galectin-3 inhibitors as a therapeutic strategy for PCOS (Figure 2).

4.2 Limitations

While our study offers valuable insights, it is limited by the relatively small sample size and cross-sectional design, which may affect the generalizability of the findings. Additionally, our reliance on real-world data introduces some variability in patient characteristics, particularly in terms of metformin adherence and BMI distribution. Importantly, androgen levels were not assessed in this study, precluding us from investigating potential correlations with galectin-3 levels. Despite these limitations, our findings provide a foundation for future research to explore the pharmacodynamics of metformin and galectin-3 interactions in a larger, more controlled cohort. Further studies should also assess the long-term effects of metformin dosage on galectin-3 modulation and its clinical outcomes in PCOS.

5 Conclusion

Our study provides important insights into the modulation of galectin-3 levels by metformin dosage and hyperprolactinemia in women with PCOS, highlighting the relevance of patient-specific treatment strategies for addressing the complex metabolic and hormonal challenges of this condition. By demonstrating the critical role of metformin dosage and hormonal factors, particularly hyperprolactinemia, in modulating galectin-3 levels, these findings challenge traditional paradigms focused solely on pharmacokinetics and pave the way for exploring galectin-3 as a novel therapeutic target in PCOS.

The potential clinical application of galectin-3 inhibitors, in conjunction with personalized metformin dosing, represents a promising therapeutic avenue. Future research should focus on large-scale clinical trials to validate the utility of galectin-3 as both a biomarker and a therapeutic target. Additionally, exploring the pharmacodynamics of metformin in relation to galectin-3 will be crucial for advancing PCOS treatment protocols and optimizing patient outcomes.

While our study provides valuable insights, further research with larger cohorts is needed to confirm these findings and explore the broader implications of galectin-3 modulation in PCOS.

Data availability statement

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

Ethics statement

The studies involving humans were approved by Review Boards of the University Clinical Center Nis (approval number 38212). 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

VN: Conceptualization, Data curation, Formal Analysis, Project administration, Supervision, Validation, Writing–original draft, Writing–review and editing. MS: Data curation, Investigation, Resources, Validation, Writing–review and editing. DM: Data curation, Investigation, Resources, Validation, Writing–review and editing. SS: Formal Analysis, Investigation, Methodology, Writing–review and editing. VS: Data curation, Investigation, Methodology, Writing–review and editing. HT: Data curation, Investigation, Methodology, Writing–review and editing. AI: Data curation, Formal Analysis, Validation, Writing–review and editing. DS: Conceptualization, Formal Analysis, 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.

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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Metformin as a disease-modifying therapy in osteoarthritis: bridging metabolism and joint health

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Background: Osteoarthritis (OA) and impaired glucose tolerance (IGT) frequently coexist, leading to compounded clinical and metabolic challenges. This study investigates the effects of metformin in improving both clinical outcomes (pain, stiffness, physical function) and metabolic parameters (inflammatory markers, lipid profile, BMI) in patients with knee OA and IGT.

Methods: The study included 60 patients diagnosed with knee OA and IGT. Participants were divided into two groups: 26 patients received standard OA treatment without metformin (Without Metf), while 34 received metformin (500 mg twice daily) for 3 months, in addition to standard treatment (With Metf). Clinical assessments (WOMAC, Lequesne Algofunctional Index, KOOS, VAS) and metabolic markers (CRP, NLR, SOD, lipid profile, BMI) were measured before treatment, after 1 month, and after 3 months.

Results: The With Metf group showed significantly greater improvements in pain, stiffness, physical function, and quality of life compared to the Without Metf group. Metformin also led to significant reductions in inflammatory markers and improvements in lipid profiles and metabolic health indicators. The With Metf group demonstrated enhanced BMI, waist-to-hip ratio, and waist-to-height ratio. Furthermore, the need for increased NSAID doses was predicted by factors such as pain severity and inflammatory markers.

Conclusion: Metformin effectively alleviates osteoarthritis symptoms and improves metabolic health in patients with both OA and IGT. Further research is needed to explore its long-term effects on joint health, inflammatory markers, and its potential role in OA management in patients without IGT.

KEYWORDS

osteoarthritis, impaired glucose tolerance, metformin, inflammatory markers, disease-modifying therapy

1 Introduction

The investigation of pleiotropic drugs in osteoarthritis (OA) is focused on identifying agents that simultaneously reduce symptoms and target underlying pathogenic mechanisms, potentially slowing disease progression (Fazio et al., 2024; Coppola et al., 2024; Halabitska et al., 2024a). Metformin, a first-line pharmacological agent widely used for managing type 2 diabetes mellitus (T2DM), has garnered considerable attention for its effects beyond glycemic control (Baker et al., 2021; Bailey, 2024). As an insulin-sensitizing agent, metformin is also frequently prescribed for individuals with impaired glucose

tolerance (IGT), a prediabetic condition characterized by disrupted glucose homeostasis, systemic inflammation, and metabolic dysfunction (Ping et al., 2024; Hostalek et al., 2015; Rojas and Gomes, 2013). While its primary therapeutic role is in improving insulin sensitivity and reducing hepatic gluconeogenesis, growing evidence suggests that metformin exerts pleiotropic effects, including anti-inflammatory, antioxidant, and metabolic regulatory properties (Dutta et al., 2023; Apostolova et al., 2020; Drzewoski and Hanefeld, 2021). These attributes position metformin as a promising candidate for addressing a range of metabolic and degenerative disorders (Rotermund et al., 2018; Isop et al., 2023; Petrie, 2024).

OA, a chronic and progressive degenerative joint disease, has traditionally been associated with mechanical factors such as joint overload and injury (He et al., 2020; Felson, 2013; Hall et al., 2016; Halabitska and Babinets, 2021). OA is one of the most prevalent musculoskeletal disorders worldwide (Allen et al., 2022; Global, 2023). IGT affects a significant portion of the population, particularly in older adults (Kalyani and Egan, 2013; Fang et al., 2019; Hermans et al., 2005). Their comorbidity is common and poses challenges due to overlapping inflammatory and metabolic pathways (Aziz et al., 2024; Berenbaum and Walker, 2020). However, emerging evidence highlights the critical role of metabolic and inflammatory mechanisms in its pathogenesis, particularly in individuals with metabolic comorbidities such as obesity, T2DM, and IGT (Chandrasekaran and Weiskirchen, 2024; Rohm et al., 2022; Ruze et al., 2023; Redkva et al., 2021; Zemlyak et al., 2023). The comorbidity of osteoarthritis and obesity highlights a complex interplay of systemic inflammation and metabolic disturbances, exacerbating the progression of both conditions (Halabitska et al., 2021; Nedunchezhiyan et al., 2022; Halabitska et al., 2024b). These comorbidities create a vicious cycle (Li B. et al., 2024; Swain et al., 2022; Repchuk et al., 2021). This interaction not only accelerates joint degeneration but also contributes to chronic low-grade inflammation, insulin resistance, and impaired overall metabolic homeostasis (Halabitska et al., 2024c; Vinuesa et al., 2021; Roberts et al., 2013). OA is characterized by cartilage degradation, subchondral bone remodeling, and synovial inflammation, processes that are exacerbated by systemic metabolic dysfunction (He et al., 2020; De Roover et al., 2023; Halabitska et al., 2024d). In this context, the metabolic and anti-inflammatory actions of metformin may have a dual benefit: addressing systemic metabolic derangements and modulating local joint pathology (Foretz et al., 2023; Domingo et al., 2024; He, 2020).

Preliminary studies have demonstrated that metformin can mitigate key mechanisms underlying OA progression (Xu et al., 2024; Anis et al., 2012; Li et al., 2020; Lambova, 2023). These include reductions in systemic inflammation and oxidative stress, improvements in lipid metabolism, and direct modulation of chondrocyte function (Adam et al., 2024; Horváth et al., 2023; Su et al., 2022). Moreover, metformin has been shown to enhance the synthesis of extracellular matrix components, promoting cartilage repair and potentially slowing the degenerative processes associated with OA (Feng et al., 2020; Yao et al., 2023; Zheng et al., 2021; Song et al., 2022). Despite these promising findings, the clinical application of metformin in OA remains underexplored, and robust evidence from longitudinal studies and clinical trials is necessary to validate its therapeutic potential.

This article aims to explore the potential role of metformin in managing OA in patients with IGT. By addressing both systemic and local pathological mechanisms, metformin may offer a novel therapeutic approach for patients with these comorbid conditions. Further elucidation of its disease-modifying properties could pave the way for integrating metformin into broader treatment paradigms for OA, particularly in the context of metabolic health optimization.

2 Materials and methods

2.1 Subjects

The study included 60 patients diagnosed with knee OA and IGT. Inclusion criteria required participants to have a confirmed diagnosis of OA based on the American College of Rheumatology (ACR), EULAR, and National Institute for Health clinical and radiographic criteria (Peat et al., 2006; Wang et al., 2024), which include the presence of pain, stiffness, or functional limitation in at least one joint, along with radiographic evidence of joint space narrowing, osteophytes, and subchondral sclerosis. Additionally, participants had to meet the criteria for IGT, defined by a fasting blood glucose level between 5.6 and 6.9 mmol/L (100–125 mg/dL) or a 2-h postprandial glucose level between 7.8 and 11.0 mmol/L (140–199 mg/dL), in accordance with the World Health Organization (WHO) criteria (Bergman et al., 2024).

Exclusion criteria included the presence of other metabolic or systemic conditions that could confound the results, such as uncontrolled diabetes mellitus, cardiovascular disease, or inflammatory rheumatic diseases. Patients with a history of joint surgery, joint replacement, or other significant comorbidities such as malignancies were also excluded from the study.

The study was conducted in accordance with the core principles outlined in the Council of Europe's Convention on Human Rights and Biomedicine, as well as the ethical guidelines set forth in the World Medical Association's Declaration of Helsinki on medical research involving human subjects, including its subsequent revisions (World Medical Association Declaration of Helsinki, 2014). Additionally, the research adhered to the regulations specified in Ministry of Health of Ukraine Order No. 690, dated 23 September 2009. All participants provided written informed consent before their participation. Ethical approval for the study was granted by the Bioethics Committee of I. Horbachevsky Ternopil National Medical University, Ministry of Health of Ukraine (Protocol No. 78, 18 August 2024).

The study cohort was divided into two groups: 26 patients in the Without Metf group and 34 patients in the With Metf group, with matched characteristics in terms of age, gender, severity, and disease progression of osteoarthritis (Table 1). The Without Metf group received OA treatment according to the established protocol, along with recommendations for improving glucose tolerance, including dietary modifications (e.g., reducing carbohydrate intake, increasing dietary fiber, and promoting a balanced nutritional regimen), regular physical exercise, and other lifestyle interventions. The With Metf group received OA treatment in accordance with the protocol, in addition to receiving metformin at a dose of 500 mg

TABLE 1 Demographic and OA duration characteristics.

Indicator	Without Metf (n = 26)	With Metf (n = 34)	p-value
Male	57.69%	55.88%	p = 0.432 (MW)
Age	47 (35–52.75)	48 (34–53)	p = 0.279 (MW)
Duration of OA	7 (4–9)	7 (4.5–9)	p = 0.725 (MW)
Kellgren–Lawrence Grade	2 (2–2)	2 (2–2)	p = 0.244 (MW)

Median and interquartile range (IQR) were used to summarize the data, p–Mann-Whitney U-test (MW).

twice daily for a period of 3 months. All clinical parameters were assessed before treatment, after 1 month, and after 3 months.

2.2 Laboratory and clinical data

The Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) was employed to evaluate pain, stiffness, physical function, and overall health status in patients with OA. It consists of three subscales: Pain (P), Stiffness (S), and Function (F). An overall score (OS) is calculated based on the combined results from these subscales, offering a holistic assessment of the patient's condition (Ebrahimzadeh et al., 2014).

The Lequesne Algofunctional Index (LI) was used to assess pain, functional impairment, and overall health status in patients with OA. It consists of two primary components: Pain Assessment (PA) and Functional Impairment Assessment (FIA). An Overall Score (OS) is derived from the combined results of both components, providing a comprehensive evaluation of the patient's condition (Faucher et al., 2002).

The Knee injury and Osteoarthritis Outcome Score (KOOS) scale was utilized to evaluate various aspects of knee health in patients with osteoarthritis. It includes five subscales: Pain (P), Other Symptoms (OS), Function in Daily Living (FDL), Function in Sport/Recreation (FS/R), and Knee-Related Quality of Life (KRQL). These subscales collectively provide a comprehensive assessment of pain, functionality, and quality of life related to knee osteoarthritis (Roos and Lohmander, 2003).

The Visual Analog Scale (VAS) was employed to assess pain (VAS-P) in patients with osteoarthritis. Additionally, functional limitations (VAS-FL), stiffness (VAS-S), and physical activity and mobility (VAS-PAM) were evaluated to gain a more comprehensive understanding of the impact of osteoarthritis on daily functioning and quality of life (Delgado et al., 2018).

The Timed Up and Go (TUG) test and the 6-Minute Walk Test (6MWT) assessed functional mobility and endurance in patients with OA. The TUG measures the time to rise from a chair, walk a set distance, turn, return, and sit, while the 6MWT evaluates the distance walked in 6 minutes, indicating physical endurance and functional capacity (Montgomery et al., 2020; Buisseret et al., 2020).

The SF-36 Health Survey was used to assess health-related quality of life in patients at three time points: before treatment, after 1 month, and after 3 months. The survey included the following scales: Physical Functioning (SF-36-PF), Role Limitations due to Physical Health (SF-36-RP), Bodily Pain (SF-36-BP), General Health (SF-36-GH), Vitality (SF-36-VT), Social Functioning (SF-36-SF), Role Limitations due to Emotional Health (SF-36-RE), and Mental Health (SF-36-MH) (Ware and Sherbourne, 1992; Ware, 2000).

Anthropometric measurements were taken to assess patients' metabolic health, including Body Mass Index (BMI), calculated as weight in kilograms divided by height in meters squared (kg/m^2); Waist-to-Hip Ratio (WHR), determined by dividing waist circumference by hip circumference; and Waist-to-Height Ratio (WHtR), calculated as the ratio of waist circumference to height.

Fasting plasma glucose (FPG) was measured in mmol/L using an enzymatic method on the Cobas c311 analyzer (Roche Diagnostics, Germany); sensitivity: 0.11 mmol/L; measurement range: 0.11–41.7 mmol/L; intra-assay CV <2%; analyzed in duplicate. Glycated hemoglobin (HbA1c) was determined as a percentage (%) by high-performance liquid chromatography (HPLC) using the Tosoh G8 HPLC Analyzer (Tosoh Corporation, Japan); sensitivity: 0.1%; measurement range: 3.0%–18.0%; intra-assay CV <2%; analyzed in duplicate. The Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) was calculated using the formula: HOMA-IR = (FPG × fasting insulin)/22.5. C-peptide levels were measured in ng/mL using an immunoassay method on the Architect i2000SR analyzer (Abbott, USA); sensitivity: 0.02 ng/mL; measurement range: 0.02–20 ng/mL; intra-assay CV <5%; analyzed in duplicate.

The Neutrophil-to-Lymphocyte Ratio (NLR) was calculated by dividing the neutrophil count by the lymphocyte count, both measured in cells/μL both measured in cells/μL using the Sysmex XN-1000 hematology analyzer (Sysmex Corporation, Japan); sensitivity: 0.01×10^9/L; measurement range: neutrophils $0.01–100 \times 10^9$/L, lymphocytes $0.01–50 \times 10^9$/L; intra-assay CV <3%; analyzed in duplicate. C-Reactive Protein (CRP) was quantified in mg/L using an immunoturbidimetric assay using an immunoturbidimetric assay on the Cobas c501 analyzer (Roche Diagnostics, Germany); sensitivity: 0.3 mg/L; measurement range: 0.3–350 mg/L; intra-assay CV <3%; analyzed in duplicate. Hydroxyproline (HP) levels were determined in mg/L by colorimetric analysis using the Shimadzu UV-1800 spectrophotometer (Shimadzu, Japan); sensitivity: 0.5 mg/L; measurement range: 0.5–100 mg/L; intra-assay CV <5%; analyzed in triplicate. Malondialdehyde (MA) was measured in μmol/L using a thiobarbituric acid reactive substances assay on the Agilent Cary 60 UV-Vis spectrophotometer (Agilent Technologies, United States); sensitivity: 0.1 μmol/L; measurement range: 0.1–25 μmol/L; intra-assay CV <5%; analyzed in triplicate. Superoxide Dismutase (SOD) activity was assessed in U/mL using a spectrophotometric method on the Beckman Coulter DU 730 analyzer (Beckman Coulter, United States); sensitivity: 0.05 U/mL; measurement range: 0.05–25 U/mL; intra-assay CV <5%; analyzed in triplicate. α_1-Antitrypsin (α_1-AT) concentrations were measured in

g/L by nephelometry on the BN ProSpec analyzer (Siemens Healthineers, Germany); sensitivity: 0.1 g/L; measurement range: 0.1–4.5 g/L; intra-assay CV <3%; analyzed in duplicate.

Total Cholesterol (TC) was measured in mmol/L using a colorimetric enzymatic method on the Abbott Architect c8000 analyzer (Abbott, United States); sensitivity: 0.1 mmol/L; measurement range: 0.1–15.0 mmol/L; intra-assay CV <3%; analyzed in duplicate. Low-Density Lipoprotein (LDL) cholesterol was quantified in mmol/L using the direct measurement method on the Abbott Architect c8000 analyzer (Abbott, United States); sensitivity: 0.2 mmol/L; measurement range: 0.2–10.0 mmol/L; intra-assay CV <3%; analyzed in duplicate. High-Density Lipoprotein (HDL) cholesterol levels were determined in mmol/L by a homogeneous enzymatic assay on the Abbott Architect c8000 analyzer (Abbott, United States); sensitivity: 0.1 mmol/L; measurement range: 0.1–4.0 mmol/L; intra-assay CV <3%; analyzed in duplicate. Triglycerides (TG) were assessed in mmol/L using an enzymatic colorimetric method on the Abbott Architect c8000 analyzer (Abbott, United States); sensitivity: 0.1 mmol/L; measurement range: 0.1–11.3 mmol/L; intra-assay CV <3%; analyzed in duplicate.

The OA exacerbation characteristics were assessed as follows: duration of exacerbation (≤7 days or >7 days) (OA exc. dur.), frequency of exacerbations in the last 3 months (≤2 times or >2 times) (OA exc. freq.), need for additional medical interventions during exacerbation (e.g., injections, physiotherapy) (OA exc. med. int.), requirement for work leave or exemption due to exacerbation (OA exc. work leave), occurrence of accompanying symptoms (e.g., swelling, redness) (OA exc. acc. sym.), need for increasing the dose of NSAIDs (NSAIDs dose), and need for increasing the duration of NSAID use (NSAID dur.).

2.3 Statistical analysis

Quantitative variables were first tested for normality using the Shapiro-Wilk test. Variables were described using median (Me) and lower and upper quartiles (Q1 – Q3). Categorical data were described with absolute and relative frequencies. The Mann-Whitney U-test was used to compare two groups on a quantitative variable. The comparison of frequencies in the analysis of 2 by 2 contingency tables was performed using Fisher's exact test. As a measure of the effect size when comparing groups regarding binary variables, the odds ratio (OR) with a 95% confidence interval (95% CI) was calculated. The Friedman test was used, along with the Conover-Iman test with Holm correction as a *post hoc* method. A prognostic model for the probability of a specific outcome was constructed using logistic regression. The coefficient of determination, indicating the portion of variance explained by the logistic regression, was evaluated using Nagelkerke's R^2. For assessing the discriminatory ability of quantitative variables in predicting a specific outcome, ROC curve analysis was performed. The cut-off point for the quantitative variable was determined by the highest value of the Youden index. Differences were considered statistically significant at $p < 0.05$.

Statistical analyses were conducted using commercially available software packages, including IBM SPSS Statistics (version 25), R (version 4.0.3), and GraphPad Prism (version 9.3). These programs were used for data management, statistical testing, and generating visual representations of the results.

3 Results

3.1 Analysis of clinical outcomes across treatment groups

In both studied groups, statistically significant differences were observed. The analysis of WOMAC index dynamics revealed significant improvements in the WOMAC-P, WOMAC-S, WOMAC-F, and WOMAC-OS, with notable changes observed both before treatment *versus* after 3 months and after 1 month *versus* after 3 months across all scales. However, for the WOMAC-F, a significantly greater difference in the dynamics of the indicators was found in the With Metf group compared to the Without Metf group (Table 2).

In both studied groups, significant changes were observed in the Lequesne Algofunctional Index. In the Without Metf group, the analysis demonstrated significant changes in the LI-PA, LI-FIA, and LI-OS scales. Significant differences in the LI-FIA scale were observed between before treatment and after 1 month. Additionally, notable changes were identified in the LI-FIA and LI-OS scales between after 1 month and after 3 months (Table 3).

In the With Metf group, significant changes were observed across all Lequesne Algofunctional Index scales during treatment. Notable differences were found in the LI-PA and LI-OS scales between before treatment and after 1 month, before treatment and after 3 months, and after 1 month and after 3 months. Significant changes were also observed in the LI-FIA scale between before treatment and after 3 months, and between after 1 month and after 3 months (Table 3).

A significantly greater difference in the dynamics of the LI-PA and LI-FIA scales was found in the With Metf group compared to the Without Metf group (Table 3).

Significant statistical differences between the groups were observed in the Lequesne Algofunctional Index scales, particularly in the LI-FIA and LI-OS, after 3 months (Table 3).

In the Without Metf group, the KOOS scales analysis revealed significant changes throughout treatment, particularly in the KOOS-FS/R and KOOS-KRQL scales. Significant changes were observed in the KOOS-FS/R scale and the KOOS-KRQL scale between before treatment and after 3 months, and after 1 month and after 3 months (Table 4).

In the With Metf group, significant changes were observed across the KOOS scales – KOOS-P, KOOS-OS, KOOS-FDL, KOOS-FS/R, and KOOS- KRQL – during treatment. Notable changes were found between before treatment and after 1 month in the KOOS-OS and KOOS-FDL scales, and between before treatment and after 3 months in the KOOS-OS, KOOS-FDL, KOOS-FS/R, and KOOS- KRQL scales. Additionally, significant changes in the KOOS-P, KOOS-OS, and KOOS-FDL scales were observed between after 1 month and after 3 months (Table 4).

Additionally, a higher statistical significance of changes in the indicators was observed in the With Metf group for the KOOS-P, KOOS-OS, KOOS-FDL, and KOOS-FS/R scales, compared to the Without Metf group (Table 4).

In the Without Metf group, significant changes were noted in the VAS-P, VAS-FL, VAS-S, VAS-PA, and VAS-M scales throughout treatment. Notable differences were observed in the VAS-FL, VAS-S, and VAS-PA, and VAS-M scales between after 1 month and after 3 months, as well as in the VAS-PA and VAS-M scales between between before treatment and after 1 month (Table 5).

TABLE 2 Dynamics of WOMAC index indicators in the with and without Metf groups.

Indicator	Group	Before treatment	After 1 month	After 3 months	p-value
Pain Scale (P)	Without Metf (n = 26)	7 (5–8)	6 (6–7)	6 (5.25–7)	**p < 0.001** (F)
	With Metf (n = 34)	7 (6–8)	6 (5–7.75)	6 (5–7)	**p < 0.001** (F)
Stiffness Scale (S)	Without Metf (n = 26)	4 (3–4)	4 (3–4)	4 (3–4)	**p = 0.003** (F)
	With Metf (n = 34)	4 (3–4)	4 (3–4)	4 (3–4)	**p < 0.001** (F)
Function Scale (F)	Without Metf (n = 26)	18 (16–19.75)	18 (16–20)	16 (15–18.75)	**p < 0.001** (F)
	With Metf (n = 34)	19 (17–20)	17 (16–20)	17 (15–18)	**p < 0.001** (F)
Overall Score (OS)	Without Metf (n = 26)	28 (28–31.75)	27.5 (25–31)	26 (22.25–28)	**p < 0.001** (F)
	With Metf (n = 34)	29.5 (27–32.75)	28 (24–31)	26 (23.25–28.75)	**p < 0.001** (F)

Median and interquartile range (IQR) were used to summarize the data.
p - the statistical difference observed within a single group before treatment, after 1 month, and after 3 months [Friedman test (F)].
Statistically significant p-values are highlighted in bold.

TABLE 3 Lequesne algofunctional index dynamics in the with and without Metf groups across treatment periods.

Indicator	Group	Before treatment	After 1 month	After 3 months	p-value
Pain Assessment (PA)	Without Metf (n = 26)	4 (4–4)	4 (4–4)	4 (3–4)	**p = 0.045** (F)
	With Metf (n = 34)	4 (4–5)	4 (3–4)	4 (3–4)	**p < 0.001** (F)
Functional Impairment Assessment (FIA)	Without Metf (n = 26)	4 (4–4)	4 (4–4)	4 (4–4)	**p = 0.006** (F)
	With Metf (n = 34)	4 (3–4)	3 (3–4)*	3 (3–3)***	**p < 0.001** (F)
Overall Score (OS)	Without Metf (n = 26)	8 (8–8)	8 (7–8.75)	7 (7–8)	**p < 0.001** (F)
	With Metf (n = 34)	8 (7–9)	7 (7–8)	7 (6–7)***	**p < 0.001** (F)

Median and interquartile range (IQR) were used to summarize the data.
p - the statistical difference observed within a single group before treatment, after 1 month, and after 3 months [Friedman test (F)].
* ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$) – the statistical difference between the Without Metf and With Metf groups in a single observation period [Mann-Whitney U-test (MW)].
Statistically significant p-values are highlighted in bold.

TABLE 4 KOOS scale dynamics in the with and without Metf groups across treatment periods.

Indicator	Group	Before treatment	After 1 month	After 3 months	p-value
Pain (P)	Without Metf (n = 26)	66 (65–69.75)	67 (64–70)	67 (64–71)	p = 0.078 (F)
	With Metf (n = 34)	67.5 (63.25–70)	68 (64–70)	68.5 (65.5–72.75)	**p = 0.002** (F)
Other Symptoms (OS)	Without Metf (n = 26)	65 (62.25–68)	65 (63–69)	65 (63–69)	p = 0.228 (F)
	With Metf (n = 34)	66 (64–68.75)	66 (64–69.75)	68 (65–70.75)	**p < 0.001** (F)
Function in Daily Living (FDL)	Without Metf (n = 26)	71.5 (69.25–74)	72 (70–74)	72 (71–74)	p = 0.102 (F)
	With Metf (n = 34)	71 (68–74.75)	71 (69–75.75)	74.5 (70.25–78.5)	**p < 0.001** (F)
Function in Sport/Recreation (FS/R)	Without Metf (n = 26)	52 (49.25–54)	52 (49.25–55)	53.5 (50.5–55)	**p = 0.016** (F)
	With Metf (n = 34)	51 (48–53)	52 (48–54.75)	54 (50.25–56.5)	**p < 0.001** (F)
Knee-Related Quality of Life (KRQL)	Without Metf (n = 26)	59.5 (55.25–62)	59.5 (55.5–62)	60.5 (57.25–62.75)	**p < 0.001** (F)
	With Metf (n = 34)	59.5 (57–63)	60.5 (58–64.75)	63 (60–67)	**p < 0.001** (F)

Median and interquartile range (IQR) were used to summarize the data.
p - the statistical difference observed within a single group before treatment, after 1 month, and after 3 months (Friedman test (F)).
* ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$) – the statistical difference between the Without Metf and With Metf groups in a single observation period (Mann-Whitney U-test (MW)).
Statistically significant p-values are highlighted in bold.

In the With Metf group, significant changes were observed throughout treatment in the VAS-P, VAS-FL, VAS-S, VAS-PA, and VAS-M scales. Statistically significant differences were found between before treatment and after 1 month, before treatment and after 3 months, as well as between after 1 month and after 3 months for all these scales (Table 5).

TABLE 5 Analysis of VAS changes in the with and without Metf groups across treatment periods.

Indicator	Group	Before treatment	After 1 month	After 3 months	p-value
Pain (P)	Without Metf (n = 26)	42.5 (37–46.25)	42.5 (39–45)	43.5 (40–47)	**p = 0.009** (F)
	With Metf (n = 34)	39 (36.25–43.5)	37 (34.25–40)**	35 (31–37.75)***	**p < 0.0010** (F)
Functional Limitations (FL)	Without Metf (n = 26)	37.5 (32–42.75)	38 (32.25–41)	36.5 (30–40)	**p = 0.008** (F)
	With Metf (n = 34)	34.5 (29–37)	32 (30–35)*	30 (27–35)**	**p < 0.001** (F)
Stiffness (S)	Without Metf (n = 26)	35.5 (34–41)	35 (32–40)	35 (30–39.5)	**p = 0.01** (F)
	With Metf (n = 34)	36.5 (31.25–40.75)	32.5 (30–36.5)	31 (27.75–35)	**p < 0.001** (F)
Physical Activity and Mobility (PAM)	Without Metf (n = 26)	35.5 (29–38.75)	32.5 (25.5–37)	32.5 (25–35)	**p < 0.001** (F)
	With Metf (n = 34)	36 (30.25–40)	33.5 (30–40)	31 (25.5–37)	**p < 0.001** (F)

Median and interquartile range (IQR) were used to summarize the data.
p - the statistical difference observed within a single group before treatment, after 1 month, and after 3 months [Friedman test (F)].
* ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$) – the statistical difference between the Without Metf and With Metf groups in a single observation period [Mann-Whitney U-test (MW)].
Statistically significant p-values are highlighted in bold.

TABLE 6 Changes in TUG and 6MWT scales across treatment periods in the with and without Metf groups.

Indicator	Group	Before treatment	After 1 month	After 3 months	p-value
Timed Up and Go (TUG)	Without Metf (n = 26)	15.5 (13–19)	14.5 (12–16)	13 (12–15)	**p < 0.001** (F)
	With Metf (n = 34)	14 (12–17)	12 (10–15)*	12 (10–15)	**p < 0.001** (F)
6-Minute Walk Test (6MWT)	Without Metf (n = 26)	345 (335.25–353.75)	345 (335.25–356)	350 (340–358.75)	**p < 0.001** (F)
	With Metf (n = 34)	349.5 (335.25–359.75)	355 (340.5–365)*	355 (346.25–366)*	**p < 0.001** (F)

Median and interquartile range (IQR) were used to summarize the data.
p - the statistical difference observed within a single group before treatment, after 1 month, and after 3 months [Friedman test (F)].
* ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$) – the statistical difference between the Without Metf and With Metf groups in a single observation period [Mann-Whitney U-test (MW)].
Statistically significant p-values are highlighted in bold.

Furthermore, a greater statistical significance in the changes of the indicators was found in the With Metf group for your VAS-P, VAS-FL, and VAS-S scales, compared to the Without Metf group (Table 5).

Statistical differences between the groups were observed in the VAS-P and VAS-FL scales after 1 and 3 months. Furthermore, a significant difference was noted in the VAS-S scale between the groups after 3 months (Table 5).

In the Without Metf group, significant changes were observed in the TUG and 6MWT scales throughout the treatment period. Statistically significant differences were found between before treatment and after 3 months, as well as between after 1 month and after 3 months for both scales. Additionally, changes in the TUG scale were significant between before treatment and after 1 month (Table 6).

In the With Metf group, significant changes were observed in the TUG and 6MWT scales throughout the treatment period. Statistically significant differences were found in all indicators of both scales between before treatment and after 1 month, before treatment and after 3 months, as well as between after 1 month and after 3 months (Table 6).

Between the groups, statistical differences were observed for the TUG and 6MWT scales after 1 month, with a further significant difference noted in the 6MWT scale after 3 months (Table 6).

In the Without Metf group, statistically significant changes were observed across the OKS questionnaire scales during treatment, including OKS-P ($p = 0.005$) (Friedman test), OKS-S ($p = 0.002$) (Friedman test), and OKS-F ($p = 0.001$) (Friedman test). Additionally, significant differences were found in the OKS-F scale before treatment and after 1 month ($p < 0.001$) (Conover-Iman test with Holm correction), in the OKS-S scale before treatment and after 3 months, and in the OKS-F scale between after 1 month and after 3 months ($p = 0.008$) (Conover-Iman test with Holm correction) (Figure 1).

In the With Metf group, statistically significant changes were observed across all OKS questionnaire scales throughout treatment ($p < 0.001$) (Friedman test). Significant differences were also found between before treatment and after 1 month for all scales of the questionnaire ($p < 0.001$) (Conover-Iman test with Holm correction). Additionally, statistically significant changes were noted between after 1 month and after 3 months for all OKS scales ($p < 0.001$) (Conover-Iman test with Holm correction) (Figure 1).

Statistically significant differences between the groups were also observed in the OKS-F scale after 1 month ($p = 0.043$) (Mann-Whitney U-test) and after 3 months ($p = 0.032$) (Mann-Whitney U-test) (Figure 1).

In the Without Metf group, statistically significant changes were observed across several SF-36 scales throughout treatment. Specifically, SF-36-RP ($p = 0.004$) (Friedman test) [before treatment vs. after 1 month: $p = 0.035$, before treatment vs. after 3 months: $p < 0.001$, after 1 month vs. after 3 months: $p = 0.035$ (Conover-Iman test with Holm correction)]. SF-36-GH ($p < 0.001$) (Friedman test) [before treatment vs. after 3 months: $p < 0.001$, after 1 month vs. after 3 months: $p < 0.001$ (Conover-Iman test with Holm

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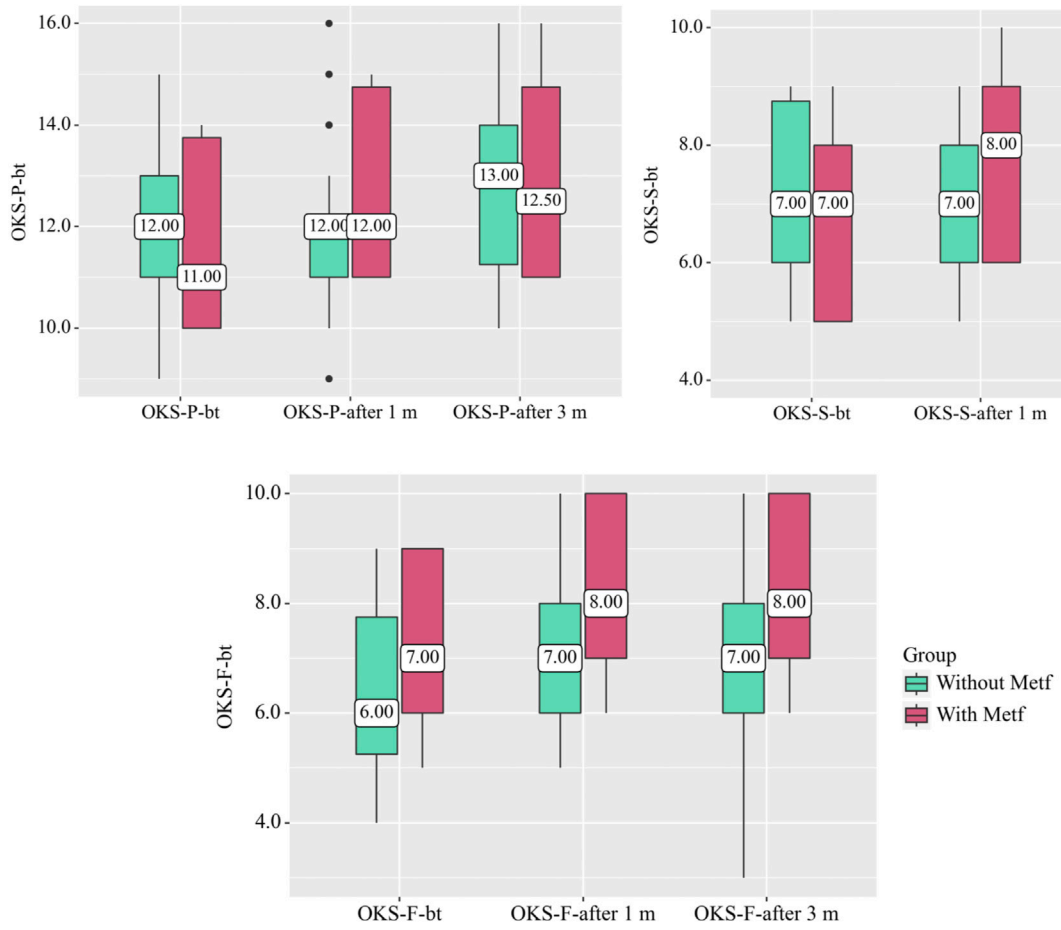

FIGURE 1
Changes in OKS Questionnaire Scales in the With and Without Metf Groups Across Treatment Periods. Statistical analysis was performed using the Friedman test for differences within groups over time, with *post hoc* comparisons conducted using the Conover-Iman test with Holm correction. Differences between groups at each time point were assessed using the Mann-Whitney U-test.

correction)], SF-36-VT ($p < 0.001$) (Friedman test) [before treatment vs. after 1 month: $p < 0.001$, before treatment vs. after 3 months: $p < 0.001$, after 1 month vs. after 3 months: $p = 0.014$ (Conover-Iman test with Holm correction)], SF-36-SF ($p = 0.015$) (Friedman test) [before treatment vs. after 3 months: $p = 0.032$, after 1 month vs. after 3 months: $p = 0.046$ (Conover-Iman test with Holm correction)], SF-36-RE showed ($p < 0.001$) (Friedman test) [before treatment vs. after 3 months: $p < 0.001$, after 1 month vs. after 3 months: $p = 0.001$ (Conover-Iman test with Holm correction)], and SF-36-MH ($p < 0.001$) (Friedman test) [before treatment vs. after 3 months: $p < 0.001$, after 1 month vs. after 3 months: $p < 0.001$ (Conover-Iman test with Holm correction)] (Figure 2).

In the With Metf group, statistically significant changes were observed across several SF-36 scales throughout treatment. Specifically, SF-36-PF ($p < 0.001$) (Friedman test) [before treatment vs. after 1 month: $p = 0.004$, before treatment vs. after 3 months: $p < 0.001$, after 1 month vs. after 3 months: $p = 0.039$ (Conover-Iman test with Holm correction)], SF-36-BP ($p < 0.001$) (Friedman test) [before treatment vs. after 1 month: $p < 0.001$, before treatment vs. after 3 months: $p < 0.001$, after 1 month vs. after 3 months: $p = 0.004$ (Conover-Iman test with Holm correction)], SF-36-GH ($p < 0.001$) (Friedman test) [before treatment vs. after 1 month: $p < 0.001$, before treatment vs. after 3 months: $p < 0.001$, after 1 month vs. after 3 months: $p = 0.007$ (Conover-Iman test with Holm correction)], SF-36-VT ($p < 0.001$) (Friedman test) [before treatment vs. after 1 month: $p < 0.001$, before treatment vs. after 3 months: $p < 0.001$, after 1 month vs. after 3 months: $p = 0.012$ (Conover-Iman test with Holm correction)], SF-36-RE ($p < 0.001$) (Friedman test) [before treatment vs. after 1 month: $p = 0.004$, before treatment vs. after 3 months: $p < 0.001$, after 1 month vs. after 3 months: $p = 0.045$ (Conover-Iman test with Holm correction)], and SF-36-MH ($p < 0.001$) (Friedman test) [before treatment vs. after 1 month: $p = 0.003$, before treatment vs. after 3 months: $p < 0.001$, after 1 month vs. after 3 months: $p = 0.023$ (Conover-Iman test with Holm correction)] (Figure 2).

A statistically significant difference was also found in the indicators between the groups on the scales SF-36-PF after 1 month ($p = 0.002$) (Mann-Whitney U-test) and SF-36-SF after 1 month ($p = 0.005$) (Mann-Whitney U-test), after 3 months ($p = 0.020$) (Mann-Whitney U-test) (Figure 2).

In the Without Metf group, significant changes in BMI and WHR were observed over the course of treatment. Furthermore, statistically significant differences in BMI and WHR were identified between before treatment and after 1 month of treatment. Similarly, significant changes in BMI and WHR were

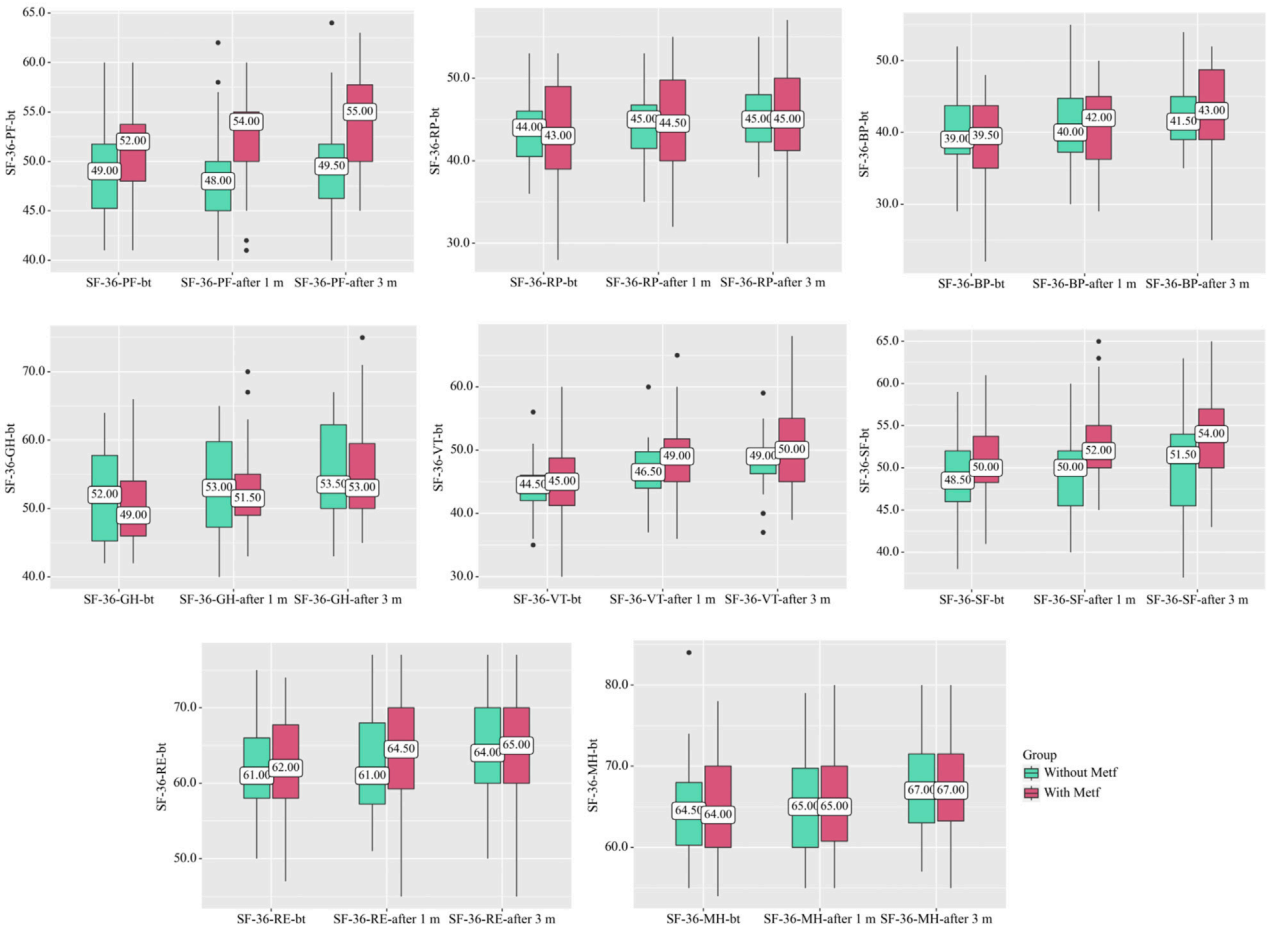

FIGURE 2
SF-36 Scale Dynamics in the With and Without Metf Groups Across Treatment Periods. Statistical analysis was performed using the Friedman test for differences within groups over time, with post hoc comparisons conducted using the Conover-Iman test with Holm correction. Differences between groups at each time point were assessed using the Mann-Whitney U-test.

observed between after 1 month and after 3 months of treatment (Table 7).

In the With Metf group, significant changes were observed in BMI, WHR, and WHtR values throughout the course of treatment. Furthermore, statistically significant differences were identified for all indicators between before treatment and after 1 month, before treatment and after 3 months, as well as between after 1 month and after 3 months within this group (Table 7).

Moreover, a more significant statistical difference in the changes of the indicators was observed in the With Metf group for the BMI and WHtR scales, compared to the Without Metf group (Table 7).

Between the groups, significant statistical differences were observed after 1 month for BMI and WHtR, and after 3 months for BMI, WHR, and WHtR (Table 7).

In the Without Metf group, significant statistical changes were observed in FPG level throughout the course of treatment. Furthermore, notable differences in FPG were found between the measurements taken at 1 month and 3 months (Table 8).

In the With Metf group, statistically significant changes were observed throughout the treatment in the levels of FPG, HbA1c, HOMA-IR, and C-peptide. Additionally, significant changes were found across all indicators when comparing measurements before treatment to after 1 month, before treatment to after 3 months, and between after 1 month and after 3 months (Table 8).

Additionally, the With Metf group showed a more pronounced statistical difference in the changes of the FPG, HbA1c, HOMA-IR, and C-peptide indicators compared to the Without Metf group (Table 8).

Statistically significant differences between the groups were observed after 1 month for all indicators, with the exception of C-peptide. After 3 months, significant differences were found across all studied parameters (Table 8).

In the Without Metf group, statistically significant changes were observed throughout the treatment in the levels of NLR, CRP, MA, and SOD. Additionally, significant changes in NLR and SOD were found between before treatment and after 1 month. Statistically significant changes in NLR and CRP were observed between before treatment and after 3 months. Furthermore, significant differences were noted in NLR, CRP, MA, and SOD between after 1 month and after 3 months (Table 9).

In the With Metf group, statistically significant changes were observed during the treatment period in the levels of NLR, CRP, HP, MA, SOD, and α1-AT. Significant changes in NLR, CRP, MA, and SOD were found between before treatment and after 1 month.

TABLE 7 Changes in BMI, WHR, and WHtR across treatment periods in the with and without Metf groups.

Indicator	Group	Before treatment	After 1 month	After 3 months	p-value
Body Mass Index, kg/m^2 (BMI)	Without Metf (n = 26)	29.87 (27.35–31.26)	29.61 (27.15–31.6)	28.99 (26.57–30.51)	**p = 0.019** (F)
	With Metf (n = 34)	28.02 (26.39–30.19)	26.52 (25.26–29.32)*	24.98 (24.68–27.54)**	**p < 0.001** (F)
Waist-to-Hip Ratio, (WHR)	Without Metf (n = 26)	0.96 (0.92–0.99)	0.94 (0.91–0.96)	0.94 (0.88–0.96)	**p < 0.001** (F)
	With Metf (n = 34)	0.99 (0.94–1.03)	0.91 (0.85–0.96)	0.81 (0.75–0.86)***	**p < 0.001** (F)
Waist-to-Height Ratio, (WHtR)	Without Metf (n = 26)	0.56 (0.51–0.6)	0.55 (0.52–0.59)	0.55 (0.51–0.59)	p = 0.762 (F)
	With Metf (n = 34)	0.54 (0.48–0.64)	0.5 (0.45–0.56)*	0.5 (0.45–0.52)**	**p < 0.001** (F)

Median and interquartile range (IQR) were used to summarize the data.
p - the statistical difference observed within a single group before treatment, after 1 month, and after 3 months [Friedman test (F)].
* ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$) – the statistical difference between the Without Metf and With Metf groups in a single observation period [Mann-Whitney U-test (MW)].
Statistically significant p-values are highlighted in bold.

TABLE 8 Changes in glucose tolerance indicators during treatment.

Indicator	Group	Before treatment	After 1 month	After 3 months	p-value
Fasting plasma glucose, mmol/L (FPG)	Without Metf (n = 26)	6.44 (6.32–6.54)	6.38 (6.24–6.51)	6.4 (6.25–6.41)	**p = 0.006** (F)
	With Metf (n = 34)	6.38 (6.18–6.45)	6.01 (5.85–6.04)***	5.53 (5.49–5.7)***	**p < 0.001** (F)
Glycated hemoglobin, % (HbA1c)	Without Metf (n = 26)	6 (5.9–6.1)	6 (6–6.1)	6 (5.9–6.1)	P = 0.162 (F)
	With Metf (n = 34)	6 (5.9–6.1)	5.8 (5.7–6)***	5.65 (5.5–5.7)***	**p < 0.001** (F)
HOMA-IR	Without Metf (n = 26)	2.74 (2.68–2.83)	2.75 (2.7–2.85)	2.79 (2.67–2.86)	P = 0.542 (F)
	With Metf (n = 34)	2.69 (2.62–2.8)	2.62 (2.52–2.71)**	2.49 (2.4-2.59)***	**p < 0.001** (F)
C-peptide, ng/mL	Without Metf (n = 26)	3.08 (2.96–3.2)	3 (2.9–3.09)	3.02 (2.93–3.18)	P^a = 0.211 (F)
	With Metf (n = 34)	3.02 (2.94–3.12)	2.94 (2.81–3.01)	2.84 (2.75–2.93)***	**p < 0.001** (F)

Median and interquartile range (IQR) were used to summarize the data.
p - the statistical difference observed within a single group before treatment, after 1 month, and after 3 months [Friedman test (F)].
* ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$) – the statistical difference between the Without Metf and With Metf groups in a single observation period [Mann-Whitney U-test (MW)].
Statistically significant p-values are highlighted in bold.

Additionally, statistically significant changes in NLR, CRP, HP, MA, and SOD were observed between before treatment and after 3 months. Furthermore, significant differences in NLR, CRP, HP, MA, SOD, and α_1-AT were noted between after 1 month and after 3 months (Table 9).

Furthermore, a more significant statistical difference in the changes of the HP, MA, and α_1-AT indicators was observed in the With Metf group compared to the Without Metf group (Table 9).

Statistically significant differences between the groups were observed in HP levels after 1 month and after 3 months (Table 9).

In the Without Metf group, statistically significant changes were observed in LDL levels throughout the treatment period. Additionally, significant changes in LDL levels were found between before treatment and after 3 months, as well as between after 1 month and after 3 months (Table 10).

In the Metf group, statistically significant changes were observed throughout the treatment period in TC, LDL, HDL, and TG levels. Additionally, significant changes were found in TC and LDL levels between before treatment and after 1 month. TC and HDL levels exhibited statistically significant changes between before treatment and after 3 months. Furthermore, TC, LDL, HDL, and TG levels showed significant changes between after 1 month and after 3 months (Table 10).

In addition, a more notable statistical difference in the changes of the TC, LDL, HDL, and TG indicators was found in the With Metf group compared to the Without Metf group (Table 10).

Statistically significant differences between the groups were found in TC and LDL levels after 1 and 3 months (Table 10).

3.2 Assessment of osteoarthritis exacerbation parameters

Over a 3-month period, various aspects of osteoarthritis (OA) exacerbations were investigated, including the duration of flare-ups (OA exc. dur., ≤7 days or >7 days), their frequency (OA exc. freq., ≤2 times or >2 times), and the need for additional medical interventions such as injections or physiotherapy (OA exc. med. int.). Other factors assessed included the requirement for work leave or exemption due to exacerbations (OA exc. work leave), the occurrence of accompanying symptoms (OA exc. acc. sym., e.g., swelling, redness), and the necessity to adjust NSAID therapy by increasing either the dose (NSAIDs dose) or duration of use (NSAID dur.). These parameters provided insight into the severity and impact of OA exacerbations on patient management (Table 11).

No statistically significant difference was found between these indicators in the studied groups during the treatment period. Additionally, an analysis of the interrelationships among the investigated indicators of Osteoarthritis Exacerbation Characteristics was conducted, and a prognostic model was developed to predict the need for increased NSAID doses in patients with osteoarthritis and impaired glucose tolerance, based on factors such as VAS-P-after 3 months, OKS-F-after 3 months, HbA1c-after 3 months, C-peptide-after 3 months, CRP-after 3 months, HP-after 3 months, and α1-AT-after 3 months using binary logistic regression. The model was constructed using 60 observations, and the relationship between these variables is described by the following equation:

$$P = 1 / (1 + e^{-z}) \times 100\%$$

$$z = -10,247 + 0,346X_{\text{VAS-P-after 3 m}} + 1,011X_{\text{OKS-F-after 3 m}} - 13,099X_{\text{HbA1c-after 3 m}} + 11,210X_{\text{C-peptide-after 3 m}} - 3,489X_{\text{CRP-after 3 m}} + 1,807X_{\text{HP-after 3 m}} + 19,109X_{\text{α1-AT-after 3 m}}$$

where P represents the probability estimate for "yes," z denotes the value of the logistic function, $X_{\text{VAS-P-after 3 m}}$ refers to VAS-P-after 3 m, $X_{\text{OKS-F-after}}$ 3 m refers to OKS-F-after 3 m, $X_{\text{hba1c-after 3 m}}$ refers to HbA1c-after 3 m, $X_{\text{C-peptide-after 3 m}}$ refers to C-peptide-after 3 m, $X_{\text{CRP-after 3 m}}$ refers to CRP-after 3 m, $XH_{\text{P-after 3 m refers}}$ to HP-after 3 m, and $X_{\text{α1-at-after 3 m}}$ refers to α_1-AT-after 3 months.

The resulting regression model, in terms of the alignment between the predicted and observed values upon the inclusion of

TABLE 9 Changes in inflammatory markers and antioxidant Enzyme levels throughout the treatment period.

Indicator	Group	Before treatment	After 1 month	After 3 months	p-value
Neutrophil-to-Lymphocyte Ratio (NLR)	Without Metf (n = 26)	2.48 (2.3–2.62)	2.46 (2.29–2.62)	2.33 (2.21–2.54)	**p < 0.001** (F)
	With Metf (n = 34)	2.47 (2.33–2.71)	2.35 (2.19–2.61)	2.29 (2.15–2.56)	**p < 0.001** (F)
C-Reactive Protein, mg/L (CRP)	Without Metf (n = 26)	4.5 (3.79–5.09)	4.46 (3.71–5.04)	4.35 (3.63–4.91)	**p < 0.001** (F)
	With Metf (n = 34)	4.56 (4.23–5.2)	4.5 (4.19–5.04)	4.2 (4.04–4.57)	**p < 0.001** (F)
Hydroxyproline, mg/L (HP)	Without Metf (n = 26)	7.71 (6.74–8.38)	7.77 (6.56–8.36)	7.71 (6.55–7.96)	p = 0.240 (F)
	With Metf (n = 34)	7.04 (6.33–7.85)	6.9 (6.29–7.66)*	6.5 (5.9–7.39)*	**p < 0.001** (F)
Malondialdehyde, μmol/L (MA)	Without Metf (n = 26)	7.09 (4.84–8.29)	6.95 (4.73–8.19)	6.86 (4.65–7.81)	**p = 0.012** (F)
	With Metf (n = 34)	6.73 (5.57–8.03)	6 (5.02–7.05)	5.32 (4.63–6.58)	**p < 0.001** (F)
Superoxide Dismutase, U/mL (SOD)	Without Metf (n = 26)	195.02 (189.05–204.3)	197.03 (190.77–208.99)	206 (196.04–209.25)	**p < 0.001** (F)
	With Metf (n = 34)	195.44 (189–210.4)	202.52 (193.98–214.46)	206.87 (201.04–217)	**p < 0.001** (F)
α_1-Antitrypsin, g/L (α_1-AT)	Without Metf (n = 26)	1.6 (1.52–1.65)	1.61 (1.52–1.65)	1.6 (1.55–1.65)	p = 0.228 (F)
	With Metf (n = 34)	1.65 (1.52–1.70)	1.64 (1.55–1.7)	1.65 (1.58–1.7)	**p = 0.013** (F)

Median and interquartile range (IQR) were used to summarize the data.
p - the statistical difference observed within a single group before treatment, after 1 month, and after 3 months [Friedman test (F)].
* ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$) – the statistical difference between the Without Metf and With Metf groups in a single observation period [Mann-Whitney U-test (MW)].
Statistically significant p-values are highlighted in bold.

TABLE 10 Changes in lipid profile parameters throughout the treatment period.

Indicator	Group	Before treatment	After 1 month	After 3 months	p-value
Total Cholesterol, mmol/L (TC)	Without Metf (n = 26)	5.6 (5.39–5.88)	5.59 (5.36–5.91)	5.58 (5.37–5.77)	P = 0.205 (F)
	With Metf (n = 34)	5.43 (5.32–5.61)	5.34 (5.23–5.53)**	5.27 (5.12–5.48)***	**p < 0.001** (F)
Low-Density Lipoprotein, mmol/L (LDL)	Without Metf (n = 26)	3.5 (3.19–3.68)	3.49 (3.19–3.76)	3.41 (3.2–3.67)	**p = 0.006** (F)
	With Metf	3.37 (2.98–3.51)	3.15 (2.95–3.39)	3.15 (2.95–3.40)**	**p < 0.001** (F)
High-Density Lipoprotein, mmol/L (HDL)	Without Metf (n = 26)	1.23 (1.1–1.4)	1.23 (1.08–1.39)	1.27 (1.13–1.39)	p = 0.218 (F)
	With Metf (n = 34)	1.19 (1.12–1.25)	1.27 (1.18–1.33)	1.31 (1.22–1.40)	**p < 0.001** (F)
Triglycerides, mmol/L (TG)	Without Metf (n = 26)	1.94 (1.86–2.02)	1.98 (1.83–2.05)	1.88 (1.78–2)	p = 0.575 (F)
	With Metf (n = 34)	2 (1.85–2.11)	1.97 (1.83–2.08)	1.98 (1.82–2.08)	**p < 0.001** (F)

Median and interquartile range (IQR) were used to summarize the data.
p - the statistical difference observed within a single group before treatment, after 1 month, and after 3 months [Friedman test (F)].
* ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$) – the statistical difference between the Without Metf and With Metf groups in a single observation period [Mann-Whitney U-test (MW)].
Statistically significant p-values are highlighted in bold.

TABLE 11 Indicators of osteoarthritis exacerbation characteristics based on group.

Variable	Categories	Group		p
		Without Metf (n = 26)	With Metf (n = 34)	
OA exc. dur	≤7 days	11 (42.3)	14 (41.2)	0.952
	>7 days	15 (57.7)	20 (58.8)	
OA exc. freq	≤2 times	13 (50.0)	16 (47.1)	0.834
	>2 times	13 (50.0)	18 (52.9)	
OA exc. med. int	no	15 (57.7)	24 (70.6)	0.414
	yes	11 (42.3)	10 (29.4)	
OA exc. work leave	no	19 (73.1)	27 (79.4)	0.759
	yes	7 (26.9)	7 (20.6)	
OA exc. acc. sym	no	20 (76.9)	29 (85.3)	0.507
	yes	6 (23.1)	5 (14.7)	
NSAIDs dose	no	18 (69.2)	27 (79.4)	0.386
	yes	8 (30.8)	7 (20.6)	
NSAID dur	no	16 (61.5)	27 (79.4)	0.156
	yes	10 (38.5)	7 (20.6)	

The number of patients (percentage) p–Fisher's exact test.

TABLE 12 Characteristics of the association between predictors of the model and the odds of the need for increased NSAID doses.

Predictors	Unadjusted		Adjusted	
	COR; 95% CI	p	AOR; 95% CI	p
VAS-P-after 3 m	1.079; 1.005–1.158	0.037*	1.414; 1.119–1.788	0.004*
OKS-F-after 3 m	1.308; 0.894–1.914	0.167	2.748; 1.105–6.828	0.030*
HbA1c-after 3 m	1.867; 0.137–25.508	0.640	0.000; 0.000–0.075	0.015*
C-peptideafter 3 m	74.040; 2.754–1990.219	0.010*	73,887.578; 5.382–1,014,843,245.924	0.021*
CRP-after 3 m	0.603; 0.253–1.436	0.253	0.031; 0.003–0.347	0.005*
HP-after 3 m	1.975; 1.139–3.425	0.015*	6.095; 1.626–22.851	0.007*
α_1-AT-after 3 m	0.058; 0.000–36.017	0.385	198,996,938.868; 8.406–4,712,523,809,095,451.000	0.027*

* – the effect of the predictor is statistically significant ($p < 0.05$).

predictors compared to the model without predictors, is statistically significant ($p < 0.001$). The Nagelkerke pseudo-R^2 was 67.9% (Table 12).

An increase of 1 in VAS-P-after 3 months increased the odds of the need for increased NSAID doses by a factor of 1.414. An increase of 1 in OKS-F-after 3 months increased the odds of the need for increased NSAID doses by a factor of 2.748. An increase of 1 in HbA1c-after 3 months decreased the odds of the need for increased NSAID doses by a factor of 488,307.202. An increase of 1 in C-peptide-after 3 months increased the odds of the need for increased NSAID doses by a factor of 73,887.578. An increase of 1 in CRP-after 3 months decreased the odds of the need for increased NSAID doses by a factor of 32.756. An increase of 1 in HP-after 3 months increased the odds of the need for increased NSAID doses by a factor of 6.095. An increase of 1 in α1-AT-after 3 months increased the odds of the need for increased NSAID doses by a factor of 198,996,938.868 (Figure 3).

The following curve was obtained when assessing the discriminatory ability of the regression model using ROC analysis (Figures 4, 5).

The probability estimate P is a statistically significant predictor of the Need for Increased NSAID Doses (AUC = 0.942; 95% CI: 0.858–1.000, $p < 0.001$). The threshold value of the probability estimate P at the cut-off point, corresponding to the highest Youden's index, was 0.191. A "yes" was predicted when the probability estimate P was greater than or equal to this value. The sensitivity and specificity of the resulting predictive model were 93.3% and 82.2%, respectively.

A statistical analysis was conducted to evaluate the relationship between BMI and the duration of osteoarthritis exacerbations (OA exc. dur.) (Table 13).

10.3389/fphar.2025.1567544

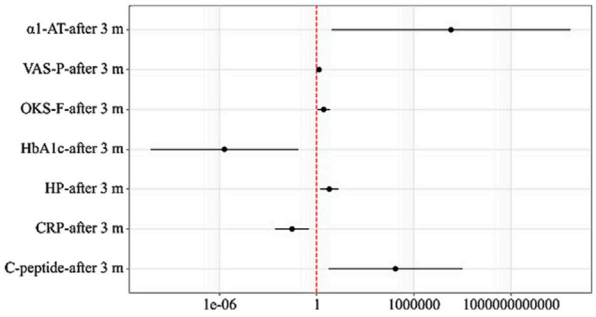

FIGURE 3
Odds ratios with 95% CI estimates for the studied predictors of the Need for Increased NSAID Doses.

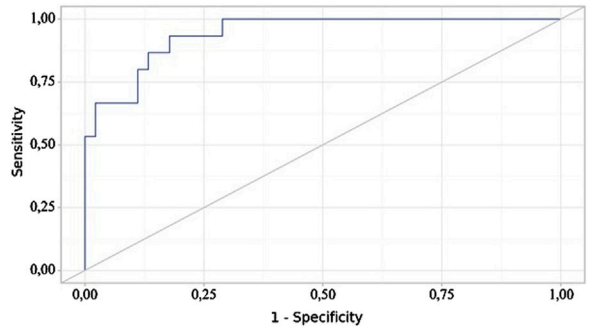

FIGURE 4
ROC curve illustrating the discriminatory ability of the regression model in predicting NSAIDs dose.

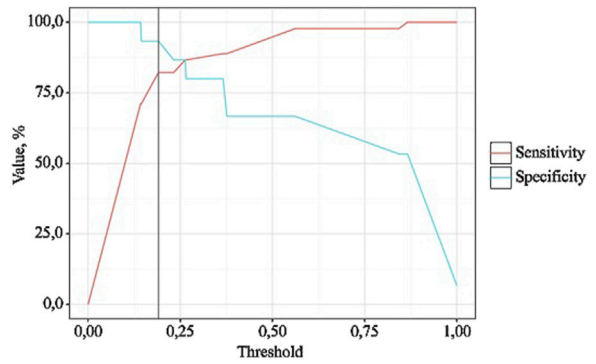

FIGURE 5
Analysis of model sensitivity and specificity depending on the threshold values of NSAIDs dose probability estimates.

According to the presented table, when analyzing BMI depending on OA exc. dur., statistically significant differences were found (p = 0.015) (Figure 6).

The discriminatory capacity of >7 days from BMI was evaluated using ROC analysis, which yielded the following curve (Figures 7, 8).

BMI is a statistically significant predictor of OA exc. dur (AUC = 0.686; 95% CI: 0.553–0.820, p = 0.015). The threshold value of BMI at the cut-off point, corresponding to the highest Youden index, was

TABLE 13 Analysis of BMI depending on OA exacerbation duration.

Indicator	Categories	BMI-after 1 m			p
		Me	$Q_1 - Q_3$	n	
OA exc. dur	≤7 days	27.01	24.84–28.16	25	0.015*
	>7 days	29.54	25.81–31.28	35	

* – the effect of the predictor is statistically significant (p < 0.05).

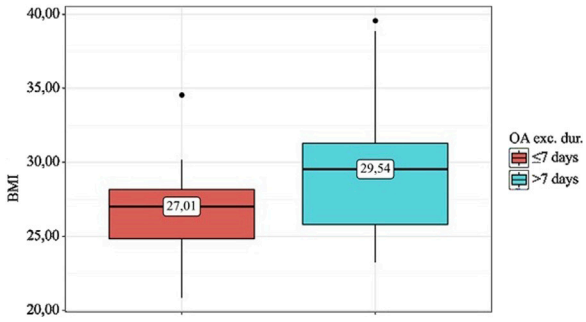

FIGURE 6
Analysis of BMI depending on OA exc. dur.

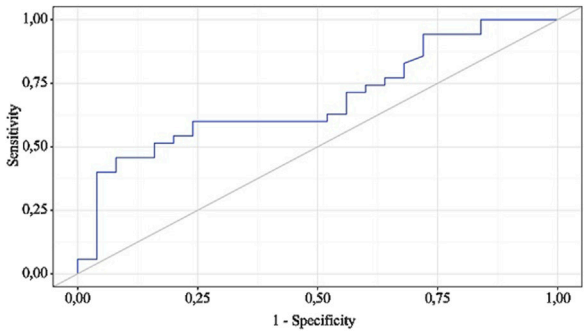

FIGURE 7
ROC curve characterizing the discriminatory ability of BMI-after 1 m in predicting OA exc. dur.

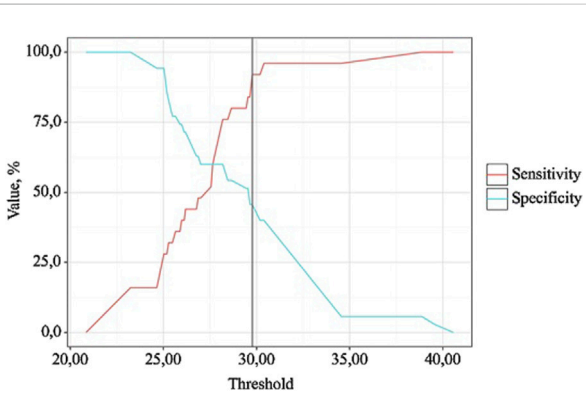

FIGURE 8
Analysis of the sensitivity and specificity of the model depending on the threshold values of the probability assessments for OA exc. dur.

29.770. >7 days was predicted when BMI was equal to or greater than this value. The sensitivity and specificity of the obtained predictive model were 45.7% and 92.0%, respectively.

4 Discussion

The application of metformin in OA has garnered attention for its metabolic and anti-inflammatory properties, with additional implications for oxidative stress and lipid profile modulation (Song et al., 2022; Lai et al., 2022; Chen et al., 2022; Ragab et al., 2024). Previous studies have demonstrated that metformin exerts a beneficial effect on oxidative stress, an important factor in OA pathogenesis (Alimoradi et al., 2025; Zuliani et al., 2020; Arinno et al., 2023). Oxidative stress plays a significant role in the degeneration of cartilage and the progression of OA by promoting inflammation and joint tissue damage (Ansari et al., 2020; Liu et al., 2022). Our findings support this, as metformin treatment resulted in reduced levels of markers such as superoxide dismutase, which is associated with oxidative stress. This is consistent with previous research indicating that metformin can reduce oxidative stress and protect against joint degeneration in OA patients, potentially contributing to a slower progression of the disease (Xu et al., 2024; Ruan et al., 2022; Barnett et al., 2017; Hyun et al., 2013). Metformin exerts its therapeutic effects in OA through a combination of systemic and local mechanisms that address both metabolic and inflammatory pathways contributing to disease progression (Yao et al., 2023; Song et al., 2021; Wang et al., 2019). At the systemic level, metformin significantly reduces insulin resistance and hyperglycemia, which are strongly linked to the chronic low-grade inflammation characteristic of metabolic disorders, including IGT (Tsalamandris et al., 2019; Tizazu et al., 2019). By activating AMP-activated protein kinase (AMPK), metformin plays a pivotal role in inhibiting the mechanistic target of rapamycin (mTOR) signaling pathway (Amin et al., 2019; Nair et al., 2014; Putilin et al., 2020). This inhibition is critical, as mTOR activation is associated with chondrocyte hypertrophy, extracellular matrix breakdown, and cartilage degeneration, all of which are hallmark features of OA pathology (Fazio et al., 2024; Chawla et al., 2022; Dong and Jin, 2025).

Locally, metformin demonstrates potent anti-inflammatory properties by downregulating the production of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) (Jia et al., 2024; Scafidi et al., 2024; Tsuji et al., 2020). These cytokines are major drivers of synovial inflammation, joint swelling, and cartilage erosion (Lubberts and van den Berg, 2003; Miller et al., 2014; Lubberts et al., 2001; Sokolove and Lepus, 2013).

Metformin has also demonstrated antiviral properties, particularly in inhibiting the replication of several viruses, including COVID-19 (Halabitska et al., 2024e; Petakh et al., 2022; Buchynskyi et al., 2023). Its potential to modulate viral infections, coupled with its anti-inflammatory effects, suggests that metformin may offer therapeutic benefits beyond metabolic conditions (Petakh et al., 2023a; Martin et al., 2023; Amengual-Cladera et al., 2024). Metformin exhibits antimicrobial properties, influencing pathogens and gut microbiota (Nosulenko et al., 2014; Jauvain et al., 2021; Bilyi et al., 2015; Garg and Mohajeri, 2024). Metformin affects stress responses, hormonal balance, and gut microbiota, potentially reducing inflammation and improving metabolic resilience (Topol and Kamyshny, 2013; Bilous et al., 2021; Petakh et al., 2023b).

Additionally, metformin's effects on lipid profile are a notable area of interest (Gillani et al., 2021; Machado et al., 2012; Garimella et al., 2016). OA patients often present with metabolic disturbances, including dyslipidemia, which can exacerbate joint inflammation and cartilage degradation (Adam et al., 2024; Wei et al., 2023; Zhu et al., 2022). In our study, metformin led to significant improvements in lipid markers, including total cholesterol, LDL, HDL, and triglycerides. This is in line with other studies that have shown metformin's ability to improve lipid profiles, suggesting that it may not only mitigate inflammation and oxidative stress but also correct underlying metabolic dysfunctions that worsen OA symptoms (Xing et al., 2022; Pradas et al., 2019; Zou et al., 2024). In fact, metformin's ability to improve lipid metabolism may offer an additional mechanism for its positive effects in OA, as dyslipidemia is associated with increased risk of systemic inflammation and accelerated joint damage (Chen et al., 2022; Sobieh et al., 2023; Gkretsi et al., 2010; Mocanu et al., 2024).

When comparing our findings to other studies, the reduction in inflammatory markers such as C-reactive protein (CRP) and neutrophil-to-lymphocyte ratio with metformin use is also well-documented in the literature (Cameron et al., 2016; Hambly et al., 2023; Pitsavos et al., 2007; Rahnavard et al., 2022). These results highlight metformin's dual role in both controlling blood glucose and exerting anti-inflammatory effects, which have been linked to improved clinical outcomes in OA patients (Veronese et al., 2019; Kim et al., 2022; Lin et al., 2023). While our study demonstrates significant improvements in pain, stiffness, and functional limitations, differences in patient populations, dosages, or treatment durations align with the variability observed in the broader literature (Magni et al., 2021; Ferreira et al., 2024; Nahin et al., 2016). These discrepancies emphasize the need for further research to determine optimal dosing regimens and long-term efficacy of metformin in OA management.

Furthermore, the potential impact of metformin on neuropathic aspects of OA should not be overlooked (Zhang et al., 2024; Cao et al., 2024; Puşcaşu et al., 2024). As OA can be associated with peripheral neuropathy, particularly in patients with comorbid diabetes or metabolic dysfunction, the ability of metformin to influence glucose metabolism may offer additional therapeutic benefits (Song et al., 2022; Chen et al., 2022; Kaur et al., 2023; Li S. et al., 2024). Previous research has indicated that metformin may reduce nerve damage and improve pain perception in OA patients with diabetes, providing a rationale for its broader application in OA management (Alenazi et al., 2023; Alimoradi et al., 2023; Aiad et al., 2024). Genetic determination plays a crucial role in individual responses to medications (Sydorchuk et al., 2020; Mroziewicz and Tyndale, 2010; Chen et al., 2024). Genetic factors influence the expression and effectiveness of pleiotropic drug effects, including those of metformin, which are being actively studied by various researchers (Buchynskyi et al., 2024a; Pawlyk et al., 2014; Froldi, 2024; Buchynskyi et al., 2024b; Lyubomirskaya et al., 2020).

Finally, while metformin has shown promise in improving metabolic disturbances and reducing inflammation in OA, its efficacy may be limited in certain patient groups, particularly the elderly or those with renal impairment (Kulkarni et al., 2020; Ala and Ala, 2021; Kloppenburg et al., 2025). Our study highlights the need for careful patient selection and monitoring to avoid potential risks, such as lactic acidosis, in vulnerable populations. Further research is needed to investigate the long-term effects of metformin on joint health, its impact on oxidative stress, lipid metabolism, and

inflammatory markers, as well as its potential for combination therapy with other disease-modifying agents for OA.

5 Limitations

This study has several limitations that should be considered when interpreting the results. First, the relatively small sample size limits the generalizability of the findings to a broader population of patients with osteoarthritis (OA) and impaired glucose tolerance (IGT). Larger, multicenter studies are required to validate these results and confirm their applicability to different populations. Additionally, the study's observational nature and the lack of randomization may introduce selection bias, and further randomized controlled trials are needed to better assess the causal effects of metformin on OA symptoms and metabolic outcomes.

Moreover, the study only focused on a specific cohort of patients with both OA and IGT, without considering those with OA and normal glucose tolerance, which limits our understanding of metformin's potential effects across different metabolic states. The absence of long-term follow-up data also prevents us from fully assessing the sustained impact of metformin on joint health, pain, and inflammation over time.

Another limitation lies in the lack of detailed mechanistic data on how metformin influences the molecular pathways underlying both OA and metabolic dysfunction. Future studies should focus on elucidating these pathways and assessing the long-term effectiveness of metformin in modifying OA progression.

Despite these limitations, the findings provide valuable insights into the potential benefits of metformin for managing OA symptoms and metabolic dysfunction, and future research is needed to explore its broader application and long-term impact.

6 Conclusion

Patients receiving metformin showed significantly greater improvements in both clinical and metabolic outcomes compared to those not receiving metformin. The metformin group demonstrated reductions in pain, stiffness, and improved physical function, as measured by the WOMAC, Lequesne Algofunctional Index, KOOS, and VAS scales, with notable improvements in quality of life and mobility. In contrast, the non-metformin group showed less significant changes. Metformin also led to reduced inflammatory markers, including C-reactive protein, neutrophil-to-lymphocyte ratio, and superoxide dismutase, suggesting decreased systemic inflammation. Additionally, improvements in lipid profiles, such as reductions in total cholesterol, LDL, HDL, and triglycerides, were observed, highlighting metformin's metabolic benefits. Patients on metformin also showed significant improvements in BMI, waist-to-hip ratio, and waist-to-height ratio, indicating enhanced metabolic health. The need for increased NSAID doses in patients with osteoarthritis and impaired glucose tolerance can be predicted by factors such as pain severity, functional limitations, and inflammatory markers. Further studies are needed to confirm these findings and assess the long-term effects of dose adjustments. BMI has been identified as a potential predictor of OA exacerbation duration, with a threshold of 29.77. These findings suggest that metformin is effective in alleviating osteoarthritis symptoms and improving metabolic health in patients with osteoarthritis and impaired glucose tolerance. However, further research is needed to explore its long-term effects on joint health and inflammatory markers, as well as its potential role in managing osteoarthritis in patients without impaired glucose tolerance.

Data availability statement

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

Ethics statement

The studies involving humans were approved by the Local Ethics Committee of the I. Horbachevsky Ternopil National Medical University as protocol N78, dated 18 August 2024. 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

IH: Data curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing–original draft. PP: Writing–review and editing. OK: Supervision, Visualization, Writing–review and editing.

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Conflict of interest

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

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

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The adjunctive role of metformin in patients with mild to moderate ulcerative colitis: a randomized controlled study

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Background: Metformin, hypoglycemic medication, is recognized for its diverse properties and its capacity to influence the inflammatory pathways. Medications with anti-inflammatory and anti-oxidative characteristics have been demonstrated to be able to elicit and sustain remission in ulcerative colitis (UC), chronic inflammatory disorder of the bowel. Studies in both preclinical and clinical settings have looked into the several metabolic pathways via which metformin protects against UC.

Aim: To assess efficacy of metformin as adjunctive therapy in patients with mild to moderate UC.

Methods: This clinical research was double-blinded, randomized, controlled, and involved 60 patients with mild to moderate UC. The participants were randomly assigned to one of two groups (n = 30). The control group was given 1 g of mesalamine three times a day (t.i.d.) for a period of 6 months (mesalamine group). The metformin group was given 500 mg of metformin twice daily and 1 g of mesalamine t. i.d. For a period of 6 months. Patients with UC were assessed by a gastroenterologist using the disease activity index (DAI) both at the beginning of treatment and 6 months thereafter. To evaluate the drug's biological efficacy, measurements of fecal calprotectin, serum C-reactive protein (CRP), interleukin 10 (IL-10), and nitric oxide (NO) were taken both before and after treatment.

Study outcomes: Decrease in DAI and change in the level of measured serum and fecal markers.

Results: The metformin group displayed a statistical reduction in DAI ($p = 0.0001$), serum CRP ($p = 0.019$), NO ($p = 0.04$), and fecal calprotectin ($p = 0.027$), as well as a significant increase in IL-10 ($p = 0.04$) when compared to the mesalamine group. There was a significant direct correlation between DAI and calprotectin

($p < 0.0001$, $r = 0.551$), and between DAI and CRP ($p < 0.0001$, $r = 0.794$). There was a significant negative correlation between DAI and IL-10 ($p = 0.0003$, $r = 0.371$).

Conclusion: Metformin may be an effective adjunct drug in management of patients with mild to moderate UC by decreasing DAI and other inflammatory markers that were involved in the pathogenesis of UC.

Clinical Trial Registration: identifier NCT05553704.

KEYWORDS

ulcerative colitis, disease activity index, metformin, calprotectin, nitric oxide

1 Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are two of the many gastrointestinal tract disorders that are included in the category of inflammatory bowel diseases (IBD), which are long-term inflammatory diseases. More than 0.3% of persons in North America, Australia, and Europe have IBD, making it a very prevalent condition in western countries. This significantly burdens the medical care industry (Wanchaitanawong et al., 2022). IBD is rare (0.001%–0.05%) in Asia, Africa, and South America, but a recent study indicated that its incidence has increased yearly by 4%–15% in these newly modernized regions, raising concerns about the disease's potential impact on global health (Gajendran et al., 2019).

The pathogenesis of UC is complex and involves an impaired intestinal barrier, external factors, genetic susceptibility, bacterial imbalance in the gut, and an inappropriate immunological response (Guan 2019). The course of treatment for UC is determined by the severity and course of the disease. Currently, patients with mild to moderate cases can receive conventional treatments like amino salicylates (ASA), immunomodulators, and corticosteroids, or biologic agents like anti-interleukin (IL) 12/23, anti-tumor necrosis factor (TNF), anti-Janus kinase (JAK), and anti-integrin (Silaghi et al., 2022). Regardless of the abundance of biologic medications on the market, therapeutic outcomes are not always desirable. Patients considered to be primary non-responders to anti-TNFs ranged from 10 to 40 percent (Marsal et al., 2022). A progressive absence of response also occurred in 10–50 percent of patients (Marsal et al. 2022). Thus, between 20 and 30 percent of patients with UC and 30 to 40 percent of those with CD required intestinal surgery at some point in their lives. In addition, IBD patients find it challenging to take several currently prescribed drugs due to their negative effects (Burisch et al., 2022). Clinicians have therefore been searching for novel therapies or strategies to repurpose drugs that are successful in treating UC.

Cytokines play a major role in the inflammatory processes that promote inflammation and the pathophysiology of UC by, among other things, producing inflammatory mediators and activating inflammatory pathways. In UC, they directly result in tissue destruction and mucosal inflammation, which set off condition-specific immune reactions (Neurath 2024). Numerous inflammatory mediators were involved in the pathogenesis of UC like interleukins, chemokines, and other mediators (Neurath 2024).

Currently, the most fully investigated inflammatory indicators are CRP and fecal calprotectin (Yoon et al., 2014). Despite the documented association between endoscopic activity and CRP, the evidence are currently insufficient to justify its widespread application in UC (Magro et al., 2016). There are several positive findings for fecal calprotectin, which demonstrate extremely strong association with clinical response, endoscopic parameters, as well as mucosal restoration in UC (Angriman et al., 2007; Nakov et al., 2019; Yoon et al. 2014).

Reactive oxygen and reactive nitrogen species (ROS and RNS) have long been implicated in both the causes and development of UC (Muro et al., 2024). The injured lamina propria of patients with UC showed considerable neutrophil infiltration and a rise in myeloperoxidase levels, which were similar to the epithelia. In mice, inducible nitric oxide synthase (iNOS) gene deletion was demonstrated to drastically reduce the development and severity of colitis (McCafferty et al., 2000; Piechota-Polanczyk and Fichna 2014). In UC, iNOS is thought to be responsible for significantly elevated NO generation in the mucosa and in areas of inflammation in conjunction with nitrotyrosine (Tachibana et al., 2020). iNOS-derived NO enhances cytokine production in large intestine, leading to neutrophil infiltration, for example, by stimulating the production of intracellular adhesion molecule (ICAM) and P-selectin, resulting in colonic tissue injury (Piechota-Polanczyk and Fichna 2014).

Metformin, with its comparatively low cost as well as favorable safety characteristics, is the initial therapy for patients with type 2 diabetes (McCreight et al., 2016). Beyond its anti-diabetic effects, previous studies have highlighted its potential therapeutic benefits, including anticancer activity, cardiovascular protection, anti-aging effects, and anti-inflammatory properties (Anisimov 2013; Leone et al., 2014; Rena and Lang 2018). Notably, metformin has been shown to enhance goblet cell numbers in the gastrointestinal tract, suggesting a mucus-protective role (Xue et al., 2016). Additionally, multiple preclinical studies have demonstrated that metformin reduces colitis severity by inhibiting key inflammatory pathways, including p38 mitogen-activated protein kinase (MAPK), Jun N-terminal kinases (JNK), phosphorylated signal transducer and activator of transcription 3 (pSTAT3), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Deng et al., 2018; Di Fusco et al., 2018; El-Haggar et al., 2024). EL-mahdy et al., reported that metformin reduced disease activity index (DAI) and inflammatory markers in oxazolone induced colitis (El-Mahdy et al., 2021). Clinically, metformin alleviated inflammation, decreased serum inflammatory markers, and upregulated tight junction proteins in patients with mild to moderate UC (El-Haggar et al., 2024). Metformin is associated with improved IBD outcomes in patients with Type 2 diabetes mellitus in propensity-matched cohort study (Petrov et al., 2024).

Despite these promising findings, clinical evidence supporting the use of metformin as an adjuvant therapy in UC remains limited.

To the best of our knowledge, this study is among the first randomized controlled trials to evaluate the efficacy of metformin in patients with mild to moderate UC. In light of these investigations, our study aims to provide novel insights into the potential therapeutic role of metformin in UC management by assessing its effects on DAI, fecal calprotectin, and key inflammatory biomarkers such as serum CRP, NO, and IL-10.

2 Patients and methods

This study was a part of our previously published work about repurposing of metformin in patients with mild to moderate UC (El-Haggar et al., 2024). Between November 2022 and December 2023, sixty patients who satisfied the eligibility criteria were selected from the Gastroenterology Department. The ethical review committee of the Mansoura University Faculty of Medicine authorized this research. The Helsinki Declaration and its 1964 revisions were followed and adhered in the study's design and methods. The patients were told that they might withdraw from the research at any time. Both patients and physicians were kept blinded about the kind of exposure and randomization. An unblinded chemist administered study medications to participants to guarantee appropriate treatment assignment; the chemist was not involved in the assessment of research results.

2.1 Inclusion criteria

Patients above the age of eighteen, both male and female, were enrolled in this study. Effective contraception and a negative pregnancy test should be provided to female patients. This clinical study only covered mild to moderate patients of UC.

2.2 Exclusion criteria

Patients receiving systemic or rectal steroids, immunosuppressive medications, or having severed type UC were excluded. Additionally excluded were patients with renal or hepatic impairment in order to avoid the adverse metabolic consequences of metformin. Diabetic patients were excluded to specifically assess the anti-inflammatory and immunomodulatory effects of metformin in UC without the confounding influence of its glucose-lowering properties. Individuals have a history of lactic acidosis, total or partial colectomy, and colorectal malignancy were also ineligible. Lastly, women who were nursing and those who were receiving metformin treatment for polycystic ovarian syndrome either before or now were not included.

2.3 Study design

The safety and effectiveness of metformin as an adjuvant medication to mesalamine in treatment of UC were assessed in this clinical study.

Under the NCT05553704 code, this study was listed at www.Clinical.Trials.gov.in.2022.

The patients were split into two groups at random (n = 30), as Figure 1 CONSORT flow diagram illustrates. A computer random number generator was used to select randomly permuted blocks for the randomization process. Thirty patients were randomly allocated to one of two groups after fulfilling the eligibility requirements and giving their written, informed consent.

Group 1 (mesalamine group): Patients in this group received 1 g of mesalamine tablets t. i.d. (PentasaR 500 mg, Multi Pharm, Egypt) and a placebo for a period of 6 months.
Group 2 (metformin group): Patients in this group received 500 mg metformin tablets bid (GlucophageR 500 mg, Mina Pharm, Egypt) and 1 g of mesalamine tablets t. i.d. (PentasaR 500 mg, Multi Pharm, Egypt) for a period of 6 months.

2.4 Sample size calculations

The sample size for our study was determined based on recommendations by Sima and Lewis (Sim and Lewis 2012) who suggest a minimum of 22 participants per group for detecting small to medium effect sizes in pilot studies. Since there were not any studies that investigated the effect of metformin on DAI in patients with mild to moderate UC, this study was designed to be a pilot one. Given that our study serves as a preliminary investigation into the efficacy of metformin as an adjuvant therapy in UC, we aimed to ensure adequate statistical power while maintaining feasibility. To enhance the robustness of our findings, we increased the sample size to 32 patients per group, accounting for a power of 0.80, an α-error of 0.05 (two-tailed), and a 20% dropout rate. This approach aligns with standard recommendations for pilot studies, ensuring that our study provides meaningful preliminary data to inform future larger-scale trials.

2.5 Study protocol

In alongside the enrollment checks, UC patients received thorough mental, physical, and psychological assessments. Patients were randomly assigned to receive either a placebo tablet and 1 g of mesalamine tablets given t. i.d. (mesalamine group) or 1 g of mesalamine tablets taken t. i.d. Together with 500 mg of metformin tablets orally administered bid (metformin group), in accordance with the CONSORT guidelines. Zeta Pharma Company produced placebo tablets, which were identical in appearance to metformin tablets. Along with nutritional and lifestyle counseling, all medications were administered orally to the patients. Based on earlier research, the doses of metformin and mesalamine that have been chosen are 500 mg bid (Garber et al., 1997) and 1 g t. i.d. (Kruis et al., 2003), respectively. The chosen dosage of 500 mg twice a day falls within the widely used therapeutic range, and prior research examining its non-glycemic effects has demonstrated that it is well tolerated in non-diabetic people. Metformin by itself is not (Holstein and Egberts 2003; Lalau et al., 1998) or infrequently (Group UPDS, 1998) linked to hypoglycemia, which is characterized by symptoms and indicators of hypoglycemia and/or plasma glucose levels below 3.3 mmol/l as well as a clinical reaction to glucose delivery. The reported risks of hypoglycemia for metformin users ranged from 0% to 21%, in a previous review (Bolen et al., 2007). Metformin may have a lesser

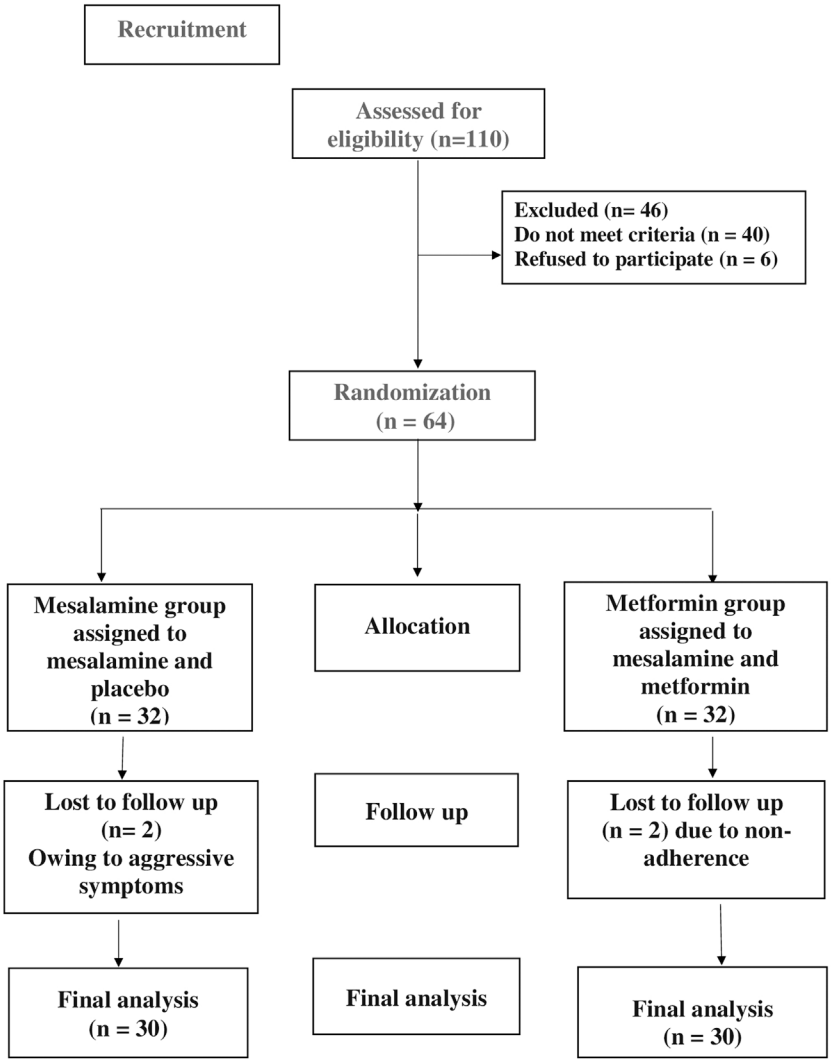

FIGURE 1
CONSORT diagram showing the flow of patients during study.

risk of hypoglycemia than other oral antidiabetic medications because it does not directly boost insulin production. However, hypoglycemia in patients using metformin may occur in association with strenuous physical activity or fasting (Bodmer et al., 2008). Furthermore, we advised all patients to take metformin after meals, this will result in reducing the incidence of hypoglycemia. Importantly, we carefully monitored all participants for any signs of hypoglycemia or adverse effects throughout the study to ensure patient safety.

2.6 Follow-up

Monthly phone calls and meetings were used to follow up with patients. At the first visit, patients received a full medical history, liver function [alanine amino-transferase (ALT) and aspartate amino-transferase (AST)] and kidney function testing (serum creatinine (SrCr), and complete blood counts in order to exclude out any organic abnormalities. Serum biomarkers (NO, CRP, IL-10) and fecal calprotectin were measured.

2.7 Study outcomes

Change in DAI and measured serum and fecal markers (IL-10, NO, CRP, calprotectin).

2.8 Evaluation of colitis

In accordance with Mitsuru Seo et al., the Disease Activity Index (DAI) was computed for every patient both before and after 6 months of treatment (Seo et al., 1992).

DAI = 13 x bowel movements +60 x blood stool +0.5 x ESR - 4 x HB - 15 x albumin +200. Index values below 150, values between 150 and 220, and values above 220 nearly corresponded to mild, moderate, and severe disease, respectively.

Bowel movements: Reflecting the frequency of diarrhea as an indicator of disease severity.

Blood in stool: A critical marker of mucosal inflammation and ulceration.

Erythrocyte Sedimentation Rate (ESR): A systemic inflammatory marker.
Hemoglobin (HB): Representing the impact of chronic inflammation and potential blood loss.
Serum albumin: A marker of nutritional status and disease severity.
Constant factor (200): Used for standardization of the scale.

2.9 Therapeutic assessments

Therapeutic assessment was done by measuring DAI, serum markers (NO, CRP, IL-10), and fecal calprotectin.

2.10 Sample collection

Before the investigation began and 6 months after the treatment, 10 mL of blood were drawn from the antecubital vein. The blood sample was then centrifuged for 10 mins at 4,500 g (Hettich Zentrifugen EBA 20) after the blood was progressively transferred into test tubes and allowed to coagulate. Two serum aliquots were taken; the first was used for routine tests on the kidney and liver, and the second one was frozen at −80°C to measure specific cytokine levels.

Stools that had been weighed were dissolved in regular saline and centrifuged. Clear supernatants were used to analyze the calprotectin in fecal material.

2.11 Biochemical analysis

Using commercially available enzyme-linked immunosorbent assay (ELISA) kits, the serum levels of IL-10 (catalogue no.: 201-12-0,103), NO (catalogue no.: 201–12–1,511), CRP (catalogue no.: DY1707), and fecal calprotectin (catalogue no.: 201-12-5,461) were measured in accordance with manufacturer's guidelines. With the exception of the CRP kits, which came from R&D Systems China Co., Ltd., the kits were supplied by Sunredio, Shanghai, China.

The human IL-10 level in samples was measured using a double-antibody ELISA kit. IL-10 was added to a monoclonal antibody enzyme well that had been pre-coated with human IL-10 monoclonal antibody for incubation; then IL-10 antibodies labelled with biotin were added and combined with Streptavidin-HRP to form an immune complex; finally, incubation and washing to remove the uncombined enzyme were carried out. Then chromogen solution A and B were added, changing the color of the liquid from blue to yellow as a result of the acid effect. All markers were measured in the same manner.

2.12 Statistical analysis

The statistical analysis was performed using Prism version 9 from GraphPadsoftware, Inc., San Diego, California, United States. Using the Shapiro-Wilk method, the normality of a continuous variable was examined. Using the Wilcoxon test and the Student's t-test before and after therapy, significant variations were observed within the group for nonparametric and parametric data, respectively. To find any statistically significant variations between groups before and after therapy, the Man Whitney test and the unpaired Student's t-test were used for nonparametric and parametric data, respectively. While the mean ± SD was utilized to convey quantitative data, numbers, median, and the interquartile range, were utilized to represent qualitative markers. The Pearson correlation test was used to find the correlation between the normally distributed parameters. The Chi-square test and Fisher exact test were utilized for categorical results. There were two tails to every p-value, with less than 0.05 p-value being regarded as statistically significant.

3 Results

3.1 Clinical and demographic characteristics

The current study found no statistically significant differences between mesalamine and metformin groups in terms of baseline characteristics, including platelet count ($p = 0.147$), SrCr ($p = 0.617$), age ($p = 0.113$), sex ($p = 0.796$), weight ($p = 0.726$), height ($p = 0.404$), ALT ($p = 0.289$), AST ($p = 0.467$), and glycated hemoglobin ($p = 0.643$). Regarding the baseline demographic data which are the same as our previously published one (El-Haggar et al., 2024), we put this table in the Supplementary Table S1. Due to non-compliance with medication, two patients were lost to follow-up in the metformin group. Two other patients were dropped from the mesalamine group and transferred to immunosuppressive combination therapy. Since sixty patients finished the trial, all measurable parameters' statistical analyses were carried out in accordance with protocol.

3.2 Effect of study medications on measured markers

At the start of the study, there were no significant differences in all measured markers between the two study groups ($p > 0.05$). (Table 1).

After treatment, mesalamine group showed significant changes in all measured markers when compared to its baseline values as followed: DAI (179.4 ± 26.37 versus 110.6 ± 33.4, $p < 0.0001$), IL-10 (170 ± 17.16 versus 178.9 ± 13.76, $p = 0.039$), NO (240 (153.5–255.5) versus 187.7 (125.3–267.3), $p = 0.007$), CRP (152.3 ± 9.521 versus 65.66 ± 8.407, $p < 0.0001$), and calprotectin (27.07 ± 3.822 versus 23.49 ± 3.718, $p = 0.0005$) (Table 1).

Metformin group displayed significant changes in all measured markers when compared to its baseline values as followed: DAI (170.9 ± 31.22 versus 81.47 ± 20.51, $p < 0.0001$), IL-10 (172.1 ± 12.04 versus 187.7 ± 18.46, $p = 0.0007$), NO (230.8 (137.5–256) versus 146 (100.5–194.2), $p = 0.0003$), CRP (154.3 ± 7.964 versus 59.80 ± 10.42, $p < 0.0001$), and calprotectin (30.44 ± 4.543 versus 21.13 ± 4.313, $p < 0.0001$) (Table 1).

Between group comparisons, unpaired t-test revealed that there were significant changes in DAI ($p = 0.0001$), IL-10 ($p = 0.04$), CRP ($p = 0.019$), and calprotectin ($p = 0.027$). ManWitney test revealed

TABLE 1 Effect of study medications on measured parameters.

Character	Mesalamine group (n = 30)			Metformin group (n = 30)			P value
	Before treatment	After treatment	P value	Before treatment	After treatment	P value	After treatment
DAI	179.4 ± 26.37	110.6 ± 33.4	<0.0001*	170.9 ± 31.22	81.47 ± 20.51	<0.0001*	0.0001**
IL-10 (pg/mL)	170 ± 17.16	178.9 ± 13.76	0.039*	172.1 ± 12.04	187.7 ± 18.46	0.0007*	0.04**
NO (μmol/L)	240 (153.5–255.5)	187.7 (125.3–267.3)	0.007#	230.8 (137.5–256)	146 (100.5–194.2)	0.0003#	0.04##
CRP (pg/mL)	152.3 ± 9.521	65.66 ± 8.407	<0.0001*	154.3 ± 7.964	59.80 ± 10.42	<0.0001*	0.019**
Calprotectin (ng/mL)	27.07 ± 3.822	23.49 ± 3.718	0.0005*	30.44 ± 4.543	21.13 ± 4.313	<0.0001*	0.027**

Data was displayed as median, interquartile range, and mean ± SD, mesalamine group; UC, patients treated with mesalamine and placebo, Metformin group; UC, patients treated with mesalamine plus metformin; DAI, disease activity index; IL-10, interleukin 10; NO, nitric oxide, CRP, C-reactive protein. (*) and (#) level of significance within the same group by paired t-test and Wilcoxon test, respectively. (**) and (##) level of significance between groups using unpaired t-test and Man Witney test, respectively. Significance at ($p < 0.05$).

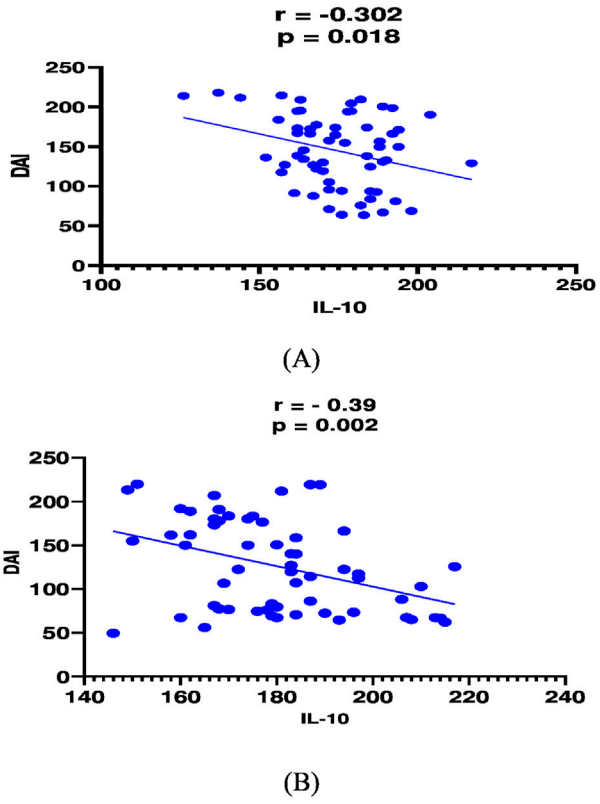

FIGURE 2
Correlation analysis between Disease activity index (DAI) and IL-10 in mesalamine group **(A)** and metformin group **(B)**.

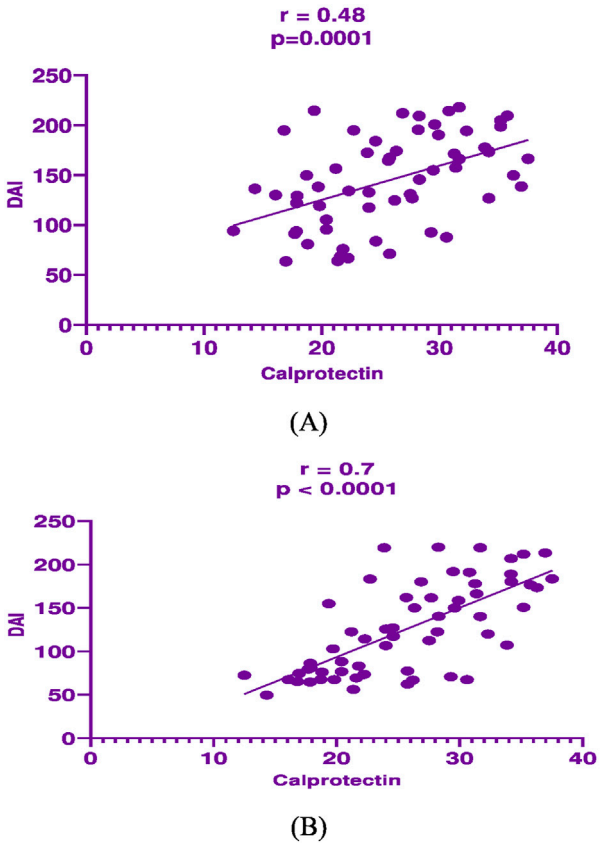

FIGURE 3
Correlation analysis between Disease activity index (DAI) and fecal calprotectin in mesalamine group **(A)** and metformin group **(B)**.

that there was a significant change in NO ($p = 0.04$) level between the two groups.

3.3 Correlation analysis between the studied parameters

In mesalamine group, there was a significant direct correlation between DAI and CRP [(p = 0.004, r = 0.36), Figure 2A], and between DAI and calprotectin [(p = 0.0001, r = 0.48), Figure 3A] and a significant negative correlation between DAI and IL-10 [(p = 0.018, r - = 0.302), Figure 4A].

In metformin group, there was a significant direct correlation between DAI and CRP [($p < 0.0001$, r = 0.854), Figure 2B], and between DAI and calprotectin [($p < 0.0001$, r = 0.7), Figure 3B] and a significant negative correlation between DAI and IL-10 [(p = 0.002, r - = 0.39), Figure 4B].

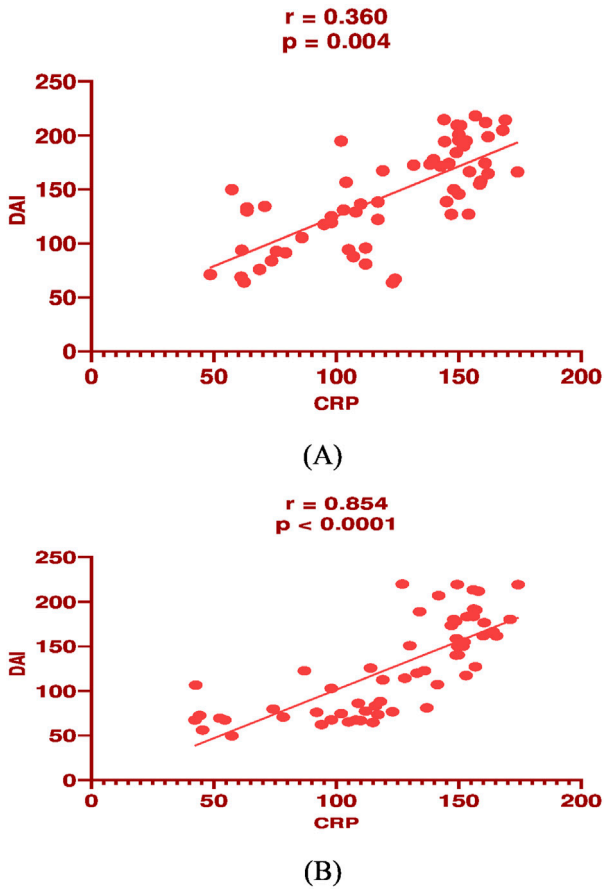

FIGURE 4
Correlation analysis between Disease activity index (DAI) and serum C-reactive protein (CRP) in mesalamine group **(A)** and metformin group **(B)**.

4 Discussion

Ulcerative colitis (UC) is a form of inflammatory bowel disease characterized by persistent and recurring inflammation in the colon, leading to symptoms such as abdominal pain, bloody diarrhea, more frequent bowel movements, and other systemic effects. Its increasing incidence has been linked to lifestyle changes in developing countries, along with several other factors (Ke et al., 2021). Reactive oxygen species and a rise in inflammatory responses have long been known to have a role in the development of this illness, even though its exact cause is unknown (El-Mahdy et al. 2021). Elevated levels of oxidative stress indicators, pro-inflammatory cells, and mediators were observed in the colonic tissue of UC patients and together they contribute to the loss of integrity and ulceration of mucosa (Wanchaitanawong et al. 2022).

We have published part of this work previously, and this is a continuation of this previous study (El-Haggar et al., 2024). Several clinical (El-Haggar et al., 2024; Kabel et al., 2017; Petrov et al. 2024) and preclinical studies (El-Mahdy et al. 2021; Ke et al. 2021; Liu et al., 2021) were conducted and evaluated the repurposing of metformin in inflammatory bowel diseases. It is worthy to note that this is the first clinical study to evaluate the effect of metformin on patients with mild to moderate UC by decreasing NO, calprotectin, CRP, DAI, and increasing anti-inflammatory cytokine (IL-10).

Metformin administration in addition to mesalamine substantially lowered oxidative stress and decreased colitis more than mesalamine monotherapy. In light of these findings, the current study offers a crucial knowledge of the part metformin plays in the medication's protective effects against oxidative stress and inflammation. The findings demonstrate that metformin lessens UC severity. There is consistent evidence that in mouse colitis, metformin has anti-inflammatory effects. Metformin decreased colonic inflammation by activating the AMP-activated protein kinase, according to research by Di Fusco et al., (Di Fusco et al. 2018). According to Lee et al., metformin reduced colitis by increasing the AMPK signaling cascade and inhibiting STAT3 activation (Lee et al., 2015). Additionally, Deng et al. demonstrated that metformin alleviated mice colitis by protecting against gastrointestinal barrier disruption through AMPKa1-dependent suppression of JNK pathway stimulation (Deng et al. 2018).

Metformin combination with mesalamine in the current study significantly reduced DAI when compared to mesalamine monotherapy. These results were in line with previous reports (Koh et al., 2014; Liu et al. 2021). Metformin (100 mg/kg and 500 mg/kg) taken orally considerably reduced the degree of severity of colitis as determined by body weight loss, DAI, and colon length (Koh et al. 2014). Dextran sulfate-(DSS)-induced acute colitis was significantly improved with metformin pretreatment (Koh et al. 2014). EL-mahdy et al., reported that metformin either alone or in combination with mesalamine significantly reduced macroscopic and microscopic scores in oxazolone induced colitis (El-Mahdy et al. 2021). All of these observations and previous findings highlighted the protective role of metformin in reducing mucosal damage in UC. When compared to the same parameters before therapy in a previous trial, metformin administration to patients with UC resulted in a significant drop in colonic endoscopic score as well as an alleviation of the histopathology (Kabel et al. 2017). Metformin reduced the symptoms of colitis brought on by the DSS, as seen by reduction in disease index, increased body weight, and improved mucosal integrity (Liu et al. 2021).

It was previously believed that oxidative stress was a major factor in the pathophysiology of UC. Persistent bowel inflammation in UC is usually attributed to an excess of ROS and RNS, respectively (Balmus et al., 2016). Oxidative stress is generated in UC when there is typically an imbalance between ROS and antioxidant capacity. Upon synthesis, ROS engage in molecular complex interactions to trigger oxidative damage within cells. This damage can impact lipids, proteins, and nucleic acids, resulting in the creation of lipid peroxides, disruption of enzyme functions, and split DNA strands (Jena et al., 2012). According to Ashabi et al.'s research, the nuclear factor erythroid 2 related factor (Nrf2) pathway is activated by metformin's promotion of AMPK activation, which in turn causes its antioxidant and anti-inflammatory actions (Ashabi et al., 2015). This was consistent with the current study's findings, which indicated that patients with UC had a markedly elevated NO level at the onset of treatment. Metformin treatment dramatically decreased NO in comparison to both the mesalamine group and its baseline value. These findings coincide with previous researches (El-Mahdy et al. 2021; Pandey et al., 2017; Sadeghi et al., 2019). Wang et al., evaluated how metformin inhibited iNOS, which in turn reduced the amount of NO produced by monocytes (Wang et al.,

2020). The present investigation found that the administration of mesalamine considerably decreased the level of NO, and these findings are consistent with prior reports (He et al., 2019). Metformin also enhanced the ability of antioxidant enzymes by raising lower glutathione levels (Sadeghi et al., 2019). Strong antioxidant and free radical scavenger properties have been demonstrated in mesalamine (Kaiser et al., 1999).

Fecal calprotectin levels were significantly lower in the metformin group as compared to the baseline and mesalamine groups. These outcomes were consistent with earlier research (Boshra 2022; Djaja et al., 2019). When there is persistent inflammation in the gut, polymorphonuclear neutrophils move from the bloodstream to the gastrointestinal mucosa. Neutrophils flow into the lumen as a result of any inflammatory process-induced damage of the mucosal architecture, releasing calprotectin, which is subsequently expelled in stool (D'Amico et al., 2020). The quantity of calprotectin found in the feces is positively correlated with the intensity of UC (Grgić et al., 2022). In comparison with mesalamine alone, the current investigation showed that metformin plus mesalamine dramatically decreased calprotectin. Grip and Olof showed that in patients with Crohn's disease (CD), there was a strong relationship between the proinflammatory mediators and the calprotectin quantity (Grip and Janciauskiene 2009). Fecal calprotectin levels in UC patients' stools are a good indicator of mucosal healing and are correlated with both histologic and endoscopic inflammation (Theede et al., 2015). In support with the current research, there was a strong positive correlation between DAI and fecal calprotectin. These results support the notion that calprotectin may be a prognostic and diagnostic non-invasive tool in UC. Taina Sipponen et al., reported that inflammatory bowel diseases activity was assessed by fecal calprotectin and lactoferrin and there was a strong correlation between Crohn's disease activity index and endoscopic findings (Sipponen et al., 2008).

The current study demonstrated that combination therapy between metformin and mesalamine significantly reduced CRP when compared to mesalamine alone. These findings were in line with previous researches in the same field (Chen et al., 2017; Vermeire et al., 2004). IL-6, IL-1β, and TNF-α stimulate the production of CRP, a pentameric protein that is virtually entirely generated by hepatocytes. (Tall 2004). The primary acute-phase protein is CRP. CRP has a baseline value of 1 mg/L, and its levels are somewhat influenced by genetics. When there is an infection or inflammation in the acute phase, CRP levels rise sharply. When the inflammatory process is managed, CRP concentrations likewise drop rapidly (Vermeire et al. 2004). CRP levels significantly decreased after metformin treatment, especially in obese women (Chen et al. 2017). The effect of metformin on acute phase reactant proteins may be due to its potent anti-inflammatory activity by decreasing NF-kB, reducing insulin resistance, and activating AMPK pathways (Ye et al., 2018). The results of the current study revealed that there was a strong correlation between DAI and CRP level. These findings were matched with previous studies (Osada et al., 2008; Schoepfer et al., 2009). The clinical and endoscopic indices were used to prospectively analyze 134 UC patients in the Schoepfer et al. study. The highest correlation ($r = 0.503$) was seen between endoscopic disease activity and CRP. When CRP was raised, the overall accuracy of detecting endoscopically active illness was 62% (Schoepfer et al. 2009). In a different Japanese investigation, the total endoscopic and histological results were connected with the CRP concentration; specifically, the activity of proximal colonic lesions was positively correlated with both CRP and erythrocyte sedimentation rate (ESR) (Osada et al. 2008). These findings were validated by Henriksen et al., who demonstrated that CRP levels at diagnosis elevated as the disease's severity increased in UC patients (Henriksen et al., 2008).

Several studies proved that metformin exhibited potent anti-inflammatory activity and boosting the levels of anti-inflammatory cytokines such as IL-10 (Jenkins et al., 2012; Sharma et al., 2013). These observations were matched with our study as there was a significant increase in serum IL-10 level by synergistic combination of metformin and mesalamine than mesalamine alone. The finding that mice lacking IL-10 and its receptor exhibit bowel inflammation on their own suggested a close relationship between IL-10 and gastrointestinal mucosal equilibrium. These factors have made IL-10's anti-inflammatory qualities an extremely interesting target for IBD treatment (Shouval et al., 2014). Several studies established that mice deficient in IL-10 developed colitis and metformin treatment significantly boosts IL-10 (Berg et al., 2002; Elliott et al., 2004). However, a conflicting result showed that in a jejunal cell model of IBD, metformin increased IL-10 transcription while decreasing it in other cases (Wu et al., 2018). The shorter duration of the investigation, which was intended to mimic an acute inflammatory response, may have contributed to this contradictory results. This notion was strongly supported by previous research showing that metformin reduced the production of IL-10 in macrophages following acute lipopolysaccharide (LPS) exposure (Postler et al., 2021). IL-10 is a cytokine with anti-inflammatory properties that is also referred to as human cytokine synthesis inhibitory factor (CSIF). Monocytes are the main producers of this cytokine, with lymphocytes producing it to a lesser degree (Sharma et al. 2013). This mediator has multiple impacts on immunoregulation and inflammatory cascades. It suppresses the expression of costimulatory molecules, Th1 cytokines, and major histocompatibility complex (MHC) class II Ags on macrophages. Additionally, it improves B cell growth, survival, and production of antibodies (Sharma et al. 2013). This cytokine is involved in the control of the JAK-STAT signaling cascade and has the ability to suppress NF-jB activation (Sharma et al. 2013). Metformin limits the generation of IL-1β and increases IL-10 in lipopolysaccharide-stimulated macrophages via inhibiting the formation of ROS from nicotinamide adenine dinucleotide hydrogen (NADH) ubiquinone oxidoreductase (Kelly et al., 2015). It has been reported that metformin has a potent anti-inflammatory activity through peroxisome proliferator-activated receptors (PPAR) dependent mechanisms (Qu and Qu 2019).

Last but not at least, the mesalamine group displayed a significant reduction in DAI, serum NO, IL-10, CRP, and fecal calprotectin when compared to its baseline values. These findings were consistent with earlier publications. (Alarfaj et al., 2024; Bahaa et al., 2024; El-Haggar et al., 2024). Mesalamine has been widely utilized in the treatment of mild to moderate cases of UC, hence it is quite likely that mesalamine itself is responsible for these observations (Bahaa et al. 2024). These results align with previous studies that examined the impact of mesalamine on UC (El-Mahdy et al. 2021; El-Haggar et al., 2024). Mesalamine possesses anti-inflammatory and apoptotic properties and suppresses inflammatory cytokines through a PPAR-gamma-dependent manner (Rousseaux et al., 2005).

The study drugs were mostly accountable for the therapeutic outcomes because at the beginning of the investigation, there were not significant differences in the groups' clinical or demographic characteristics.

5 Conclusion

While preclinical studies have suggested that metformin may exert anti-inflammatory and immunomodulatory effects in IBD through AMP-activated protein kinase (AMPK) activation, inhibition of NF-κB signaling, and modulation of gut microbiota, clinical evidence remains scarce. Our study is the first clinical trial to investigate the effects of metformin as an adjunct to mesalamine in patients with UC, with a specific focus on fecal calprotectin, NO, CRP, and IL-10 as biomarkers of inflammation. Metformin may be a potential adjunctive treatment for patients with UC. This may be brought about by the combination of metformin and mesalamine's synergistic anti-inflammatory and antioxidant properties, as well as their capacity to reduce inflammatory indicators and the DAI.

5.1 Merits of the study

1. Novelty and Clinical Relevance: Our trial is among the first to provide clinical evidence of metformin's potential benefits in UC patients, specifically evaluating its effects on inflammatory biomarkers (CRP, IL-10, calprotectin), oxidative stress markers (NO), and disease activity indices.
2. Synergistic Mechanism with Mesalamine: The combination of metformin and mesalamine may enhance anti-inflammatory effects due to their complementary mechanisms of action. While mesalamine primarily inhibits prostaglandin synthesis and suppresses local inflammation, metformin reduces systemic inflammation and oxidative stress.
3. Potential Biomarker Utility: The study highlights fecal calprotectin, NO, CRP, and IL-10 as possible indicators of metformin's therapeutic response in UC, paving the way for future biomarker-driven treatment strategies.
4. Clinical Implications for Drug Repurposing: Metformin is a well-characterized, widely available, and cost-effective drug with an established safety profile, making it an attractive candidate for adjunctive UC therapy.

5.2 Demerits and limitations of the study

1. Short Follow-up Duration: The relatively brief study period limits the ability to assess long-term effects of metformin on disease remission, relapse rates, and sustained biomarker changes.
2. Small Sample Size: The limited number of participants reduces statistical power and generalizability, necessitating larger, multicenter trials to confirm our findings.
3. Lack of Variable Dosage Analysis: The study utilized a fixed metformin dose, whereas dose-response variations could provide insights into the optimal therapeutic regimen for UC patients.
4. Absence of a Healthy Control Group and Metformin only Group: While including healthy participants and metformin only group for comparative analysis would have strengthened the findings, ethical restrictions in Egypt, particularly regarding invasive procedures such as colonic biopsies, posed challenges. IRB of Mansoura University rejected the recruitment of healthy control and metformin only groups.
5. Metformin-Related Adverse Effects Not Monitored: Given metformin's known effects on glucose metabolism, vitamin B12 levels, and weight loss. Tracking serum glucose, body weight, and vitamin B12 levels would have been beneficial. These parameters should be considered before and after treatment in future studies.

Overall, despite these limitations, our study provides preliminary clinical evidence supporting metformin as a promising adjunct to mesalamine in UC management. Further randomized controlled trials with larger sample sizes, longer follow-up periods, and dose-response evaluations are warranted to validate these findings and explore metformin's full therapeutic potential in UC.

Data availability statement

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

Ethics statement

The studies involving humans were approved by The Ethical Review Committee of the Mansoura University Faculty of Medicine. 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

AB: Conceptualization, Funding acquisition, Software, Writing–original draft. SE-H: Investigation, Project administration, Supervision, Writing–review and editing. SH: Funding acquisition, Investigation, Methodology, Validation, Writing–review and editing. MM: Methodology, Supervision, Writing–original draft. MnB: Methodology, Writing–review and editing. TE: Funding acquisition, Investigation, Supervision, Writing–review and editing. SA: Conceptualization, Formal Analysis, Funding acquisition, Software, Writing–review and editing. AA: Formal Analysis, Funding acquisition, Software, Validation, Writing–review and editing. ME: Data curation, Funding acquisition, Methodology, Writing–original draft. MsB: Data curation, Methodology, 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2025.1507009/full#supplementary-material

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Metformin inhibits pathological retinal neovascularization but promotes retinal fibrosis in experimental neovascular age-related macular degeneration

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Purpose: This study aims to investigate the effects and mechanism of action of metformin on retinal neovascularization and fibrosis in a mouse model of neovascular age-related macular degeneration (nAMD).

Methods: Very low-density lipoprotein receptor knockout (*Vldlr*$^{-/-}$) mice, a mouse model of nAMD, were used in this study. *Vldlr*$^{-/-}$ mice were administered metformin on postnatal day (P) 20 for 20 days (early stage of pathological change) or at 5.5 months of age for 45 days (late stage of pathological change). Retinal leakage was examined by fundus fluorescein angiography (FFA). Retinal neovascularization was assessed by lectin staining. Retinal fibrosis was assessed by Western blotting, immunofluorescence staining, and Masson's trichrome staining.

Results: Retinal vascular leakage and neovascularization were significantly reduced in *Vldlr*$^{-/-}$ mice treated with metformin compared to those treated with the vehicle at P40. The protein levels of inflammatory factors and phospho(p)-STAT3 were decreased, and P38 and ERK signaling were suppressed in the retinas of metformin-treated *Vldlr*$^{-/-}$ mice relative to those in the control group at P40. Fibrotic markers were upregulated in the retinas of *Vldlr*$^{-/-}$ mice treated with metformin compared to those treated with the vehicle at 7 months. Levels of the inflammatory factors and p-STAT3 were increased, and PI3K/AKT, P38, and ERK signaling were upregulated in the retinas of metformin-treated *Vldlr*$^{-/-}$ mice compared to those in the control group at 7 months.

Conclusion: Metformin inhibits pathological retinal neovascularization but promotes fibrosis in experimental nAMD. These results provide evidence and highlight important considerations for the clinical use of metformin in different stages of nAMD.

KEYWORDS

metformin, retinal neovascularization, age-related macular degeneration, neovascular AMD, very low-density lipoprotein receptor, retinal fibrosis

Introduction

Age-related macular degeneration (AMD) is the leading cause of blindness in people over 60 (Fleckenstein et al., 2024). Retinal angiogenesis is a pathological change associated with advanced AMD, also known as wet AMD or neovascular AMD (nAMD) (Pugazhendhi et al., 2021). The newly formed vessels damage the highly organized retinal structures, especially the retinal pigment epithelium (RPE) and photoreceptor layers, disrupt retinal function, and cause vision loss (Pugazhendhi et al., 2021). The mechanism of retinal neovascularization is not fully understood. The current treatment strategy relies on anti-vascular endothelial growth factor (VEGF) therapy (Fleckenstein et al., 2024). However, anti-VEGF treatment has several limitations, such as high cost, risk of infection, the need for repeated injections, and diminished efficacy (Wolf et al., 2022). Some patients exhibit incomplete response to anti-VEGF treatment (Mettu et al., 2021). Finding alternative treatments for pathological retinal angiogenesis is an urgent clinical need.

Retinal fibrosis is a pathological change that occurs in the late stages of nAMD (Tenbrock et al., 2022). It usually follows several or multiple episodes of pathological retinal angiogenesis and vascular leakage in patients with nAMD (Tenbrock et al., 2022). Extracellular matrix deposition in the lesions disrupts the normal retinal structure, leading to permanent structural damage and, ultimately, loss of function (Tenbrock et al., 2022). Unfortunately, the mechanism of retinal fibrosis is still unknown, and there is no specific treatment for it (Tenbrock et al., 2022; Armendariz and Chakravarthy, 2024).

Metformin is a clinical drug used to manage blood glucose levels in patients with type 2 diabetes (Bailey, 2017). In addition to its anti-hyperglycemic effects, recent studies have identified its protective effects in many aspects, such as anti-cancer (Vancura et al., 2018), anti-aging (Chen et al., 2022), anti-oxidative stress (Buczyńska et al., 2024), cardioprotective (Bu et al., 2022), and nephroprotective effects (Pan et al., 2020). In addition, multiple preclinical studies have shown that metformin may have therapeutic effects on retinal diseases, such as retinitis pigmentosa (Luodan et al., 2019; Athanasiou et al., 2017), diabetic retinopathy (Kim et al., 2017; Nahar et al., 2021; Yi et al., 2016), ischemic retinopathy (Joe et al., 2015), uveitis (Kalariya et al., 2012), and AMD (Qu et al., 2020; Xu et al., 2018; Ying et al., 2017). However, its effects on retinal neovascularization are not always consistent. For instance, a study showed that metformin suppressed angiogenesis by inhibiting cell proliferation, migration, and tube formation in human retinal vascular endothelial cells (Han et al., 2018). Metformin was reported to inhibit angiogenesis in a laser-induced choroidal neovascularization model (Zhang et al., 2023). However, in a mouse model of oxygen-induced retinopathy (OIR), metformin treatment didn't reduce the extent of avascular areas at the postnatal day (P)17, and the OIR pathology was remained at P21 even when the vehicle treatment showed significant improvement in OIR pathology at P21 (Joe et al., 2015). These differing effects of metformin on retinal neovascularization in different animal models suggest the complexity of its role in various pathological scenarios.

Recently, several studies have shown that metformin has anti-fibrotic effects. A study reported that metformin reversed well-established lung fibrosis in an adenosine 5′-monophosphate-activated protein kinase (AMPK)-dependent manner in a bleomycin-induced mouse model (Rangarajan et al., 2018). Another study also found that metformin reduced liver collagen deposition, inhibited liver cell apoptosis, and lowered serum malondialdehyde (MDA) levels in a CCl4-induced liver fibrosis model, indicating that metformin exerts anti-fibrotic effects in the liver (Kong et al., 2024). In addition, metformin has been shown to inhibit transforming growth factor-beta (TGF-β) and its downstream signaling, thus reducing TGF-β-induced fibrotic changes (Xiao et al., 2016; Lu et al., 2015; Lim et al., 2012). However, whether metformin has therapeutic effects on retinal fibrosis is still unknown.

In this study, we aim to investigate the effects of metformin on retinal neovascularization and fibrosis in a well-known nAMD model, the very low-density lipoprotein receptor (VLDLR) knockout ($Vldlr^{-/-}$) mice. Metformin was administered to $Vldlr^{-/-}$ mice at two-time points—the early stage of pathological change (the angiogenic stage) and the late stage of pathological change (the fibrotic stage) and the effects of metformin on retinal angiogenesis, vascular leakage, and retinal fibrosis were investigated. Our results indicate that metformin has an anti-angiogenic effect but promotes retinal fibrosis in $Vldlr^{-/-}$ mice. These dual effects of metformin may be mediated through the modulation of multiple signaling pathways, which may play opposing roles in retinal angiogenesis and fibrosis.

Materials and methods

Animals

B6; 129S7-Vldlrtm1Her/J ($Vldlr^{-/-}$) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Wild-type (WT) C57BL/6 J mice were obtained from the Laboratory Animal Center of Xiamen University (Xiamen, China). Age-matched WT mice with similar genetic backgrounds were generated by crossing C57BL/6 J and $Vldlr^{-/-}$ mice. All mice were housed in the Laboratory Animal Center of Xiamen University (Xiamen, Fujian, China). The animal experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all studies were conducted in accordance with protocols (XMULAC20220168) approved by the Experimental Animal Ethics Committee of Xiamen University.

In vivo experimental procedure

In the early angiogenic stage, $Vldlr^{-/-}$ mice were treated with metformin (200 mg/kg/day, APExBIO, United States, B1970) or a vehicle solution by daily gavage starting from P20 to P40. After the treatment, the mice underwent fundus fluorescein angiography and electroretinography (ERG), and their eyecups were collected for further experiments at P40. In the late fibrotic stage, a special animal diet containing metformin (at 1333 parts per million) and a control diet were purchased from Medicine Biomedicine Co. Ltd. (Jiangsu, Zhejiang, China). In the late fibrotic stage, $Vldlr^{-/-}$ mice were fed either the metformin-containing diet or vehicle chow starting at

5.5 months of age. After 45 days of feeding, mice were euthanized at 7 months of age, and their eyecups were collected for further experiments.

Electroretinogram

An ERG system (RetiMINER System; AiErXi Medical Equipment Co., Ltd., Chongqing, China) was used to evaluate the visual function in mice. Mice were dark-adapted overnight and anesthetized with sodium pentobarbital (40 mg/kg). The pupils were dilated with tropicamide phenylephrine eye drops (Santen Pharmaceutical Co., Ltd, Shiga plant, Japan). Full-field ERGs were recorded after subcutaneously inserting a ground electrode near the tail and a reference electrode on the back, followed by placing a golden-ring electrode on the cornea. All procedures were performed under dim red light. The a-wave and b-wave responses to flash stimuli (1.0 cd·s/m^2) were recorded and analyzed in both eyes. The amplitudes of the oscillatory potential waves were also recorded and analyzed.

Fundus fluorescein angiography

Mice were anesthetized via intraperitoneal injection of 2% tribromoethanol, and their pupils were dilated with topical 0.5% tropicamide and 0.5% phenylephrine. A 10% fluorescein sodium (Zhiyuan, Tianjin, China) was administered via intraperitoneal injection. The ocular fundus was imaged using a fundus camera (Optoprobe Science, Glamorgan, UK; OPTO-RIS). FFA images were captured 5 min after fluorescein sodium injection.

Lectin staining

Lectin staining of retinas was performed according to a published protocol (Connor et al., 2009). Briefly, eyeballs were fixed in 4% paraformaldehyde (PFA) for 1 h (h). Retinas were dissected and then incubated with 0.5% Triton X-100 (Sigma-Aldrich) at 4°C overnight. After three washes with 1X PBS, the retinas were incubated with Isolectin GS-IB4 (Thermo Fisher Scientific) overnight at room temperature. Then, the retinas were washed and flat-mounted for microscopy. Quantification of neovascularization was conducted as described previously (Connor et al., 2009).

Immunofluorescent staining

Eyecups were fixed with 4% PFA and embedded in optimal cutting temperature compound. Frozen sections of 10 μm thickness were fixed in cold acetone (−20°C) for 10 min. Sections were incubated with 0.2% Triton X-100 for 20 min and blocked with 2% BSA in PBS for 1 h. Then, sections were incubated with different primary antibodies at 4°C for 16 h. After three washes with 1X PBS, sections were incubated with Alexa Fluor 594-conjugated IgG (Abcam) or Alexa Fluor 488-conjugated IgG (Abcam) for 60 min at 37°C. Nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI, Abcam). Images were acquired using a confocal laser scanning microscope (Zeiss, Braunschweig, Germany; LSM 880).

Hematoxylin and eosin (HE) staining

Fixed eyeballs were dehydrated in ethanol, waxed, and embedded in paraffin. Sections of 6 μm thickness were cut around the optic nerve, followed by deparaffinization in xylene, and rehydration in ethanol. HE staining was performed using a staining kit (Servicebio; G1005) according to the manufacturer's instructions. The slides were observed under an optical microscope (Zeiss; Axio Lab.A1).

Western blot analysis

Total protein from eyecups was extracted using RIPA buffer supplemented with protease and phosphatase inhibitors. Protein extracts were separated by 6%–15% SDS–PAGE electrophoresis and transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with 5% non-fat milk for 2 h at room temperature (RT) and incubated with primary antibodies overnight at 4°C. After several washes, the membrane was incubated with a second ary antibody for 1 h at RT. Signal detection was performed using an enhanced chemiluminescence reagent kit (NCM Biotech, Newport, RI, United States). Bands were quantified using ImageJ and normalized to β-actin levels. The following antibodies were used: 5′-adenosine monophosphate (AM)-activated protein kinase (AMPK), phospho(p)-AMPK, p-P38, p-extracellular signal-regulated kinase (ERK), Class I phosphoinositide 3-kinase (PI3K), p-PI3K, protein kinase B (AKT), p-AKT, p-signal transducer and activator of transcription 3 (STAT3), vimentin, collagen-1, connective tissue growth factor (CTGF), glial fibrillary acidic protein (GFAP), and β-actin, which were purchased from Cell Signaling Technology (Danvers, MA, United States). Antibodies such as VEGF and vascular cell adhesion molecule (VCAM-1) were obtained from Santa Cruz Biotechnology (Dallas, TX, United States).

Statistical analysis

Prism 6 software (GraphPad, San Diego, CA, United States) was used for statistical analysis. A paired Student's t-test was used for two-group comparisons. Statistical data were expressed as mean ± SEM. $p < 0.05$ was considered statistically significant.

Results

Metformin reduces neovascularization in the retinas of *Vldlr*$^{-/-}$ mice at P40

To assess the effects of metformin on retinal neovascularization, we used *Vldlr*$^{-/-}$ mice, a mouse model of nAMD (Joyal et al., 2016). Metformin was administered daily by oral gavage from P20 to P40, which corresponds to the early stage of pathological changes dominated by angiogenesis. Vascular leakage was evaluated by

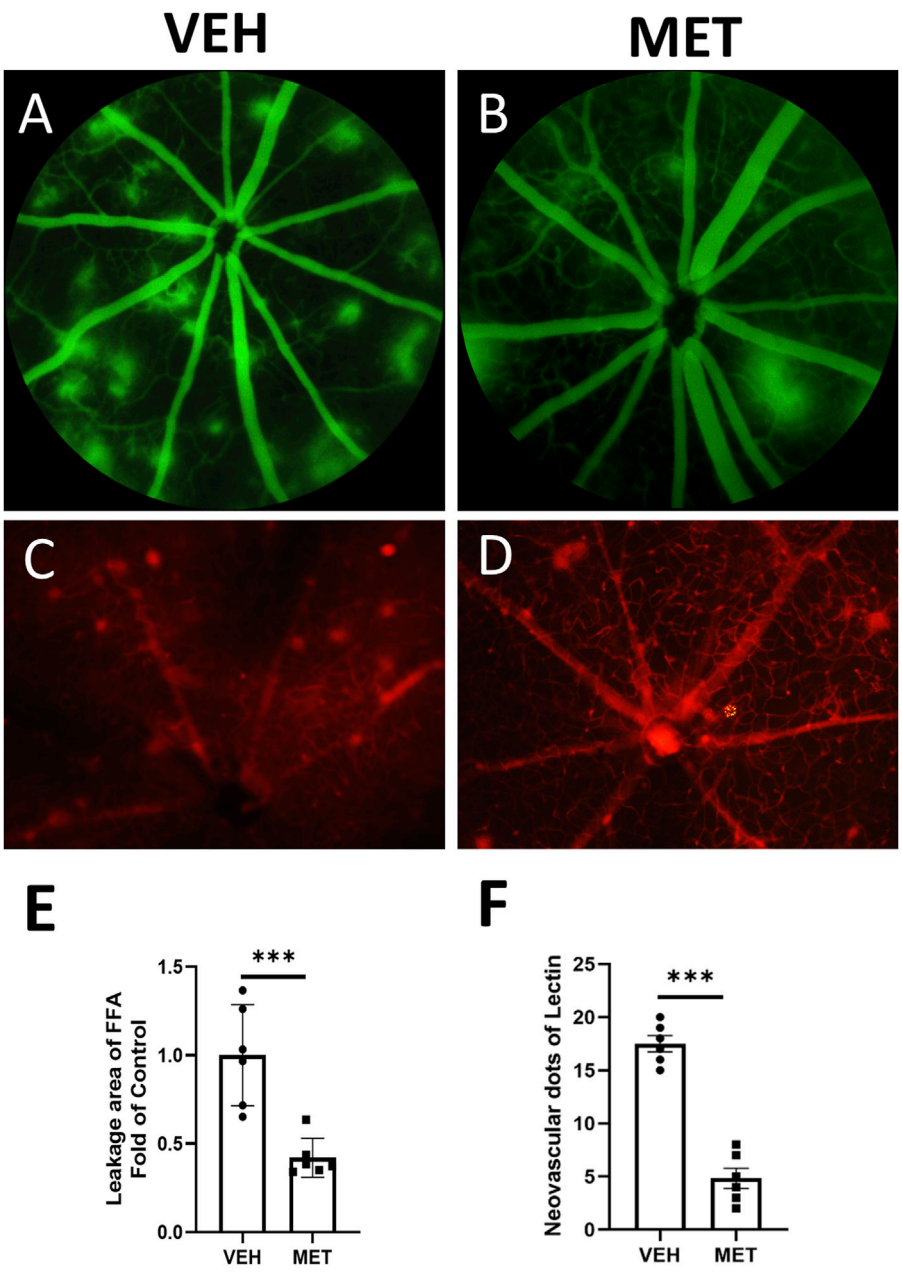

FIGURE 1
Metformin inhibits retinal vascular leakage and neovascularization in the retinas of *Vldlr*$^{-/-}$ mice at P40. *Vldlr*$^{-/-}$ mice were treated with metformin (200 mg/kg/day) or vehicle solution (control) by daily gavage from P20 to P40. The total number of retinal neovascular sprouts was quantified at P40. **(A, B)** Representative images of fundus fluorescein angiography (FFA) of *Vldlr*$^{-/-}$ mice treated with vehicle (VEH) **(A)** or metformin (MET) **(B)**. **(C, D)** Representative images of lectin staining from *Vldlr*$^{-/-}$ mice treated with vehicle (VEH) or metformin (MET). **(E, F)** Quantification of leakage areas of FFA images **(E)** or neovascular spots of lectin staining images **(F)** from vehicle and metformin-treated *Vldlr*$^{-/-}$ mice. Data are shown as mean ± SEM. N = 6, ***$p < 0.001$. A two-tailed Student's *t*-test was used.

FAA (Figures 1A, B). *Vldlr*$^{-/-}$ mice treated with metformin displayed smaller areas of retinal vascular leakage than those treated with the vehicle solution (Figure 1E). Meanwhile, lectin staining of flat-mounted retinas was performed to detect retinal neovascularization (Figures 1C, D). Metformin treatment significantly reduced the areas of retinal neovascularization in *Vldlr*$^{-/-}$ mice (Figure 1F). Taken together, these data suggest that metformin suppresses retinal neovascularization and vascular leakage in *Vldlr*$^{-/-}$ mice at P40.

Metformin improves oscillatory potentials in the retinas of *Vldlr*$^{-/-}$ mice at P40

Next, we evaluated whether metformin could improve neuronal function in *vldlr*$^{-/-}$ mice. Similarly, ERGs, which included the oscillatory potentials, were performed at P40 on *Vldlr*$^{-/-}$ mice treated with vehicle and *Vldlr*$^{-/-}$ mice treated with metformin. The a-wave and b-wave showed an upward trend but were not significantly changed after metformin treatment (Figures 2A–C).

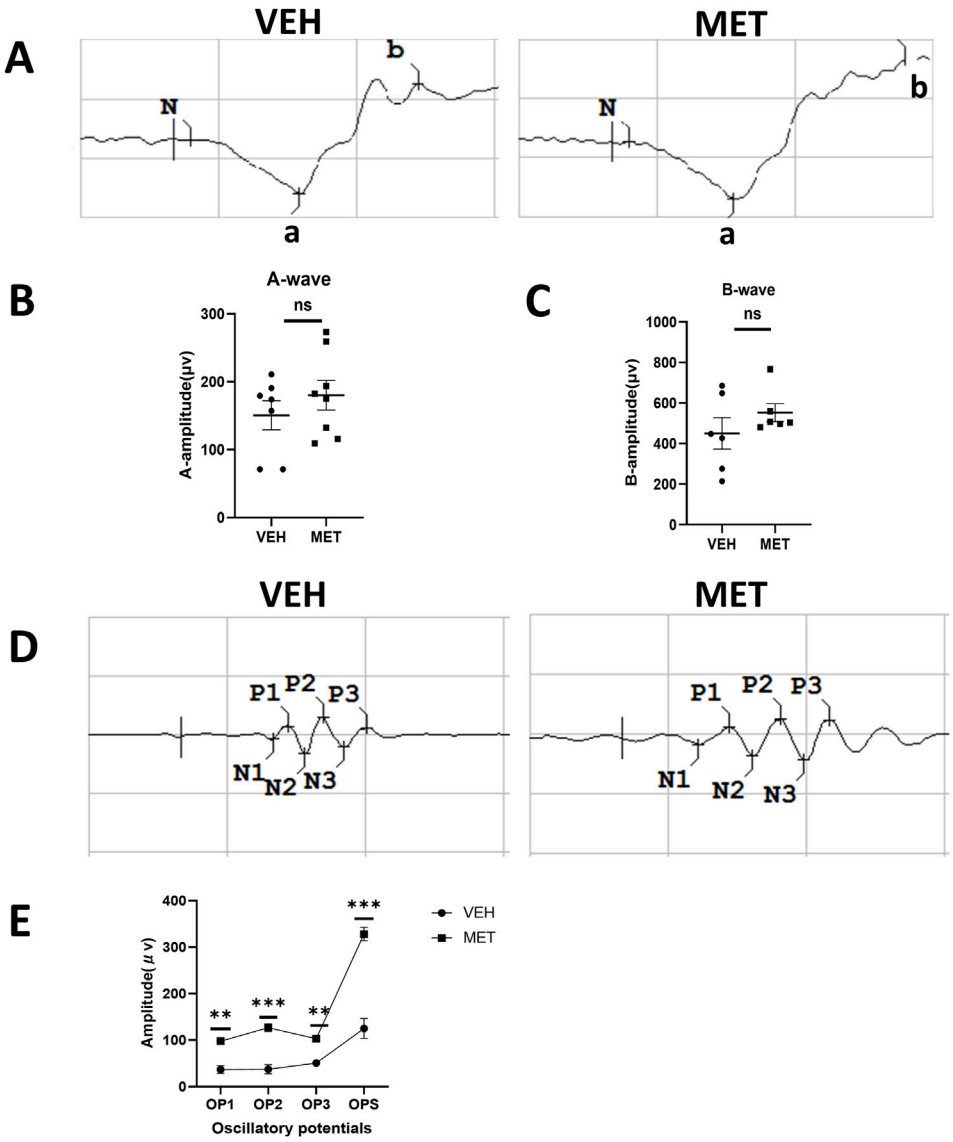

FIGURE 2
Metformin improves oscillatory potentials in the retinas of *Vldlr*$^{-/-}$ mice at P40. **(A)** Representative images of the a-wave and b-wave from *Vldlr*$^{-/-}$ mice treated with vehicle (VEH) or metformin (MET). ERGs were obtained by averaging three responses to 1.0 cd·s/m^2 flashes. **(B, C)** Amplitudes of ERG a-wave **(B)** and b-wave **(C)** of the two groups were analyzed and quantified. **(D)** Representative images of oscillatory potentials from *Vldlr*$^{-/-}$ mice treated with vehicle (VEH) or metformin (MET). **(E)** Oscillatory potentials from the two groups were analyzed and quantified. Data are shown as mean ± SEM; n = 6–8, *p < 0.05, **p < 0.01, and ***p < 0.001. A two-tailed Student's *t*-test was used.

However, the oscillatory potentials were partially rescued by metformin treatment (Figures 2D, E). Overall, metformin improves the oscillatory potentials in the retinas of *Vldlr*$^{-/-}$ mice, suggesting a potential improvement in the blood supply to the inner retina.

Metformin reduces pro-inflammatory factors and inhibits P38 and ERK signaling in the eyecups of *Vldlr*$^{-/-}$ mice at P40

Multiple studies have shown that metformin acts as an AMPK agonist, exerting its effects through the activation of AMPK signaling (Zhou et al., 2001; He and Wondisford, 2015). However, many studies suggest that metformin may play its role independent of AMPK signaling (Foretz et al., 2010; Bridges et al., 2014). To determine whether metformin activates AMPK in this study, we assessed the protein levels of p-AMPK and AMPK by Western blot analysis (Figure 3A). The levels of p-AMPK were significantly increased after metformin administration (Figure 3B), while the levels of total AMPK remained unchanged (Figure 3C). These findings suggest a possible mechanism by which metformin activates AMPK signaling in the retina. Furthermore, we investigated the specific mechanism of action of metformin in the eyecups of *Vldlr*$^{-/-}$ mice. The protein levels of VEGF, VACM-1, and p-STAT3 were significantly decreased in the retina of metformin-treated *Vldlr*$^{-/-}$ mice (Figures 3D–G), while the levels of total STAT3 were not significantly changed (Figures 3D, H). This

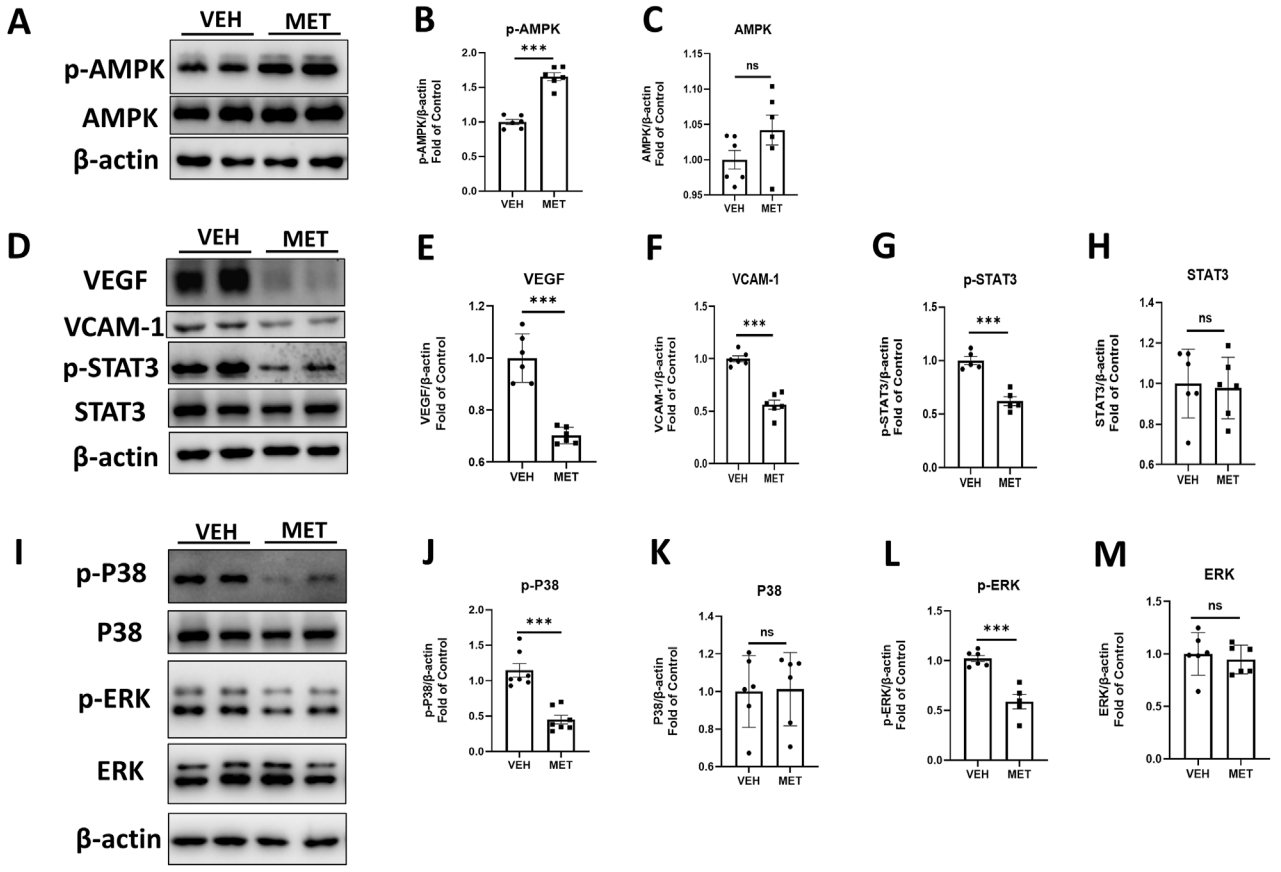

FIGURE 3
Metformin reduces the retinal pro-inflammatory cytokines, p-P38 and p-ERK, in an AMPK-dependent manner in the eyecups of *Vldlr*$^{-/-}$ mice at P40. **(A–C)** The protein levels of p-AMPK **(A, B)** and AMPK **(A, C)** in the eyecups of *Vldlr*$^{-/-}$ mice treated with vehicle (VEH) or metformin (MET) were determined by Western blot analysis and quantified by densitometry. **(D–H)** The protein levels of VEGF **(D, E)**, VCAM-1 **(D, F)**, p-STAT3 **(D, G),** and STAT3 **(D, H)** in the eyecups of *Vldlr*$^{-/-}$ mice treated with vehicle (VEH) or metformin (MET) were determined by Western blot analysis and quantified by densitometry. **(I–M)** Protein levels of p-P38 **(I, J)**, P38 **(I, K)**, p-ERK **(I, L),** and ERK **(I, M)** in the eyecups of the two indicated groups were determined by Western blot analysis and quantified by densitometry. Data are shown as mean ± SEM; n = 6. *p < 0.05, **p < 0.01, and ***p < 0.001. A two-tailed Student's *t*-test was used.

suggests that metformin may inhibit retinal inflammation and reduce p-STAT3 in *Vldlr*$^{-/-}$ mice. In addition, protein levels of p-P38 and p-ERK were reduced in *Vldlr*$^{-/-}$ retinas (Figures 3I, J, L), while the levels of P38 and ERK were unchanged (Figures 3I, K, M).These findings suggest that the P38 and ERK pathways may play a role in the metformin-mediated effects in the eyecups of *Vldlr*$^{-/-}$ mice.

Metformin promotes subretinal fibrosis in *Vldlr*$^{-/-}$ mice at 7 months of age

To further explore the function of metformin, we tested whether metformin could suppress retinal fibrosis in the eyecups of *Vldlr*$^{-/-}$ mice. The *Vldlr*$^{-/-}$ mice at the age of 5.5 months were treated with metformin for 45 days, and the expression levels of fibrotic markers were assessed. HE staining of retinal sections showed that *Vldlr*$^{-/-}$ mice treated with metformin have more lesion sites than those treated with the vehicle (Figure 4A). Masson's staining showed that more blue-stained collagens were deposited in the sub-retinal area of *Vldlr*$^{-/-}$ mice treated with metformin (Figure 4B). In addition, immunostaining showed that the signals of collagen-1 (Figure 4C), α-SMA (Figure 4D), and GFAP (Figure 4E) were stronger in *Vldlr*$^{-/-}$ mice treated with metformin than those in the control group. The protein levels of fibrotic markers, collagen-1 and vimentin, were significantly upregulated in the eyecups of metformin-treated *Vldlr*$^{-/-}$ mice (Figures 4F–H). Expression of GFAP, a glial activation marker, was elevated in the eyecups of *Vldlr*$^{-/-}$ mice treated with metformin (Figures 4F, I). Taken together, these data suggest that metformin promotes subretinal fibrosis in *Vldlr*$^{-/-}$ mice.

Metformin increases inflammation in the eyecups of *Vldlr*$^{-/-}$ mice at 7 months of age

Next, we explored the possible mechanism by which metformin promotes retinal fibrosis in *Vldlr*$^{-/-}$ mice. Immunostaining showed an increased signal for VCAM-1 in the retinal cryosections of *Vldlr*$^{-/-}$ mice treated with metformin (Figure 5A). Furthermore, the protein levels of VEGF (Figures 5B, C) and p-STAT3 (Figures 5B, D) were elevated in the eyecups of metformin-treated *Vldlr*$^{-/}$ mice, while the total protein levels of STAT3 remained unchanged

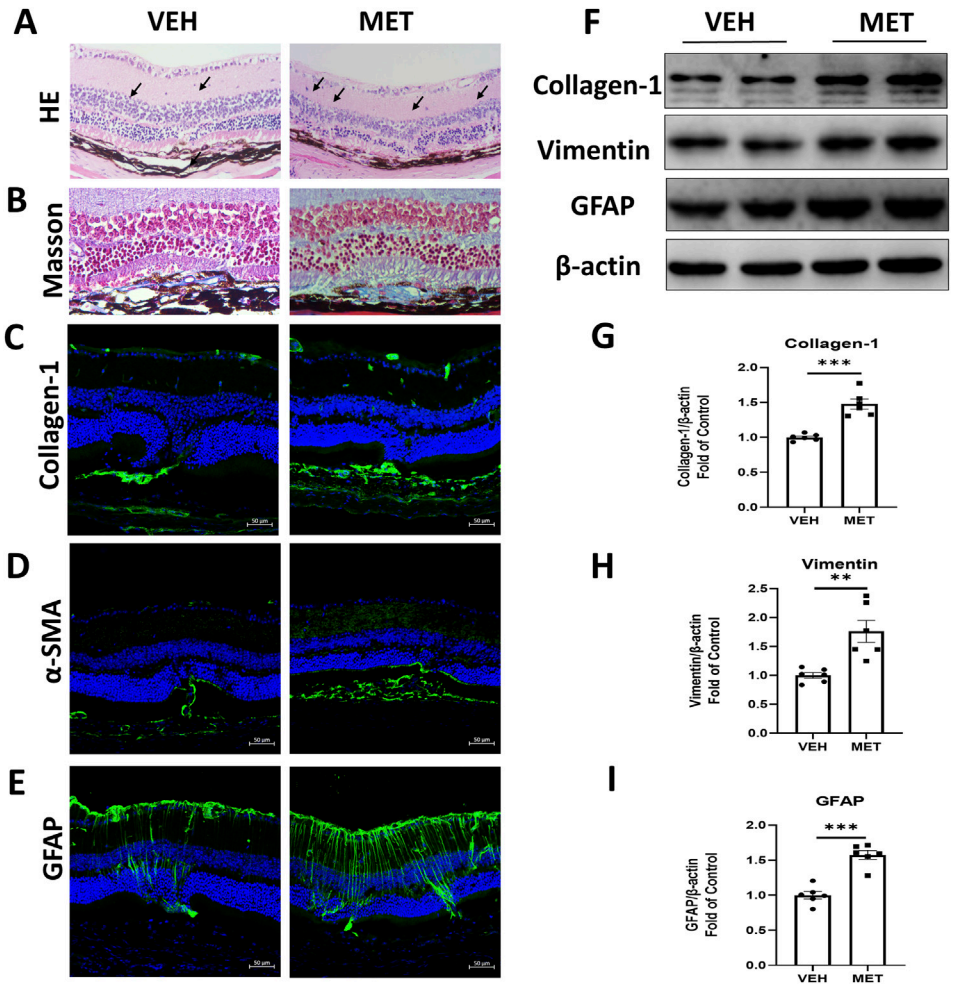

FIGURE 4
Metformin promotes subretinal fibrosis in *Vldlr*$^{-/-}$ mice at 7 months of age. *Vldlr*$^{-/-}$ mice were fed with a diet containing metformin from the age of 5.5 months to 7 months. The mice were euthanized at 7 months of age. **(A, B)** Representative retinal images of H&E staining **(A)** and Masson's staining **(B)** of collagen deposition in the retinal paraffin sections of *Vldlr*$^{-/-}$ mice fed with control chow (VEH) or metformin chow (MET). **(C–E)** Representative images of immunostaining show the expression of collagen-1 **(C)**, α-SMA **(D)**, and GFAP **(E)** in the retinal cryosections of the two indicated groups. **(F–I)** The protein levels of collagen-1**(F, G)**, vimentin **(F, H)**, and GFAP **(F, I)** were determined by Western blot analysis and quantified by densitometry in the two indicated groups. Data are shown as mean ± SEM; n = 6. *p < 0.05 and **p < 0.01. A two-tailed Student's *t*-test was used.

(Figures 5B, E). These findings suggest that metformin may increase retinal inflammation in *Vldlr*$^{-/-}$ mice at 7 months of age.

Metformin activates PI3K/AKT, P38, and ERK signaling in the eyecups of *Vldlr*$^{-/-}$ mice at 7 months of age

Furthermore, we examined several signaling pathways in the eyecups of *Vldlr*$^{-/-}$ mice treated with vehicle or metformin. Interestingly, the protein levels of p-PI3K were significantly increased, while the total PI3K levels remained unchanged in the eyecups of *Vldlr*$^{-/-}$ mice treated with metformin (Figures 6A–C). Similarly, the protein levels of p-AKT were increased, whereas the total AKT levels remained unchanged (Figures 6D–F). In addition, the protein levels of p-P38 (Figures 6G, H) and p-ERK (Figures 6G, J) were elevated in the eyecups of *Vldlr*$^{-/-}$ mice treated with metformin, with no changes observed in the protein levels of P38 and ERK (Figures 6G, I, K). Taken together, these results suggest that metformin activates the PI3K/AKT, P38, and ERK signaling pathways in the eyecups of *Vldlr*$^{-/-}$ mice.

Discussion

Metformin has been reported to have therapeutic effects in non-diabetic diseases such as cancer, cardiovascular disease, and lung fibrosis (Foretz et al., 2023). In this study, we investigated the effects of metformin on retinal neovascularization and retinal fibrosis in *Vldlr*$^{-/-}$ mice, a model of nAMD (Joyal et al., 2016; Chen et al., 2020). In the early pathological stage, metformin inhibited retinal vascular leakage and neovascularization in *Vldlr*$^{-/-}$ mice by suppressing inflammatory factors and modulating P38 and ERK signaling pathways. In contrast, during the late pathological stage, metformin promoted retinal fibrosis in *Vldlr*$^{-/-/-}$ mice by enhancing inflammation and activating the PI3K/AKT, P38, and

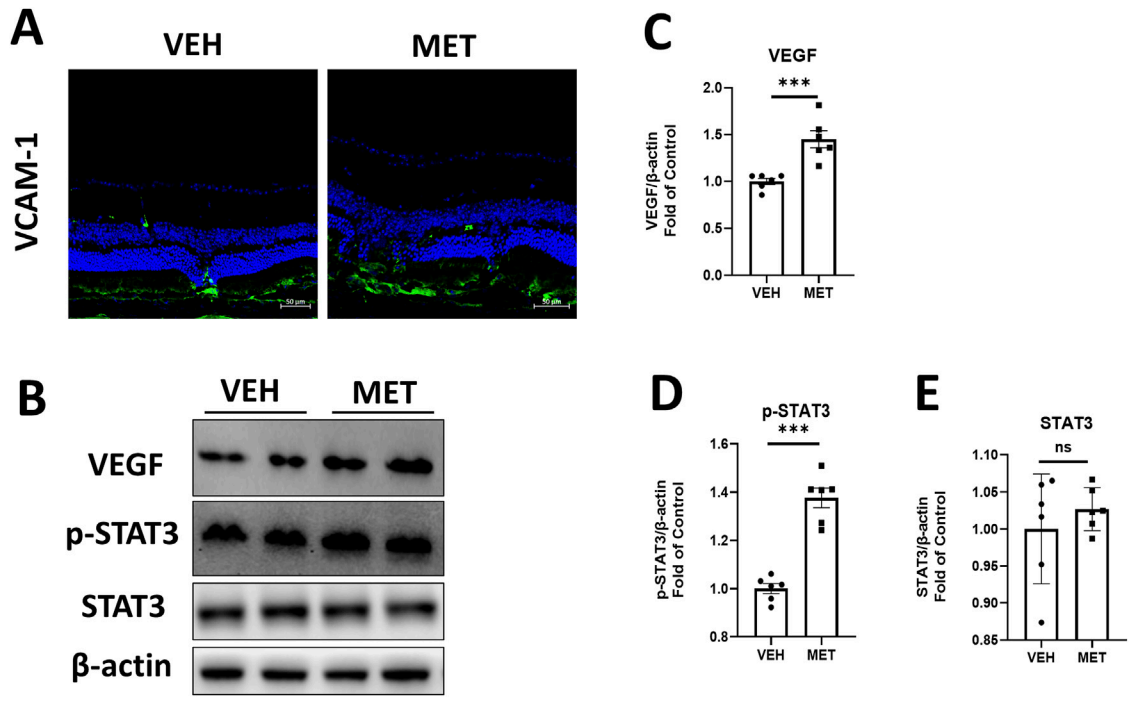

FIGURE 5
Metformin increases inflammation in the eyecups of *Vldlr*$^{-/-}$ mice at 7 months of age. **(A)** Representative images of immunostaining show the expression of VCAM-1 in the cryosection of *Vldlr*$^{-/-}$ mice fed with vehicle (VEH) or metformin (MET). **(B–E)** The protein levels of VEGF **(B, C)**, p-STAT3 **(B, D)**, and STAT3 **(B, E)** in the *Vldlr*$^{-/-}$ mice fed with control chow (VEH) or metformin chow (MET) were determined by Western blot analysis and quantified by densitometry. Data are shown as mean ± SEM; n = 6. *p < 0.05 and **p < 0.01. A two-tailed Student's *t*-test was used.

ERK signaling pathways. These differential effects of metformin on retinal angiogenesis and fibrosis highlight the complex role of metformin in retinal diseases, providing valuable insights and considerations for its clinical use at different stages of AMD.

According to the pathological changes, AMD can be divided into two types: dry AMD and wet AMD (Fleckenstein et al., 2024; Boopathiraj et al., 2024). Wet AMD, also known as neovascular AMD (nAMD), is an advanced stage of AMD characterized by retinal neovascularization (Pugazhendhi et al., 2021; Boopathiraj et al., 2024). The *Vldlr*$^{-/-}$ mouse model is considered an animal model for retinal angiomatous proliferation (RAP), a special type of nAMD (Hu et al., 2008). In our previous study, we observed elevated fibrotic markers such as collagen-1, vimentin, and fibronectin, along with collagen deposits in the eyes of 6-month-old *Vldlr*$^{-/-}$ mice (Chen et al., 2020). These findings suggest that *Vldlr*$^{-/-}$ mice could be used as a mouse model for studying retinal fibrosis. Moreover, *Vldlr*$^{-/-}$ mice have also been used as a model for retinal fibrosis in many other reports (Yang et al., 2024; Ma et al., 2024).

Based on our long-term investigation and other reports, we have artificially divided the two stages according to the pathological vascular changes in *Vldlr*$^{-/-}$ mice in this study. The first stage is from P20 to P40, and the second stage spans from 5.5 months to 7 months. The P20–P40 period in *Vldlr*$^{-/-}$ mice is considered an early stage of pathological change, dominated by angiogenesis. In contrast, the 5.5–7-month period is regarded as the late stage of pathological changes, characterized by predominant fibrosis. These two time periods were specially chosen to mimic the different stages of nAMD observed in clinical settings.

In the early stage of the pathological process, metformin could inhibit retinal vascular leakage and neovascularization in *Vldlr*$^{-/-}$ mice, indicating its inhibitory effects on retinal angiogenesis. These results were consistent with those of several studies that reported the ability of metformin to suppress retinal neovascularization (Han et al., 2018; Zhang et al., 2023). For instance, a study showed that metformin suppressed angiogenesis by inhibiting cell proliferation, migration, and tube formation in human retinal vascular endothelial cells while also reducing inflammatory molecules induced by tumor necrosis factor α (Han et al., 2018). Zhang et al. (2023) showed that oral metformin inhibited laser-induced choroidal neovascularization and decreased macrophage/microglia infiltration. Metformin has also been found to reduce the stability of hypoxia-inducible factor-1α (HIF-1α), decreasing its accumulation under hypoxic conditions and lowering VEGF expression (Wang et al., 2015).

Metformin has been implicated in patients with AMD. For instance, metformin use in AMD patients without diabetes has been explored (Aggarwal et al., 2024; Brown et al., 2019). In a retrospective case-control study, Emily et al. reported that patients who had taken metformin showed decreased odds of developing AMD (Brown et al., 2019). Similarly, another study found that exposure to metformin was associated with reduced odds of developing AMD, and its use was also associated with decreased odds of developing dry AMD (Aggarwal et al., 2024). However, the majority of the reported retrospective case-control studies did not distinguish between dry and wet AMD. Therefore, it is very challenging to assess the effects of metformin on wet AMD in

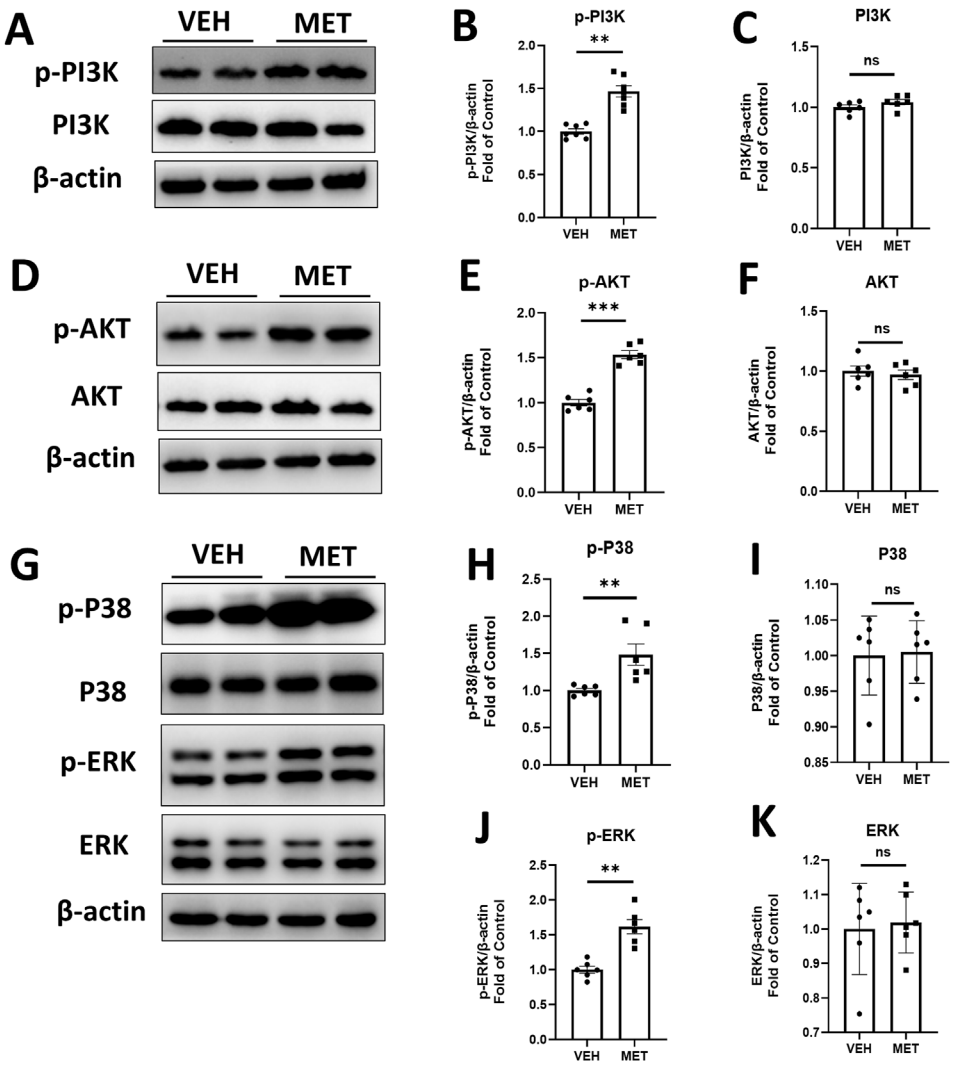

FIGURE 6
Metformin activates PI3K/AKT, p-P38, and p-ERK pathways in the eyecups of *Vldlr*$^{-/-}$ mice at 7 months of age. (A–C) The protein levels of p-PI3K **(A, B)** and PI3K **(A, C)** in the *Vldlr*$^{-/-}$ mice fed with control chow (VEH) or metformin chow (MET) were determined by Western blot analysis and quantified by densitometry. **(D–F)** The protein levels of p-AKT **(D, E)** and AKT **(D, F)** in the two indicated groups were determined by Western blot analysis and quantified by densitometry. **(G–K)** The protein levels of p-P38 **(G, H)**, P38 **(G, I)**, p-ERK **(G, J)**, and ERK **(G, K)** in the two indicated groups were determined by Western blot analysis and quantified by densitometry. Data are shown as mean ± SEM; n = 6. *p < 0.05 and **p < 0.01. A two-tailed Student's *t*-test was used.

the literature. Using an animal model of nAMD, our study showed that metformin may benefit patients in the early stage of wet AMD when retinal angiogenesis is predominant. More studies, especially prospective clinical trials, are needed to further investigate the protective role of metformin in wet AMD.

More importantly, our study demonstrated the effects of metformin on retinal fibrosis. Surprisingly, we found that metformin promoted retinal fibrosis in *Vldlr*$^{-/-}$ mice at 7 months of age. These findings are in contrast with other studies that have shown the protective effects of metformin against fibrosis in other diseases. For instance, Kheirollahi et al. (2019) reported that metformin exerts potent protective effects against lung fibrosis by inhibiting TGF-β-mediated fibrosis, suppressing collagen production, and modulating lipogenic differentiation. In addition, studies have shown that metformin has anti-fibrotic effects in the kidney, liver, and heart (Wu et al., 2021). Since our study was based on *Vldlr*$^{-/-}$ mice, further studies are warranted to include additional animal models of retinal fibrosis to better validate the effects of metformin.

The mechanisms of metformin on retinal angiogenesis and retinal fibrosis were investigated in this study. In the angiogenic stage, metformin inhibited VEGF and p-STAT3 and suppressed P38 and ERK signaling. The inhibitory effects of metformin on STAT3, P38, and ERK signaling pathways have been supported by many studies (Deng et al., 2012; Xia et al., 2021; Rice et al., 2009; Zhao et al., 2022). For example, Deng et al. (2012) reported that metformin inhibits STAT3 activation (p-STAT3) and downstream signaling in parental cell lines. Metformin has been reported to promote NK cell activity in a p38 MAPK-dependent manner (Xia et al., 2021). Studies have also shown that metformin could regulate

the ERK-mediated pathway (Rice et al., 2009; Zhao et al., 2022). However, in our study, we found that metformin promoted VEGF and p-STAT3 and increased PI3K/AKT, P38, and ERK signaling in the fibrotic stage. The opposite effects on inflammation and multiple signaling pathways in two different stages of nAMD may indicate the complexity of the role of metformin in treating eye diseases. The possible mechanism is that metformin may help maintain homeostasis by balancing the upregulated and downregulated signaling pathways throughout the pathological process of nAMD. This assumption is supported by many other studies showing that metformin may have opposing effects. For example, studies indicate that metformin promotes endothelial cell proliferation and increases the density of new blood vessels in the spinal cord (Zhao et al., 2023). It has also been found to enhance the angiogenic capacity and autophagy of human adipose tissue-derived stem cells (Tao et al., 2023). In contrast, other studies have shown that metformin inhibits VEGF and angiogenesis. For example, a study showed that metformin inhibited tumor angiogenesis and reduced VEGF expression in implanted murine breast cancer models (Wang et al., 2018). The paradoxical effects of metformin on angiogenesis suggest that its actions may vary in an organ-dependent or disease-dependent manner.

In summary, our study demonstrates that metformin inhibits retinal neovascularization in the early stage and promotes retinal fibrosis in the late stage of nAMD by differentially regulating PI3K/AKT, P38, and P-ERK signaling pathways. These results provide evidence and highlight important considerations for the clinical use of metformin in different stages of nAMD.

Data availability statement

The raw data supporting the conclusions of this article will be made available by authors on request.

Ethics statement

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

Author contributions

XW: conceptualization, data curation, formal analysis, investigation, methodology, software, and writing–original draft. XuL: conceptualization, data curation, formal analysis, methodology, and writing–original draft. SH: data curation, methodology, and writing–original draft. MW: data curation, methodology, and writing–review and editing. YX: data curation, methodology, and writing–review and editing. XC: data curation and writing–review and editing. YM: data curation and writing–review and editing. RZ: methodology, project administration, and writing–review and editing. XiL: data curation, methodology, and writing–review and editing. SL: funding acquisition, resources, and writing–review and editing. ZL: funding acquisition, resources, and writing–review and editing. QC: conceptualization, funding acquisition, resources, supervision, validation, writing–original draft, 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.

Generative AI statement

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

Publisher's note

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Metformin attenuates diabetic osteoporosis by suppressing ferroptosis via the AMPK/Nrf2 pathway

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Background: Ferroptosis is a critical factor in the impairment of osteoblast function in osteoporosis. Metformin (Met), a biguanide antidiabetic drug, has demonstrated anti-osteoporotic effects and has been confirmed to exert therapeutic benefits in diabetic osteoporosis (DOP). Nevertheless, the underlying mechanisms through which Met affects bone metabolism remain ambiguous.

Objective: This study seeks to elucidate the function of Met in DOP and to explore the potential mechanisms through which it mediates treatment effects.

Methods: *In vitro*, we utilized osteoblasts to explore the impact of Met on osteoblast differentiation and anti-ferroptosis in a high glucose and palmitic acid (HGHF) environment. *In vivo*, we developed a DOP model utilizing a high-fat diet along with streptozocin injections and evaluated the bone-protective effects of Met through micro-CT and histomorphological analyses.

Results: Met inhibits HGHF-induced ferroptosis in osteoblasts, as indicated by the elevation of ferroptosis-protective proteins (GPX4, FTH1, and SLAC7A11), along with decreased lipid peroxidation and ferrous ion levels. Furthermore, Met augmented the levels of osteogenic markers (RUNX2 and COL1A1) and enhanced alkaline phosphatase activity in osteoblasts under HGHF conditions. Mechanistic investigations revealed that Met activates the AMPK/Nrf2 pathway, effectively preventing ferroptosis progression. Additionally, *in vivo* results demonstrated Met alleviates bone loss and microstructural deterioration in DOP rats.

Conclusion: Met can activate the AMPK/Nrf2 pathway to prevent ferroptosis, thereby protecting against DOP.

KEYWORDS

metformin, diabetic osteoporosis, ferroptosis, osteoblast, Nrf2/AMPK

1 Introduction

According to the latest data from the International Diabetes Federation, the number of adults living with diabetes worldwide amounted to 536.6 million in 2021 and is expected to increase to 783.2 million by 2045, with type 2 diabetes mellitus (T2DM) accounting for 95% of all cases (American Diabetes Association Professional Practice Committee, 2022; Sun et al., 2022). Diabetic osteoporosis (DOP), a chronic musculoskeletal complication of diabetes, primarily leads to trabecular structural deterioration and increased bone fragility (Napoli et al., 2017). Osteoporosis-related fractures exceed 9 million worldwide Each year, with the majority closely associated with DOP, posing significant challenges to human health and socioeconomic stability (Johnell and Kanis, 2006). Emerging evidence suggests that ferroptosis in osteoporosis-targeted cells, such as osteoblasts and osteocytes, represents a potential underlying mechanism of osteoporosis (Gao et al., 2022; Liu et al., 2022).

Ferroptosis, a novel mode of programmed cell death, is associated with a range of diseases, including retinal degeneration and Alzheimer's disease (Stockwell, 2022). Its core mechanism arises from unregulated lipid peroxidation processes, which are closely dependent on the involvement of iron (Stockwell et al., 2017). Compared to other types of cell death, ferroptosis exhibits distinct biological characteristics, including iron overload, elevated lipid peroxides, and dysregulation of ferroptosis-related proteins (Stockwell, 2022). Key ferroptosis-protective proteins such as glutathione peroxidase 4 (GPx4), ferritin heavy chain 1 (FTH1), and solute carrier family 7, member 11 (SLC7A11), are regulated by the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) (Dodson et al., 2019b; Dong et al., 2021). Under stress conditions, Nrf2 moves into the nucleus and stimulates genes featuring antioxidant response elements (Wang Y. et al., 2022). Research indicates ferroptosis contributes to the impairment of osteogenic differentiation and mineralization capacity of osteoblasts in the diabetic microenvironment (Ma et al., 2020). However, the mechanisms that ferroptosis operates in DOP have yet to be completely clarified. Therefore, targeting the ferroptosis pathway to suppression lipid peroxidation and ferroptosis may represent a promising intervention approach for osteoporosis.

Metformin (Met) is a conventional antihyperglycemic agent that is not only effective in managing T2DM but also demonstrates potential efficacy in metabolic disorder-related conditions, such as obesity (Zheng et al., 2015; Lv and Guo, 2020). One of the mechanisms of Met is the inhibition of oxidative phosphorylation, which leads to a reduction in ATP production (Pollak, 2017), subsequently activating AMP-activated protein kinase (AMPK) (Xian et al., 2021). AMPK serves as a crucial sensor of cellular energy status, influencing cellular fate by regulating the phosphorylation of various downstream substrates, such as Nrf2 (Bootman et al., 2018; Lu et al., 2021). Furthermore, studies suggest energy stress-mediated AMPK activation may be one of the mechanisms regulating ferroptosis (Lee et al., 2020). However, there is limited research on how Met reverses osteoblastic ferroptosis through AMPK signaling. Alternatively, while Met has been shown to improve osteogenesis, the relationship between this improvement and ferroptosis remains largely unexplored.

In this research, we developed a model of osteoblastic injury caused by high glucose and high palmitic acid (HGHF) *in vitro*, as well as a type 2 DOP rat model, to systematically investigate the mechanisms by which Met inhibited osteoblastic ferroptosis and improved osteoporosis in the diabetic microenvironment.

2 Materials and methods

2.1 Antibodies and reagents

Palmitic acid (PA) and control group BSA were sourced from Kunchuang Biotechnology (Xian, China). Primary antibodies for COL1A1, RUNX2, AMPK, p-AMPK, GPX4, FTH, β-ACTIN, and Histone H3 were acquired from Cell Signaling Technology (United States), while primary antibodies for SLC7A11 and Nrf2 were supplied by Proteintech Group (Wuhan, China). Streptozocin (STZ) and Met were sourced from Beyotime Biotechnology (Shanghai, China). Ferrostatin-1, AICAR, and Compound C were all obtained from MedChemExpress (Shanghai, China). Fetal bovine serum (FBS) was obtained from Cell-Box (Hong Kong, China), and the dual antibiotics (penicillin-streptomycin) were offered by NCM Biotech (Suzhou, China). Multicolor prestained protein ladders were provided by NCM Biotech (Suzhou, China) and Epizyme Biomedical Technology (Shanghai, China).

2.2 Animal experiments

Six-week-old SD rats were supplied by Beijing Vital River Laboratory Animal Technology Company (SCXK2021-0006), weighing 180 ± 20 g. The rats were casually allocated into two groups: control group (Con; n = 6) and T2DM model group (T2DM; n = 6). The T2DM rat model was established using an 8-week high-fat diet (HFD) along with low-level STZ injections (30 mg/kg) (Skovsø, 2014; Jin et al., 2023). At the conclusion of the experiment, the rats were euthanized with CO2, and femurs were harvested for subsequent evaluation.

To verify the impact of Met on DOP in rats, the animals were arbitrarily placed into four different groups: control group (Con, n = 6); DOP group (DOP, n = 6); Met treatment group (DOP + Met, n = 6), which received a daily dose of 200 mg/kg of Met via gavage (Jeyabalan et al., 2013; Loh et al., 2022); and Met + Compound C (Cc) group (DOP + Met + Cc, n = 6), which received daily doses of 200 mg/kg of Met via gavage and 0.2 mg/kg of Cc via intravenous injection (Kim et al., 2011). The Con and DOP groups received an equal volume of physiological saline through oral administration. Treatment with Met continued for 8 weeks. Following euthanasia via

Abbreviations: Met, metformin; DOP, diabetic osteoporosis; MDA, malondialdehyde; AMPK, AMP-activated protein kinase; FTH1, ferritin heavy chain 1; GPx4, glutathione peroxidase 4; Nrf2, nuclear factor erythroid 2-related factor 2; SLC7A11, solute carrier family 7, member 11; ALP, alkaline phosphatase; FFAs, free fatty acids; Tb.BV/TV, trabecular bone volume/tissue volume; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; Tb.BMD, trabecular bone mineral density; Ct.BMD, cortical bone mineral density; Ct.Th, cortical bone thickness; Ct.Ar, cortical bone area.

CO^2 asphyxiation, the femurs were gathered for subsequent experiments. All surgical procedures and animal handling were authorized by the Animal Ethics Committee of the First Hospital of Jilin University (SYXK2022-0001).

2.3 Serum insulin and free fatty acids (FFAs)

Serum insulin concentration was evaluated utilizing an insulin ELISA kit (Solarbio, Shanghai, China) following the protocols. The concentration of FFAs in rat serum was determined following the instructions provided with the FFAs quantitative assay kit (Solarbio, Beijing, China).

2.4 Micro-CT analysis

Micro-CT scanning was conducted with equipment from PINGSENG Healthcare (Jiangsu, China) to analyze the microstructural characteristics of the rat femur. Regions of interest (ROI) were defined as 2 mm segments located below the growth plate at the distal femur and at the femoral diaphysis, where three-dimensional reconstruction and measurement were conducted.

2.5 Histomorphological and immunohistochemical (IHC) analyses

Hematoxylin and eosin (H&E) staining is employed for examining the pathological changes in bone tissue, including adipocytes and collagen fibers, and Masson's trichrome staining allows for the examination of bone trabecular integrity and the thickness of collagen fibers. The femurs were fixed in 4% paraformaldehyde for 24 h, followed by decalcification in EDTA decalcification solution for 4 weeks. After decalcification, the femurs were dehydrated through a graded ethanol series and cleared in xylene. Subsequently, the samples were encapsulated in paraffin, and serial sections of 4 μm thickness were obtained along the coronal plane of the femur. H&E staining and Masson's trichrome staining were conducted on the tissue sections, which were then mounted with neutral resin.

For IHC analysis, the 4 μm-thick tissue sections were first deparaffinized and subjected to antigen retrieval. Endogenous peroxidase activity was quenched in the sections. The sections were then incubated overnight at 4°C with a primary antibody against p-AMPK, GPX4, SLC7A11, followed by incubation with an HRP-conjugated secondary antibody at room temperature the next day. Finally, the sections were developed with DAB, counterstained with hematoxylin, dehydrated, and mounted with neutral resin for microscopic observation.

2.6 Cell culture

The osteoblast cell line MC3T3-E1 was obtained from Shanghai Enzyme Research Biotechnology Company. Cells were grown in a medium containing 10% FBS and 1% antibiotics in a 5% CO_2 incubator at 37°C. MC3T3-E1 were pretreated with Met, Compound C (10 μmol/L) (Zhang et al., 2018; Wang X. et al., 2022) and AICAR(1 mM) (Yang et al., 2017; Lu et al., 2023) for 1 h prior to HGHF treatment, as required.

2.7 Cell viability analysis

To assess cell viability, a CCK-8 assay kit was purchased from NCM Biotech (Suzhou, China) and used following the guidelines. Absorbance readings were subsequently taken at a wavelength of 450 nm using a microplate reader from BMG LABtech (Germany).

2.8 Malondialdehyde and ferrous levels

Intracellular malondialdehyde (MDA) and ferrous levels are key indicators of ferroptosis. MDA levels were assessed with the MDA assay kit provided by Beyotime Biotechnology (Shanghai, China). The intracellular ferrous ion content was determined using a ferrous ion assay kit from Sigma-Aldrich (MO, United States), with strict adherence to the provided protocol.

2.9 Transmission electron microscopy

We utilized transmission electron microscopy (TEM) to examine the morphological changes in the mitochondria of the samples. The samples underwent a range of procedures, including fixation, dehydration, embedding, sectioning, and staining. Finally, imaging analysis of the samples was performed using a Hitachi 7800 TEM (Japan).

2.10 Alkaline phosphatase (ALP) analysis

MC3T3-E1 cells used for osteogenic differentiation were plated in a six-well plate and cultured under various treatment conditions. One portion of the samples was treated with the BCIP/NBT ALP color development kit (Beyotime, Shanghai, China) and photographed with a camera. Another portion of the samples was analyzed for ALP activity following the protocols provided by Beyotime (Shanghai, China), with absorbance taken at 405 nm using a microplate reader for semi-quantitative analysis.

2.11 Western Blot (WB) analysis

The extraction of total cellular protein and nuclear protein was carried out using kits from Beyotime Biotechnology. (Shanghai, China). Subsequently, gels were prepared using a PAGE rapid preparation kit (NCM Biotech, Suzhou, China) and subjected to electrophoresis. After electrophoresis, the gel was transferred onto a 0.45 μm PVDF membrane. This membrane was treated with 5% non-fat milk for 1 h and subsequently placed overnight at 4 °C with primary antibodies. Next, the PVDF membrane was washed five times with TBST and incubated with fluorescent secondary antibodies for 1 h. Finally, protein imaging was conducted utilizing FUSION Solo6S imaging system (VILBER, France).

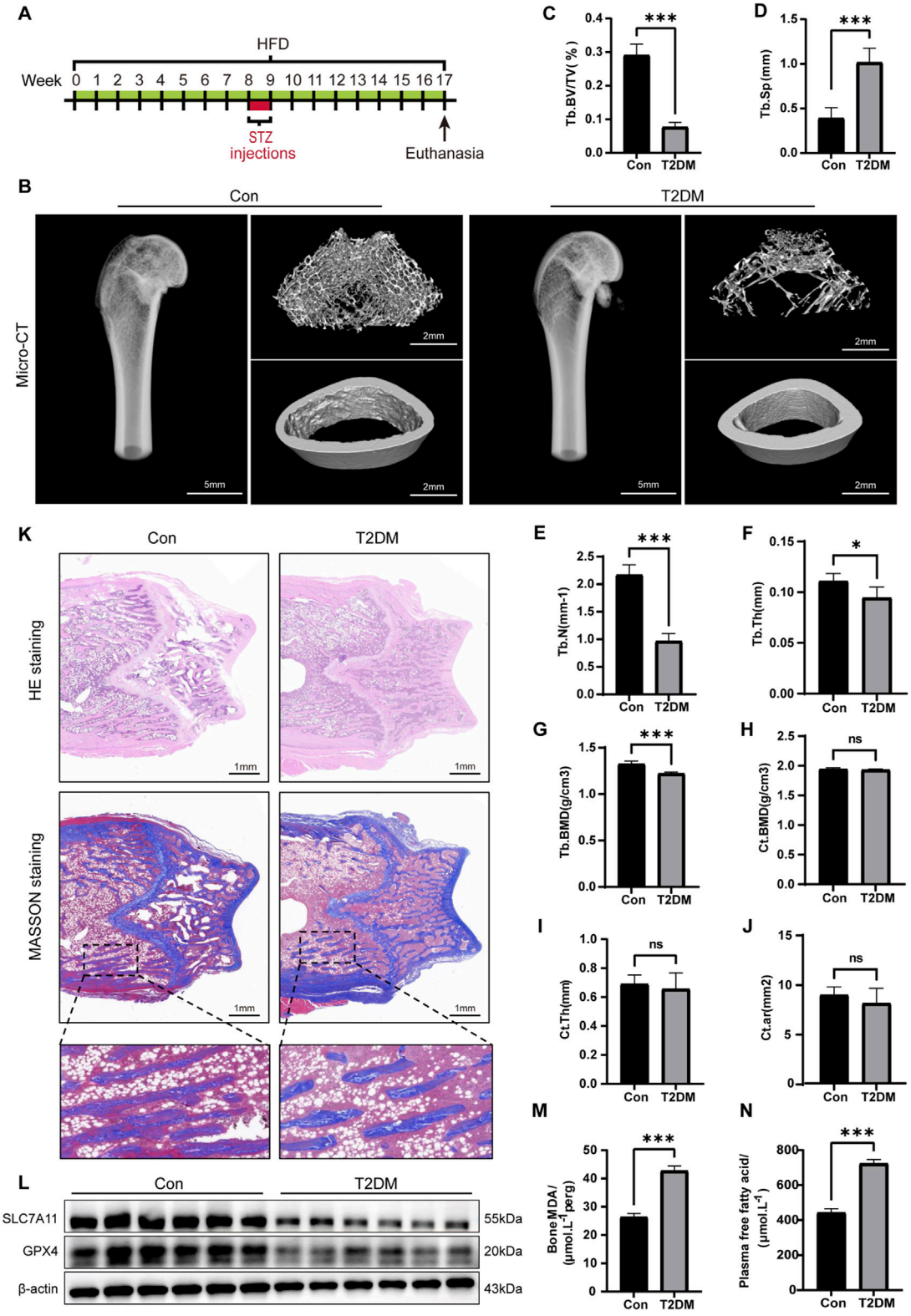

FIGURE 1
Ferroptosis in bone tissue induced by the diabetic microenvironment. **(A)** Illustrative diagram of the DOP model by administering a HFD alongside low-level STZ injections. **(B)** Representative micro-CT images depicting the microstructure of the femur. **(C–G)** Quantitative analysis of trabecular bone parameters. **(H–J)** Analysis of cortical bone parameters. **(K)** Representative images of H&E and Masson staining on the coronal section of the femoral metaphysis. **(L)** WB analysis of GPX4 and SLC7A11 in bone tissue. **(M)** MDA levels in bone tissue. **(N)** Serum FFA levels. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Each group contained six rats.

2.12 Statistical analysis

All data are presented as mean ± standard deviation (SD). Each experiment was conducted a minimum of three times. Statistical analyses were performed utilizing GraphPad Prism 9 software (CA, United States). The Student's t-test was applied for comparisons between two groups. When assessing multiple groups, one-way or two-way analysis of variance (ANOVA) with Tukey's *post hoc* test was utilized. A p-value below 0.05 was deemed statistically significant.

3 Results

3.1 Ferroptosis in bone tissue was triggered by the diabetic microenvironment

To explore the potential mechanisms implicated in DOP, we initially developed a DOP rat model by administering an 8-week HFD alongside low-level STZ injections (30 mg/kg) administered once daily for three consecutive days (Figure 1A). Following STZ injections, the plasma glucose levels in the model group were markedly higher than those in the control group (Supplrmrntary Figure S1A). The increased fasting insulin levels further indicated the existence of insulin resistance in the model group (Supplrmrntary Figure S1B). These findings validated the effective creation of the T2DM rat model.

To assess the impact of T2DM on bone microstructure, we conducted micro-CT on the femurs of the rats. The results manifested that the trabecular bone structure at the distal femur of the T2DM group was significantly sparse compared to the Con (Figure 1B). Specifically, the trabecular bone parameters in the T2DM group, including Tb. BV/TV, Tb. N, Tb. BMD, and Tb. Th, were all significantly decreased, while Tb. Sp exhibited a notable increase (Figures 1C–G). However, there were no significant differences in the cortical bone parameters (Ct. BMD, Ct. Th, and Ct. Ar) between the Con and T2DM groups (Figures 1H–J). These findings suggested T2DM leads to a deterioration in bone density and microstructure of the trabecular bone at the distal femur, while the impact on cortical bone is not significant. We also conducted H&E and Masson staining on the trabecular bone at the distal femur to evaluate the morphological changes in bone tissue within the diabetic microenvironment. The results demonstrated the arrangement of trabecular bone was sparse, with widened spacing and thinning, along with fractures in the T2DM group. There was a notable increase in adipocytes within the trabecular cavities, and the collagen fibers in the local trabecular bone had disappeared (Figure 1K). These results collectively validated the effective creation of the DOP model.

Subsequently, we further investigated whether ferroptosis occurred in the bone tissue of DOP rats. We performed WB analysis on the expression of GPX4 and SLC7A11 (Figure 1L). The semi-quantitative analysis showed a significant decrease in the expression of GPX4 and SLC7A11 in the DOP rats (Supplrmrntary Figure S2). MDA is the terminal product of lipid peroxidation. Results showed the MDA levels in the DOP group were significantly elevated (Figure 1M), suggesting a high level of lipid peroxidation. Additionally, the serum levels of free fatty acids (FFAs) in the T2DM group were significantly increased (Figure 1N). This finding suggested elevated blood glucose and high FFA levels were major characteristics of the diabetic microenvironment. Palmitic acid (PA) is the most abundant saturated FFA and is known to be lipotoxic to osteoblasts (Al Saedi et al., 2020). Therefore, in subsequent *in vitro* cell experiments, we will simulate the type 2 diabetes microenvironment by exogenously adding glucose and PA (HGHF treatment). In summary, these findings collectively suggested the diabetic microenvironment induced ferroptosis in bone tissue *in vivo*.

3.2 Ferroptosis was induced by HGHF treatment in osteoblasts

Building on previous studies and our findings on rat blood glucose levels, we applied a high concentration of glucose (HG, 25.5 mmol·L^{-1}) to MC3T3-E1 cells to simulate the diabetic microenvironment, whereas the Con group was administered low glucose (LG, 5.5 mmol·L^{-1}) (Ma et al., 2020). Additionally, MC3T3-E1 cells were exposed to a range of different concentrations of PA to determine the optimal concentration for inducing changes in osteoblasts (Figure 2A). Specifically, HGHF treatment markedly decreased osteoblast viability in a time-and dose-dependent manner, particularly at a PA concentration of 300 μmol L^{-1}, where cell viability dropped to 66.7%. Therefore, this study established the experimental conditions of treating cells with 25.5 mM glucose and 300 μmol L^{-1} PA for 48 h to induce osteoblast death. Furthermore, to evaluate the rescue effect of different concentrations of Ferrostatin-1 (Fer-1, a ferroptosis inhibitor) on osteoblast viability, we introduced Fer-1 at concentrations ranging from 1 to 10 μmol L^{-1}. The results indicated 10 μmol L^{-1} Fer-1 was the optimal concentration for following experiments (Figure 2B).

Ferroptosis is characterized by a range of morphological and biochemical changes, including alterations in mitochondrial morphology, iron overload, and lipid peroxidation. The results indicated HGHF treatment resulted in a notable elevation of MDA levels (Figure 2C), while Fer-1 treatment could partially restore this alteration. Moreover, the levels of ferrous iron (Fe^{2+}) in each treatment group were analyzed using a Fe^{2+} detection kit. The findings indicated HGHF treatment significantly elevated the levels of Fe^{2+}, with Fer-1 treatment showing some degree of improvement in this damage effect (Figure 2D).

Mitochondrial changes are regarded as key features of ferroptosis and are believed to act as amplifiers of this process. TEM observations indicated mitochondria in osteoblasts treated with HGHF exhibited shrinkage and outer membrane rupture, along with a deepening of mitochondrial color. In contrast, treatment with Fer-1 led to a partial recovery of mitochondrial structure (Figure 2E). Ultimately, WB analysis was performed to evaluate the expression of GPX4, SLC7A11, and FTH. The results demonstrated HGHF treatment markedly downregulated the expression of these proteins, while Fer-1 treatment was able to partially reverse this downregulation (Figures 2F–I). Together, these findings further confirm the occurrence of ferroptosis and elucidate its underlying mechanisms. In summary, ferroptosis was essential for HGHF-induced osteoblast ferroptosis *in vitro*.

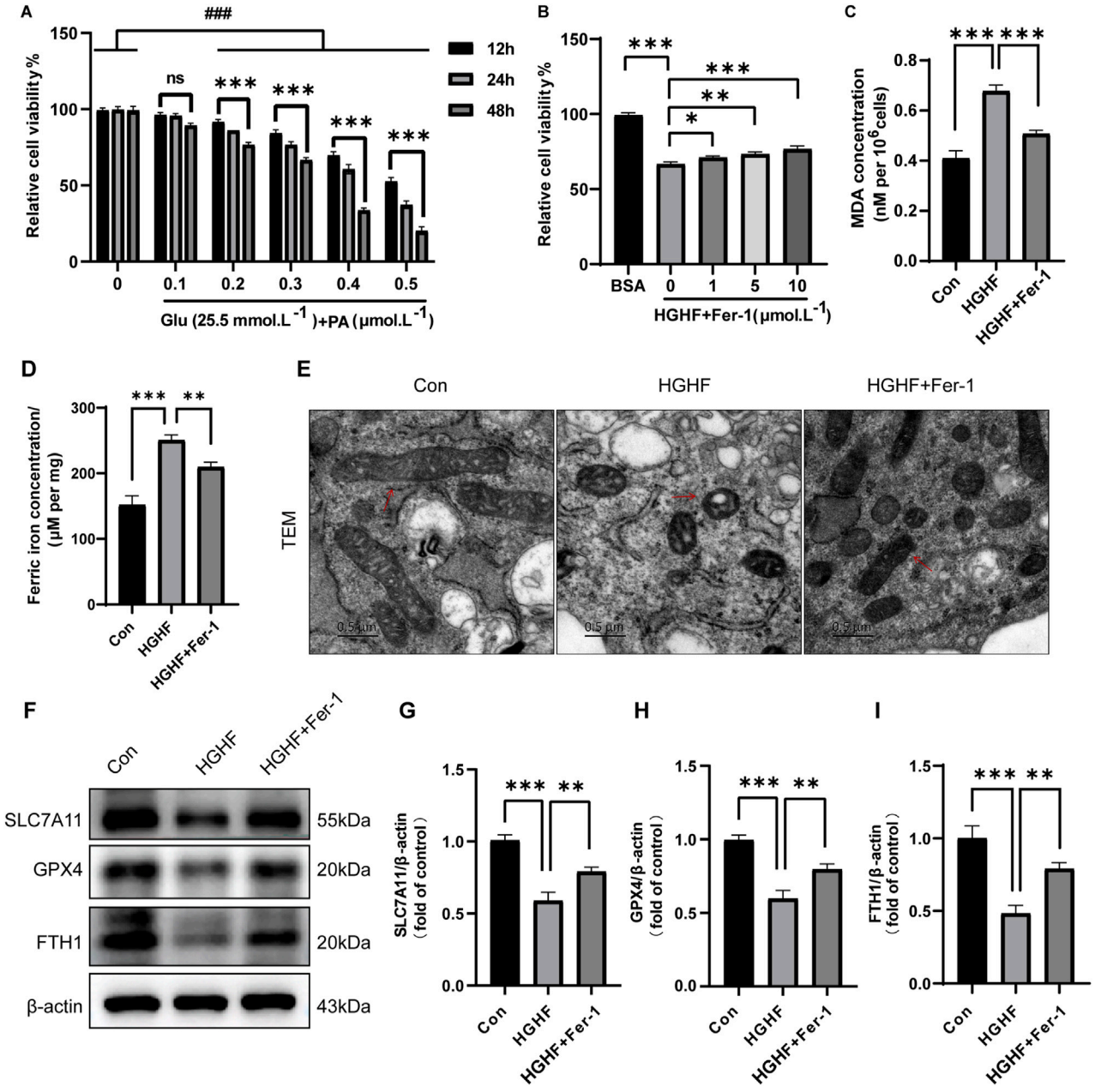

FIGURE 2
Ferroptosis was induced by HGHF treatment in osteoblasts. **(A)** Osteoblasts were cultured in a low glucose (5.5 mmol·L^{-1}) environment or in the presence of various concentrations of PA along with 25.5 mmol·L^{-1} glucose, and cell viability was measured at different time points (n = 3). **(B)** After pre-treating osteoblasts with different concentrations of Fer-1, the cells were exposed to either BSA or HGHF for 48 h, followed by assessment of cell viability (n = 3). **(C)** Measurement of MDA levels in osteoblasts (n = 3). **(D)** Analysis of Fe^{2+} content in osteoblasts (n = 3). **(E)** Representative images of mitochondrial changes in osteoblasts observed via TEM (n = 3). **(F)** WB analysis of ferroptosis-related proteins (n = 3). **(G–I)** Semi-quantitative analysis of different ferroptosis markers (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\#\#\#}P < 0.001$.

3.3 Met inhibited HGHF-induced ferroptosis and improves osteogenic differentiation

We exposed MC3T3-E1 cells affected by HGHF to varying concentrations of Met. The results indicated that treatment with 100 μmol L^{-1} Met significantly enhanced cell viability (Figure 3A). Therefore, we selected this concentration for subsequent cell experiments. Given that Met did not negatively impact cell viability or proliferation, we investigated its possible function in preventing HGHF-induced ferroptosis.

We first evaluated the effect of Met on intracellular MDA and ferrous ion levels under HGHF stimulation. HGHF treatment induced a marked elevation in MDA and ferrous ion levels, which was effectively decreased by post-treatment with Met or Fer-1, mitigating the associated oxidative damage (Figures 3B, C). Subsequent Western blot analysis revealed Met's regulatory effects on key ferroptosis-

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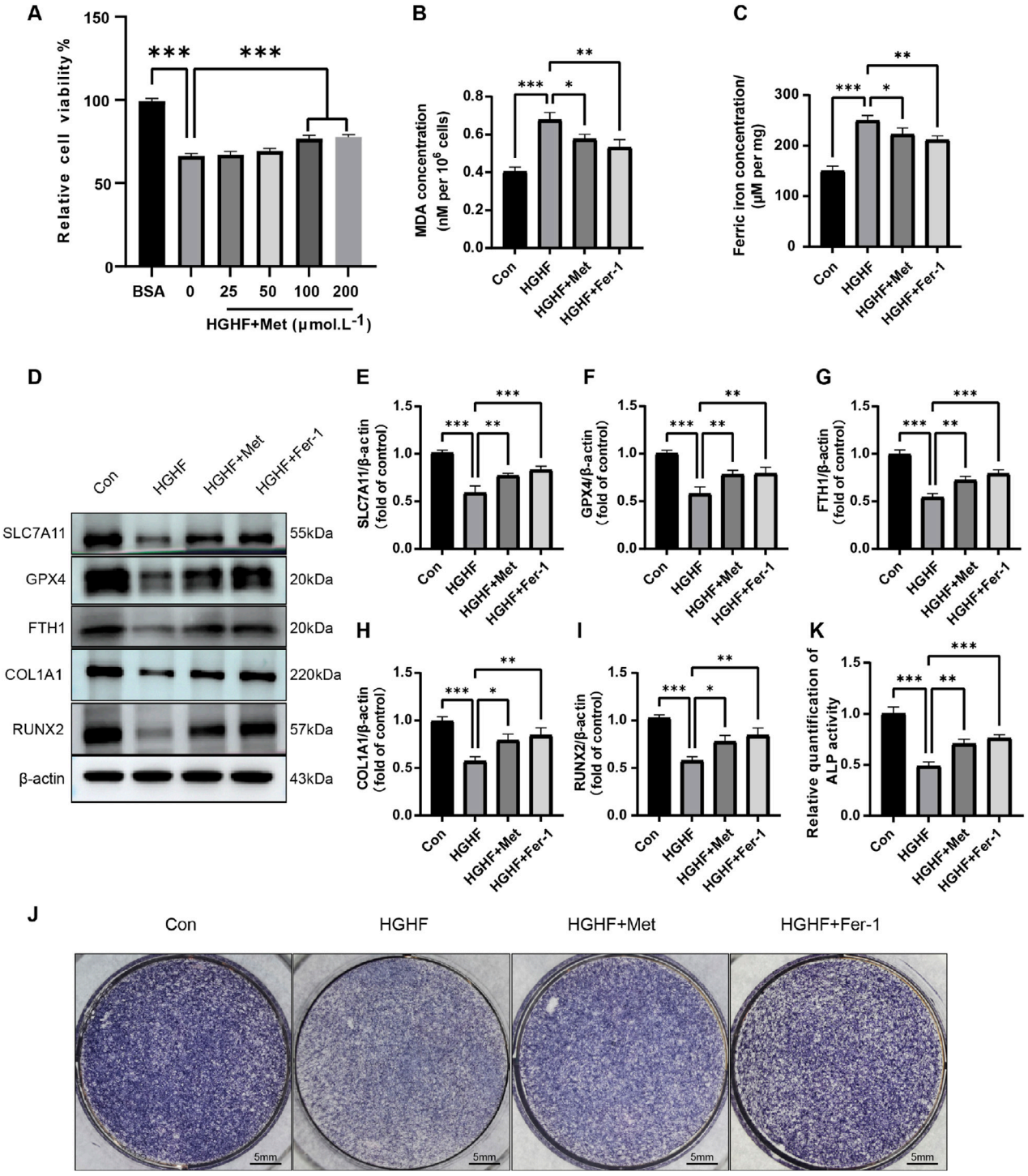

FIGURE 3
Met inhibited HGHF-induced Ferroptosis and improves osteogenic differentiation. **(A)** Changes in cell viability following 48 h of treatment with different concentrations of Met (n = 3). **(B)** Measurement of MDA levels in different treatment groups (n = 3). **(C)** Assessment of Fe^{2+} levels. **(D)** WB analysis of proteins associated with ferroptosis and osteogenic markers (n = 3). **(E–G)** Semi-quantitative analysis of ferroptosis-related proteins (n = 3). **(H, I)** Semi-quantitative analysis of osteogenic markers (n = 3). **(J)** Measurement of ALP in MC3T3-E1 cells on the seventh day of osteogenic induction (n = 3). **(K)** Semi-quantitative assessment of ALP (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

related proteins. Met treatment partially reversed the downregulation of GPX4, SLC7A11, and FTH1 expression compared to the HGHF group (Figures 3D–G). These findings suggested Met effectively inhibited ferroptosis induced by HGHF in MC3T3-E1 cells.

Ferroptosis induced by HGHF hindered the osteogenic differentiation capacity of osteoblasts. Specifically, HGHF reduced the levels of osteogenic markers (COL1A1 and RUNX2), while both Met and Fer-1 effectively attenuated these HGHF-induced

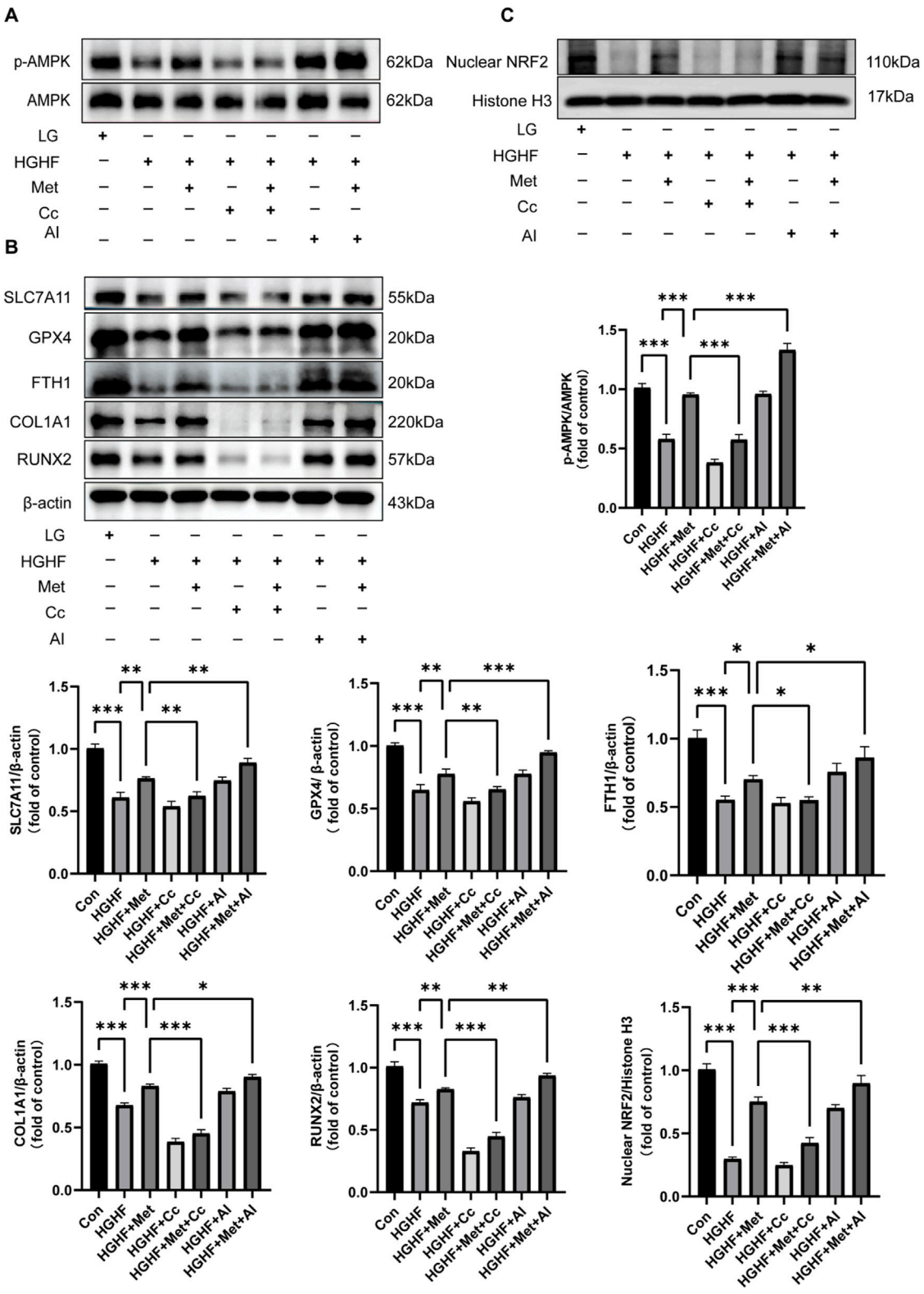

FIGURE 4
Met inhibited ferroptosis and osteogenic impairment via the AMPK/Nrf2 pathway. **(A)** WB analysis of p-AMPK and AMPK across various treatment groups (n = 3). **(B)** Analysis of changes in ferroptosis protective proteins and osteogenic markers (n = 3). **(C)** Expression of Nrf2 protein in the nucleus (n = 3). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

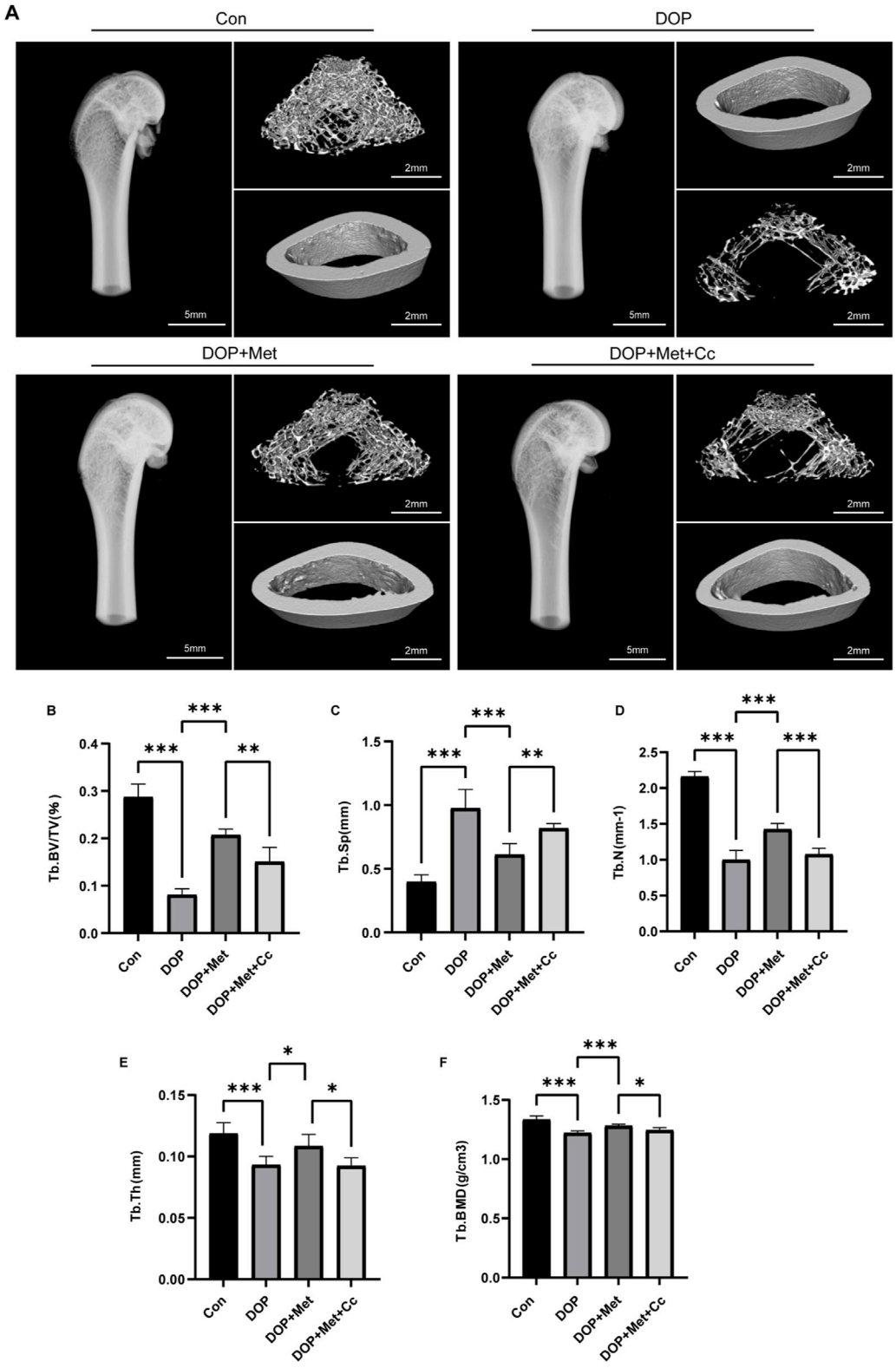

FIGURE 5
Met improved the microstructure of bone in the DOP model. **(A)** Representative micro-CT images from various treatment groups. **(B–F)** Quantitative analysis of trabecular bone parameters, including Tb. BV/TV, Tb. BMD, Tb. N, Tb. Th, and Tb. Sp. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Each group contained six rats.

impairments (Figures 3D, H, I). Alkaline phosphatase (ALP), a crucial enzyme mediating bone mineralization, showed reduced activity following HGHF exposure. However, Met or Fer-1 treatment partially restored ALP activity, suggesting their protective effects on osteogenic function (Figures 3J, K). These results underscored the significant role of Met in counteracting the adverse impacts of HGHF on the osteogenic differentiation in MC3T3-E1 cells.

3.4 Met activated the AMPK/Nrf2 pathway to inhibit ferroptosis and osteogenic impairment in osteoblasts

AMPK is a crucial metabolic regulator that plays a significant role in cellular energy homeostasis (Madhavi et al., 2019). Given its potential involvement in suppressing ferroptosis, AMPK may serve as a therapeutic target for diseases associated with ferroptosis (Lee et al., 2020). To further investigate this mechanism, we conducted experiments using Compound C (Cc, an AMPK inhibitor), alongside AICAR (AI, an AMPK activator). WB analysis revealed Met significantly upregulated the expression of p-AMPK compared to the HGHF group (Figure 4A). As shown in Figure 4B, the upregulation of ferroptosis-protective proteins and osteogenic markers induced by Met was reversed following Cc intervention, whereas AI enhanced the protective effects of Met. These results demonstrated a critical connection between AMPK activation and ferroptosis.

Given the promoting effect of the AMPK pathway on Nrf2 nuclear accumulation and Nrf2's antioxidant properties, we further investigated whether the inhibitory effect of Met on ferroptosis was associated with Nrf2 activation (Han et al., 2018). Our findings indicated Met treatment ameliorated the decline in nuclear Nrf2 protein expression induced by HGHF. The addition of Cc blocked the enhancing effect of Met on Nrf2 translocation, while AI strengthened this effect (Figure 4C). Collectively, these findings showed Met exerted a protective role against HGHF-induced ferroptosis and osteogenic differentiation in MC3T3 cells via the AMPK-dependent Nrf2 pathway.

3.5 Met inhibited ferroptosis and rescues DOP through the AMPK/Nrf2 pathway

In the DOP rat model, we further investigated the effects of Met. Micro-CT revealed that an 8-week Met treatment significantly promoted the recovery of trabecular bone structure in the distal femur of DOP rats (Figure 5A). Specifically, parameters such as Tb. BV/TV, Tb. BMD, Tb. N, Tb. Th, and Tb. Sp showed significant improvement, although this enhancement was blocked by Cc intervention (Figures 5B–F).

H&E and Masson staining were conducted on the distal femur. In comparison to the DOP group, the DOP + Met demonstrated enhanced trabecular bone density and a notable reduction in trabecular separation. In contrast, the Cc group diminished the protective effects of Met (Figure 6A). Met significantly reduced MDA levels in the DOP rats, while Cc partially attenuated this protective effect (Figure 6B). WB and IHC analyses demonstrated Met notably elevated the levels of Nrf2 and p-AMPK within the bone tissue of DOP rats, while the Cc group showed downregulation of these proteins (Figures 6C, D, F). Semi-quantitative analyses of these results are presented in Supplementary Figure S3A, B, 4A. Additionally, Met treatment enhanced the levels of ferroptosis-protective proteins like SLC7A11, GPX4, and FTH in DOP rats; however, these proteins were partially inhibited in the Met + Cc group (Figures 6E, F; Supplementary Figure S4B, C). Collectively, our findings demonstrated Met alleviated DOP through suppression of ferroptosis, highlighting its therapeutic potential for metabolic bone disorders.

4 Discussion

In this study, we revealed ferroptosis is critical in the pathogenesis of DOP. Met, as a potential therapeutic intervention, effectively inhibited the process of lipid peroxidation and ferroptosis by activating the AMPK/Nrf2 signaling axis, thereby improving osteoporosis (Figure 7).

The concept of DOP was initially introduced in 1984 and is now widely acknowledged as a prevalent form of secondary osteoporosis (Takamoto and Kadowaki, 2004). In comparison to type 1 DOP, type 2 DOP exhibits more harsh deterioration in bone microstructure, marked by elevated cortical porosity and heightened bone fragility (2022). Recent studies further support the detrimental impacts of the diabetic microenvironment on bone strength and bone mass (Ma et al., 2020). Throughout the progression of DOP, excessive accumulation of glycotoxic metabolites occurs within bone tissue, leading to a reprogramming of osteoblast metabolism (Martiniakova et al., 2024). Additionally, the toxic effects of saturated FFAs on osteoblast function represent a critical factor in this context (Kim et al., 2008; Martiniakova et al., 2024).

In this research, we developed the type 2 DOP rat model using a HFD coupled with STZ injections. Micro-CT analysis revealed no significant changes in the cortical parameters of the rat femur (Ct. BMD, Ct. Th, and Ct. Ar), consistent with previous studies (Zhang et al., 2015). This lack of significant alteration may be attributed to the short time frame of the HFD modeling period, which could limit the impact on cortical bone remodeling. Additionally, we observed significant reductions in trabecular parameters, including Tb. BV/TV, Tb. N, Tb. BMD, and Tb. Th, while Tb. Sp showed a marked increase. This might be attributed to the greater impact of glycotoxicity on the actively remodeling trabecular regions of bone, a finding that aligns with previous conclusions (Botolin and McCabe, 2007; Motyl and McCabe, 2009). The deterioration of trabecular bone in DOP is associated with the suppression of osteogenesis and/or increased bone absorption. Numerous studies have demonstrated hyperglycemia and hyperlipidemia can directly suppress the osteogenic activity of osteoblasts, although the specific mechanisms remain unclear (Yamaguchi, 2010; Takanche et al., 2020). There is considerable divergence in the findings regarding the impact of the diabetic microenvironment on osteoclasts. A prolonged HFD alone does not significantly affect osteoclastogenesis; however, when combined with elevated blood glucose levels, there is a marked increase in both the quantity and activity of osteoclasts (An et al., 2019; Kim et al., 2021). However, it

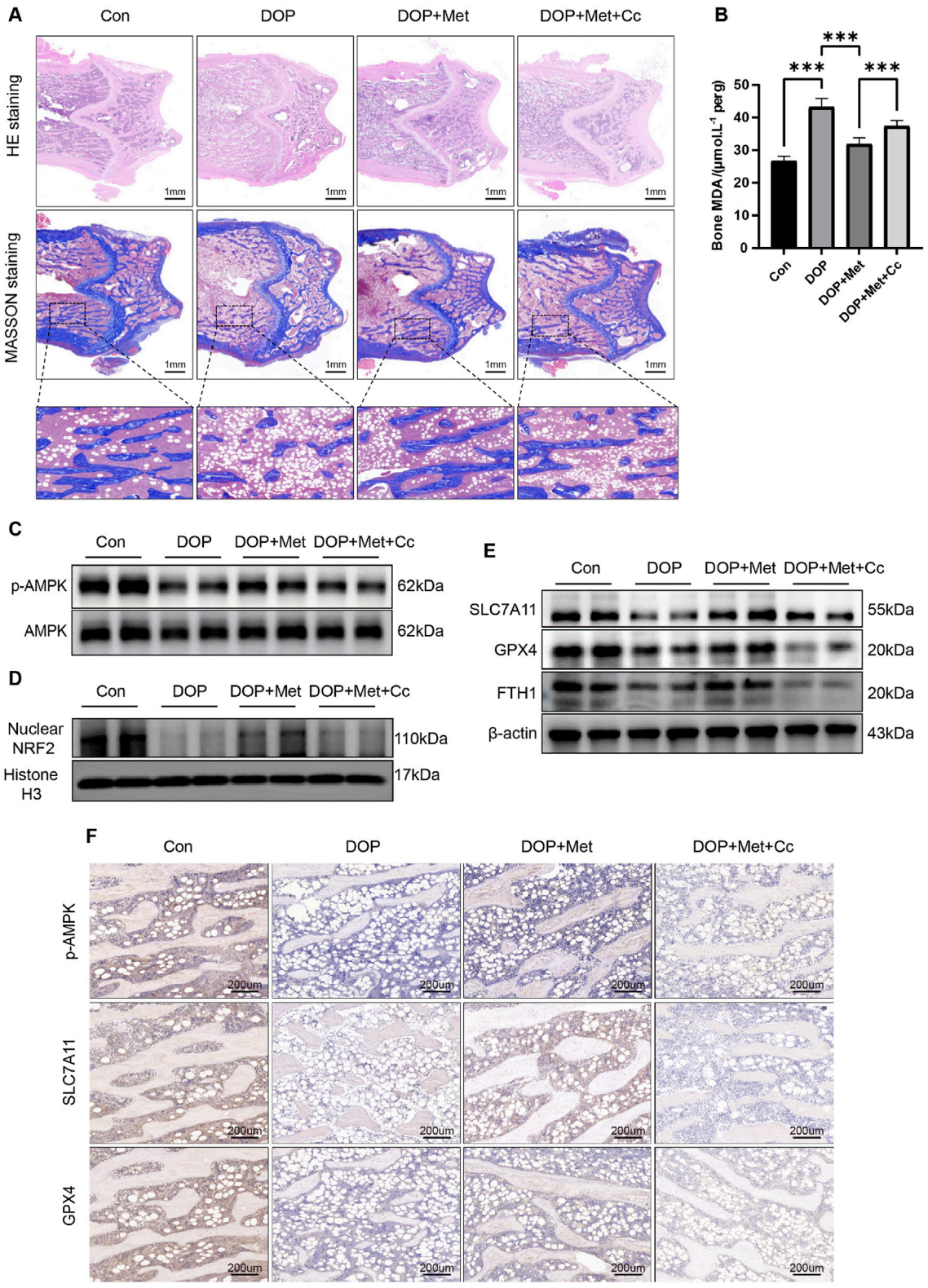

FIGURE 6
Met activated the AMPK/Nrf2 pathway to inhibit ferroptosis, rescuing DOP. **(A)** Representative coronal sections of the femoral metaphysis, displaying H&E and Masson staining images. **(B)** Measurement of MDA levels in bone tissue. **(C)** WB analysis of p-AMPK and AMPK expression in bone tissue. **(D)** Expression of Nrf2 protein in bone tissue. **(E)** WB analysis of ferroptosis-associated proteins in bone tissue. **(F)** IHC analysis of ferroptosis-associated proteins and p-AMPK in bone tissue. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Each group contained six rats.

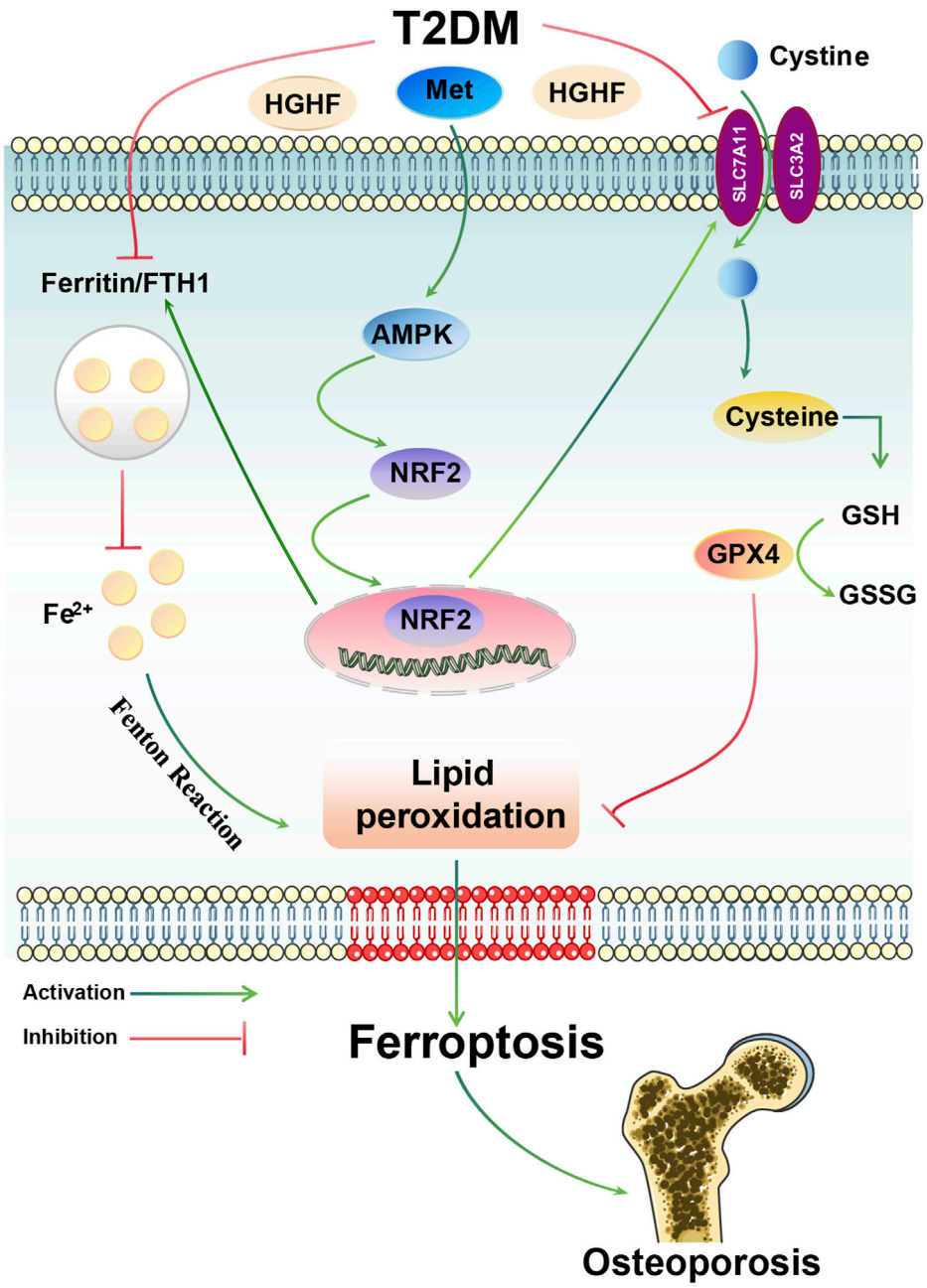

FIGURE 7
Overview of the mechanisms by which Met inhibits HGHF-induced ferroptosis and DOP. Met can attenuate HGHF-triggered ferroptosis, enhancing bone microarchitecture in DOP through the activation of the AMPK/Nrf2 signaling pathway.

remains challenging to determine whether this osteoclast-promoting effect is directly caused by glycolipid metabolites or whether it results from the release of damage-associated factors within the diabetic microenvironment (Andreev et al., 2020).

Ferroptosis is a newly identified type of iron-dependent cell death, distinguished by excessive iron accumulation (Verma et al., 2020). Classical inducers of ferroptosis promote intracellular iron accumulation primarily by inhibiting antioxidant systems. The uptake of iron through transferrin receptor 1 increases free iron concentrations, while the storage of iron by FTH1 reduces free iron levels (Brown et al., 2019). Excessive free iron can lead to increased lipid peroxidation through Fenton reactions (Stockwell, 2022). In the process of ferroptosis, FTH1, GPX4 and SLC7A11 serve as key inhibitory factors. GPX4, as a central suppressor of ferroptosis, is an antioxidant enzyme that plays a crucial role in inhibiting lipid peroxidation (Mai et al., 2017; Zhang et al., 2021). SLC7A11 facilitates the uptake of extracellular cystine (the oxidized dimer form of cysteine), which is subsequently reduced to cysteine, participating in protein synthesis and other metabolic processes (Koppula et al., 2018). The correlation between ferroptosis and osteoporosis remains adequately underexplored, particularly osteoporosis induced by type 2 DOP. Studies have shown the Nrf2/

HO-1 signaling pathway demonstrates potential in inhibiting high glucose-induced ferroptosis in type 2 DOP (Ma et al., 2020). Consistent with these findings, we observed that Fer-1 exhibited significant protective effects against ferroptosis triggered by HGHF conditions. Another important hallmark of ferroptosis is the alteration in mitochondrial morphology. Upon cellular stress, mitochondrial apoptosis typically initiates a cascade of cellular death. Given the crucial role of mitochondria in oxidative metabolism and apoptosis regulation, there exists a profound interplay between ferroptosis and mitochondrial function (Li et al., 2022). Our study revealed that osteoblasts treated with HGHF exhibited mitochondrial shrinkage and outer membrane rupture, alongside deepening mitochondrial coloration. In contrast, Met treatment partially restored mitochondrial structure. Furthermore, we observed that Met significantly mitigated lipid peroxidation induced by HGHF while markedly reducing the expression levels of SLC7A11, GPX4, and FTH1, suggesting Met exerts a beneficial effect on countering ferroptosis.

Met is a traditional and cost-effective first-line hypoglycemic agent (Shin et al., 2020). Numerous studies have demonstrated that Met can improve diabetes and its complications (Hu et al., 2020; Ala and Ala, 2021). Met significantly enhances the phosphorylation levels of AMPK in osteoblasts, which aligns with the findings of this study (LaMoia and Shulman, 2021). Additionally, research suggests that Met-induced AMPK activation may serve as an effective therapeutic strategy to prevent osteoblast apoptosis (Jang et al., 2011). However, the particular function of Met regarding type 2 DOP warrants further investigation. AMPK, as one of the action targets of Met, is an enzyme that is widely expressed in various tissues, including the heart, kidneys, liver, brain, bone, and skeletal muscle (Lee et al., 2020). As a sensor of cellular energy status, dysfunction of AMPK can lead to a variety of human diseases, particularly metabolic disorders (Peng et al., 2024). Recent studies have indicated that cancer cells exhibit resistance to ferroptosis due to high levels of AMPK activity, while cancer cells with inactivated AMPK are more prone to ferroptosis (Yang et al., 2021). However, whether Met exerts its inhibitory effect on HGHF-induced ferroptosis through AMPK activation has yet to be investigated. To confirm this hypothesis, we evaluated the impact of AMPK phosphorylation on ferroptosis. We observed the expression of p-AMPK was observably reduced in MC3T3-E1 cells treated with HGHF and in bone tissue from DOP rats, a condition that was partially ameliorated by Met treatment. Furthermore, co-treatment with Met and the AMPK activator AI enhanced the antioxidant capacity against ferroptosis in MC3T3-E1 cells exposed to HGHF. In contrast, the application of the AMPK inhibitor Cc under HGHF conditions diminished these protective benefits and heightened the susceptibility of MC3T3-E1 cells to ferroptosis. Collectively, these findings demonstrated Met ameliorated DOP through ferroptosis inhibition. Unlike conventional anti-osteoporotic agents such as bisphosphonates, which primarily act by suppressing osteoclastic bone resorption (Coe et al., 2015), Met exhibits dual therapeutic benefits: glycemic control and osteogenic enhancement. Although bisphosphonates increase bone mineral density in elderly women with T2DM, they concomitantly reduce osteogenic marker, potentially leading to secondary suppression of bone formation (Keegan et al., 2004; Gangoiti et al., 2008).

Nrf2 acts as a vital transcription factor responsible for maintaining redox balance within cells. Under normal physiological conditions, Nrf2 is highly unstable in the cytoplasm, where it is rapidly ubiquitinated and degraded via the proteasome pathway (Adelusi et al., 2020). In response to stress, Nrf2 can move into the nucleus and stimulate the transcription of genes associated with antioxidant response elements (Zhao et al., 2022). However, following prolonged oxidative stress, Nrf2 expression levels gradually decrease, exacerbating oxidative damage (Mathur et al., 2018; Wang et al., 2020). Activation of Nrf2 can upregulate the expression of various target genes, including GPX4, FTH1, and SLC7A11 (Dodson et al., 2019a). The Nrf2/SLC7A11/GPX4 axis has been shown to inhibit ferroptosis and oxidative stress induced by cerebral ischemia-reperfusion, thus exerting neuroprotective effects (Yuan et al., 2021). Research has indicated that activation of the AMPK signaling pathway can promote the accumulation of Nrf2 in the nucleus, enhancing its antioxidant activity in models of renal ischemia-reperfusion injury and diabetic cardiomyopathy (Wang X. et al., 2022; Kuang et al., 2023). Nevertheless, the contribution of the AMPK/Nrf2 pathway to the effects of Met on ferroptosis in DOP remains ambiguous. Our study found HGHF treatment reduced the antioxidant defense capacity of Nrf2 in MC3T3-E1 cells and diminished its nuclear translocation. Met activated AMPK and promoted the movement of Nrf2 into the nucleus, leading to upregulation of protein expression for FTH1, SLC7A11, and GPX4, which alleviated lipid peroxidation and the following ferroptosis. Furthermore, Met treatment enhanced the ability for osteogenic differentiation in MC3T3-E1 cells. *In vivo* experiments further demonstrated that Met administration reduced MDA levels in the femur and elevated the levels of GPX4, SLC7A11, and FTH1 in the distal femur, ultimately improving bone microstructure.

Our study has several limitations. First, while clinical bone specimens from DOP patients are challenging to obtain, utilizing primary human osteoblasts could provide additional validation of our findings. Second, the absence of genetic models targeting key ferroptosis regulators (such as GPX4) limits our mechanistic understanding of Met's effects on ferroptosis pathways. Finally, our investigation focused on the 8-week therapeutic window, leaving the long-term efficacy and dose-dependent effects of Met in DOP management an important area for future research.

5 Conclusion

This research demonstrates the important role of ferroptosis in the development of DOP. In the DOP microenvironment, Met prevents ferroptosis and enhances osteogenic differentiation by activating the AMPK/Nrf2 pathway. Our results emphasize the promise of Met as a therapeutic agent to inhibit ferroptosis and highlight its prospective value in osteoporosis treatment.

Data availability statement

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

Ethics statement

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

Author contributions

YL: Conceptualization, Methodology, Supervision, Writing – review and editing, Software, Validation, Visualization, Writing – original draft. ZF: Formal Analysis, Software, Visualization, Writing – review and editing. XW: Methodology, Software, Validation, Visualization, Writing – review and editing. QY: Data curation, Formal Analysis, Software, Visualization, Writing – review and editing. SL: Data curation, Formal Analysis, Visualization, Writing – review and editing. DZ: Conceptualization, Funding acquisition, Methodology, 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.

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.2025.1527316/full#supplementary-material

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Metformin in gynecological disorders: pathogenic insights and therapeutic implications

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Metformin, the most widely used anti-diabetic drug, has been demonstrated to exert various effects, including antioxidant, anti-inflammatory, anti-tumor, and cardioprotective properties. Due to its affordability and low toxicity profile, metformin is increasingly used to prevent or treat a wide range of gynecological disorders, as evidenced by epidemiological studies, clinical trials, and animal and *in vitro* studies. Trial findings for non-cancer conditions such as endometriosis, premature ovarian failure (POF), and uterine fibroids remain controversial and insufficient. However, most current clinical trials for polycystic ovarian syndrome (PCOS) and gynecological malignancies are ongoing phase II–III trials. The pharmacological effects of metformin have been shown to target the insulin-like growth factor (IGF), AMP-activated protein kinase (AMPK), phosphatidylinositol 3-kinase (PI3K)/AKT, MAPK, NF-κB, and other signal transduction pathways, highlighting its potential in the treatment of gynecological disorders. In this review, we discuss the biological impacts of metformin and the mechanisms of action pertinent to the treatment of different gynecological disorders.

KEYWORDS

metformin, polycystic ovary syndrome, endometriosis, premature ovarian failure, gynecologic malignancies, ovary, uterus

1 Introduction

Gynecological conditions often determine a woman's reproductive health and quality of life. However, there is a very high prevalence of gynecological disease worldwide, particularly in developing countries (Bigambo et al., 2022). In China, 45.96% of women suffer from dysmenorrhea, a prevalence lower than that reported in countries such as Ghana (68.1%) and Greece (89.2%). However, the prevalence of ovarian dysfunction in China (11.16%) is notably higher than the global prevalence (3.7%). Most women with gynecological disorders are treated with combination therapies, including surgery, chemotherapy, radiotherapy, and endocrine therapy, which could have serious side effects (He et al., 2024). Thus, it is important to reduce the burden of disease and improve global women's health through an effective intervention using cheap and widely available drugs (Mousa et al., 2021).

Metformin (N,N-dimethylbiguanide), a standard clinical drug for type 2 diabetes mellitus (T2DM) for over 60 years (Foretz et al., 2023), has been shown to have multiple biological effects beyond its hypoglycemic properties, such as antioxidant, anti-inflammatory, anti-tumor, anti-fibrotic, and antiviral activities (Du et al., 2022; Petrasca et al., 2023; Wang et al., 2023). Recently, metformin has been increasingly acknowledged for its efficacy in the treatment of non-diabetic conditions, such as obesity, cirrhosis of the liver, heart failure, brain damage, and various cancers, including gynecological cancers (Top et al., 2022; Wang et al., 2023). Mechanistically, metformin specifically inhibits mitochondrial respiratory chain complex 1 in a range of tissues, leading to the activation of AMP-activated protein kinase (AMPK) in various tissues, including hepatocytes, muscles, and neurons (Top et al., 2022). Although it is considered an activator of AMPK, evidence supports the involvement of alternative AMPK-independent pathways, suggesting that further research is warranted (Top et al., 2022).

Furthermore, several studies have reported that metformin is effective for the treatment of gynecological disorders, including polycystic ovarian syndrome (PCOS) (Li, Wu et al.), endometriosis, premature ovarian failure (POF), and uterine fibroids. In addition, metformin contributes to a reduction in the risk of gynecologic malignancies, including ovarian, cervical, and endometrial cancers (Shi et al., 2019; Kim et al., 2022; Drevinskaite et al., 2023). Notably, metformin has been established to have treatment efficacy, with several ongoing phase II–III clinical trials in gynecological disorders (Bae-Jump et al., 2020; Garcia-Beltran et al., 2023). This review aims to comprehensively delineate the effects of metformin on gynecological diseases, focusing on its biological mechanisms and therapeutic implications in clinical practice.

2 Effects of metformin on PCOS

PCOS is a common disorder of hormonal and metabolic imbalance, characterized by hyperandrogenism, dysfunctional ovulation, and polyfollicular ovaries and accompanied by metabolic abnormalities, such as insulin resistance (IR) and obesity (Zhao et al., 2023), affecting women during their reproductive years, with potential long-term cardiovascular, metabolic, and reproductive health outcomes (Zhu et al., 2022; Anbar et al., 2023). Although the exact cause of PCOS remains unclear, factors such as genetics, oxidative stress, chronic inflammation, and metabolic dysfunction are believed to be involved (Gu et al., 2016; Zhao et al., 2023). Oral contraceptives and hormone therapy are commonly used to manage PCOS, but they carry higher risks of side effects such as venous thromboembolism and reduced bone density, especially in postmenopausal women (Beksinska et al., 2018; Bachrach, 2020). Due to the interconnected nature of IR, obesity, and hyperandrogenism in PCOS, insulin-sensitizing agents, especially metformin, have been frequently studied (Cassar et al., 2016) (Figure 1).

2.1 Effects of metformin on IR and hyperandrogenemia in PCOS

PCOS is frequently associated with IR and hyperandrogenemia, affecting 65%–95% of women with the condition (Cassar et al., 2016; Zhao et al., 2023). IR, a defect in insulin signaling that impairs glucose utilization, is a key contributor to metabolic disturbances in PCOS. In addition, sex-hormone-binding globulin (SHBG) is a glycoprotein that transports androgens and estrogens. Androgen excess, a common feature of PCOS, exacerbates IR and is linked to elevated testosterone levels and a decrease in SHBG levels (Xing et al., 2021). Metformin primarily targets IR by enhancing insulin sensitivity, reducing hepatic gluconeogenesis, and increasing muscle glucose uptake (Cassar et al., 2016). It also lowers androgen levels by increasing SHBG, addressing both IR and hyperandrogenemia in PCOS (Diamanti-Kandarakis et al., 1998; Xing et al., 2021).

Metformin also reduces pregnancy-related risks for women with PCOS, such as miscarriage, preeclampsia, and preterm delivery, by improving menstrual regularity and promoting ovulation. A meta-analysis of randomized trials has demonstrated that metformin lowers the incidence of miscarriage (RR = 0.86, 95% CI: 0.67–1.12), preeclampsia (RR = 0.45, 95% CI: 0.24–0.83), and preterm delivery (RR = 0.37, 95% CI: 0.23–0.61) in PCOS patients (Moghetti et al., 2000; Zhu et al., 2022). In addition, metformin treatment greatly reduced gestational diabetes mellitus (GDM) incidence (RR = 0.59, 95% CI 0.43–0.80) compared to placebo, which was attributed to improved β-cell responsiveness and reduced hepatic gluconeogenesis (Yu et al., 2024). Notably, the GDM trial demonstrated that metformin had comparable efficacy to insulin in managing established GDM cases, with additional benefits including attenuated maternal weight gain (mean difference [MD] −2.3 kg, $p < 0.001$) and a 76% reduction in the incidence of preeclampsia (Syngelaki et al., 2016).

Metformin's effects also extend to cardiovascular risk reduction as it influences key metabolic pathways to improve lipid metabolism and endothelial function, potentially lowering the risk of cardiovascular events (Lan et al., 2015). It has been suggested that metformin displays potential beneficial effects by reducing several cardiovascular dysfunction risk factors, such as body mass index (BMI) (MD: −0.53 kg/m^2, 95% CI −0.95, −0.12), insulin-resistance (HOMA-IR) (MD:−0.50, 95% CI −0.91, −0.09), triglyceride (MD: −0.11 mmol/L, 95% CI −0.20, −0.02), plasma plasminogen activator inhibitor-1 (Ruth, Day et al.) (MD: −4.99 ng/mL, 95% CI −6.78, −3.21) (Li and Li, 2023), carotid intima-media thickness (CIMT), and flow-mediated dilation (FMD) (Wang et al., 2024) in PCOS patients. Taken together, these data suggest that metformin may help reduce cardiovascular events in PCOS patients. At the molecular level, metformin activates the AMPK or PI3K/mammalian target of rapamycin (mTOR) signaling pathways via oxidative stress, thereby improving glucose and lipid metabolism, and it also contributes to improved endothelial function, which is associated with higher levels of high-density lipoprotein cholesterol (HDL-C); lower levels of low-density lipoprotein cholesterol (LDL-C), triglycerides, and total cholesterol; and reduced risk of cardiovascular events (Anbar et al., 2023; Wang et al., 2024). Animal models reveal that metformin upregulated placental growth factor (PlGF) while suppressing soluble fms-like tyrosine kinase-1 (sFlt-1) production through AMPK-mediated pathways (Tong et al., 2022).

Hyperandrogenism, characterized by elevated androgen levels and typical of PCOS, is often exacerbated by increased ovarian androgen production. Metformin has been shown to directly reduce ovarian androgen levels, independent of its effects on insulin sensitivity (Kurzthaler et al., 2014). This provides a dual therapeutic

benefit for both insulin-sensitive and insulin-resistant women with PCOS (la Marca et al., 1999; Vrbikova et al., 2001). Long-term metformin therapy reduces both steroidogenic and metabolic enzyme activities in the ovaries and the adrenal response to adrenocorticotrophin (ACTH), helping mitigate hyperandrogenism in women with PCOS (Lan et al., 2015; LaMoia and Shulman, 2021).

In addition, decreased activity of ERK1/2 with elevated androgens was found in women with PCOS (Guney et al., 2022). *In vitro*, metformin appears to activate ERK1/2 and decrease the activity of CYP19 (P450 aromatase) for estrogen production (Hirsch et al., 2012). The ERK1/2 pathway is activated by luteinizing hormone (LH) in the ovaries, and this activation increases androgen synthesis in theca cells. However, metformin suppresses ERK1/2 activation, reducing androgen levels and improving hormonal balance (Li et al., 2025). In addition, in PCOS rat models, metformin also improves hormonal balance by upregulating the phosphatidylinositol 3-kinase (PI3K) pathway, further demonstrating its multifaceted action on IR and metabolic dysregulation (Guo et al., 2022).

2.2 Effects of metformin on inflammation in PCOS

PCOS has been recognized as a low-degree chronic inflammation disease, evidenced by elevated cytokine levels and macrophage infiltration. The inflammatory markers include C-reactive protein (CRP), interleukin-6 (IL-6), interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α), neutrophil-to-lymphocyte ratio (NLR), and platelet-to-lymphocyte ratio (PLR) (Duleba and Dokras, 2012). The increased inflammatory markers are associated with IR-related metabolic dysfunction in PCOS (Gonzalez et al., 2014). Specifically, metformin ameliorates IR and enhances clinical outcomes by reducing the expression of inflammatory markers IL-6 and TNF-α in PCOS in humans (Victor et al., 2015) and animal models (Rabah et al., 2023).

Although the exact etiology of the chronic inflammation process associated with PCOS is not known, it is believed that in adipose tissue, adipocytes and immune cells are the primary sources of cytokine release (Lonardo et al., 2024). Obesity and IR are common risk factors for inflammation, and chronic low-grade inflammation may be more severe in obese and IR women with PCOS (Gonzalez et al., 2014). Macrophages produce inflammatory cytokines that could trigger IR in metabolic tissues. The suppression of macrophages may reduce inflammatory cytokine levels, which are associated with a decrease in IR (Hyun et al., 2013). Furthermore, there is a high prevalence of excess adiposity in PCOS, characterized by enlarged adipocytes and the accumulation of macrophages within adipose tissue (Echiburu et al., 2018).

Previous studies have indicated that metformin exerts anti-inflammatory effects through the inhibition of several pro-inflammatory signaling pathways, such as MAPK, nucleotide-binding oligomerization domain (NOD)-like receptors, leucine-rich repeats (LRRs), and NLR family pyrin domain containing 3 (NLRP3) inflammasome (Jia et al., 2020; Jin et al., 2022). NF-κB is a key factor in determining the inflammatory condition in PCOS, and the activation of NF-κB leads to increased inflammation. Metformin suppresses the PI3K-AKT-NF-κB signaling pathway and reduces the expression of inflammatory genes in PCOS-like rats (Zhang et al., 2017). In addition, Toll-like receptors (TLRs) mediate inflammatory responses associated with increased interleukin accumulation and contribute to the pathogenesis of PCOS (Gu et al., 2016). Metformin treatment effectively attenuates the release of inflammatory cytokines in endometrial tissues via the TLR4/NF-κB signaling pathway (Hu et al., 2021). Additionally, metformin reduces inflammatory cytokines by decreasing leukocyte–endothelium interactions in PCOS (Victor et al., 2015).

2.3 Effects of metformin on oxidative stress in PCOS

Oxidative stress, an imbalance between oxidants and antioxidants, is caused by excessive reactive oxygen species (ROS) production. It has been suggested that ROS levels are elevated in women with PCOS (Duleba and Dokras, 2012). Mitochondrial dysfunction is the main source of ROS, and it is especially important for ovarian function and metabolic activity (Rabah et al., 2023; Wu et al., 2023; Chen et al., 2024). ROS may directly lead to IR and hyperandrogenism through the $p47^{phox}$ (a NADPH oxidase subunit) component, which is related to the response of immune cells within adipose tissue in women with PCOS (Gonzalez et al., 2019). On the other hand, ROS activates the NF-κB signaling pathway, which stimulates an inflammatory response that further increases IR and hyperandrogenism (Rudnicka et al., 2022). IR in the skeletal muscle of women with PCOS is associated with reduced expression of genes involved in mitochondrial oxidative metabolism and reduced expression of peroxisome proliferator-activated receptor γ coactivator α (PGC-1α), which interacts with AMPK or proliferator-activated receptor γ (PPARγ) and controls proteins involved in the regulation of mitochondrial function and metabolism, including oxidative phosphorylation (*OXPHOS*) gene and mitochondrial DNA (mtDNA) replication (Skov et al., 2007; Abu Shelbayeh et al., 2023).

Metformin, with its antioxidant properties, reduces ROS levels and improves mitochondrial function and insulin sensitivity (Udono and Nishida, 2022). In clinical trials, metformin therapy in PCOS women improves oxidative stress markers, including a decrease in malondialdehyde (MDA) levels and an increase in glutathione (GSH) levels and superoxide dismutase (SOD) enzyme activity (Xu et al., 2022). Moreover, metformin improves mitochondrial function and contributes to helping restore hormonal balance and insulin sensitivity in PCOS patients (Rabah et al., 2023). The PI3K/AKT/mTOR signaling pathway has been demonstrated to be the most disturbed pathway in ovarian granulosa cells (GCs) under oxidative stress and in PCOS (Abuelezz et al., 2020). Metformin decreases ROS levels, resulting in reduced autophagy via the PI3K/AKT/mTOR signaling pathway in H_2O_2-induced GCs and a PCOS rat model (Xu et al., 2022). Studies in the PCOS–IR rat model demonstrate that metformin reduces ROS levels, resulting in improved mitochondrial function and insulin sensitivity and upregulated PI3K and AKT gene expression (Rabah et al., 2023).

3 Effects of metformin on endometriosis

Endometriosis is a common gynecological condition affecting 5%–15% of women of reproductive age worldwide, leading to pain,

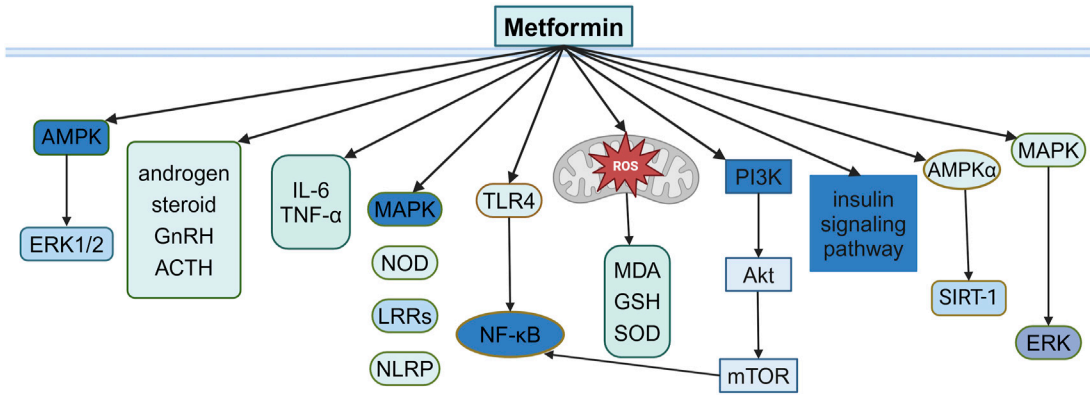

FIGURE 1
Illustration of the signaling pathway by which metformin affects PCOS.

infertility, and systemic inflammation (Taylor et al., 2021). The condition is characterized by altered lipid metabolism in the liver and adipose tissue, contributing to elevated levels of inflammatory markers such as IL-6, TNF-α, and CRP (Taylor et al., 2021). The recommended first-line treatments for endometriosis, such as nonsteroidal anti-inflammatory drugs (NSAIDs) and hormonal therapies, are often poorly tolerated due to their side effects and limited efficacy in reducing ectopic lesions (Brown et al., 2017; Peitsidis et al., 2023). Although studies in women with endometriosis treated with metformin are scarce, it has been demonstrated that metformin may avoid the main side-effects of other treatments and exert anti-inflammatory, anti-proliferative, antioxidative, and anti-angiogenic effects on endometriosis, suggesting its potential role in the management of endometriosis (Figure 2).

3.1 Effects of metformin on hormonal regulation in endometriosis

Endometriosis is a hormone-dependent condition with estrogen dependency and progesterone resistance. Elevated estrogen production and marked progesterone resistance are the key events that promote the ectopic implantation of endometrial cells (Vannuccini et al., 2022). Metformin modulates steroid hormone levels, inhibiting the secretion of follicle-stimulating hormone (FSH), LH, estradiol, progesterone, and androstenedione in ovarian GCs (Rice et al., 2013). Endometriosis is characterized by the ectopic growth of endometrial stromal cells and glands. Metformin has been shown to have inhibitory effects on the growth of progesterone-resistant endometrial epithelial cells and peritoneal adhesions in animal models (Zhuo et al., 2016; Hu et al., 2018).

Importantly, aromatase is a member of the cytochrome P450 (CYP) superfamily and induces the aromatization of androgens into estrogens. High levels of steroidogenic acute regulatory protein and aromatase are expressed in endometriotic stromal cells (ESCs), contributing to the growth of endometrial implants (Xu et al., 2014). The inhibition of aromatase is one of the targets of available and emerging drugs for endometriosis. Several findings have shown that metformin could suppress aromatase activity by inhibiting prostaglandin E2 (PGE2)-induced CYP19A1 and StAR expression in endometriotic stromal cells; thus, it appears to be effective in the medical management of endometriosis (Xu et al., 2014; Zhou et al., 2015).

3.2 Effects of metformin on inflammation in endometriosis

In patients with endometriosis, aberrant inflammatory responses are observed, with elevated levels of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, found in the serum, peritoneal fluid, and ectopic lesions (Zhou et al., 2019; Sapmaz et al., 2022). Metformin therapy may relieve the clinical symptoms of endometriosis (pelvic pain and menstrual disorders), accompanied by a reduction in the levels of inflammatory cytokines in the serum and peritoneal fluid in women with endometriosis due to its anti-inflammatory effects (Omer, 2016).

NF-κB has been extensively reported to play a role in the progression of endometriosis, particularly in lesion development, cell proliferation, and angiogenesis. In patients with endometriosis, activation of NF-κB is found in endometrial cells, macrophages, and peritoneal fluid (Liu et al., 2022), contributing to the proliferation of endometriotic cells (Wang et al., 2022) and the polarization of peritoneal macrophages (Lousse et al., 2008). In addition to inflammatory cells, other cell types, such as stromal cells, fibroblasts, and endothelial cells, could produce inflammatory cytokines and contribute to pro-inflammatory effects (Mohebbi et al., 2022). Studies on endometriosis have shown that metformin exerts potent anti-NF-κB effects, alleviating disease progression, and it has demonstrated the potential to target pro-inflammatory molecules in endometriotic cells (Takemura et al., 2007), macrophages, and vascular smooth muscle cells *in vitro* (Isoda et al., 2006), in obese mice (Hyun et al., 2013), and in rat models of endometriosis (Jamali et al., 2021; Sapmaz et al., 2022). These findings indicate the significant therapeutic potential of metformin in endometriosis management by targeting inflammatory pathways.

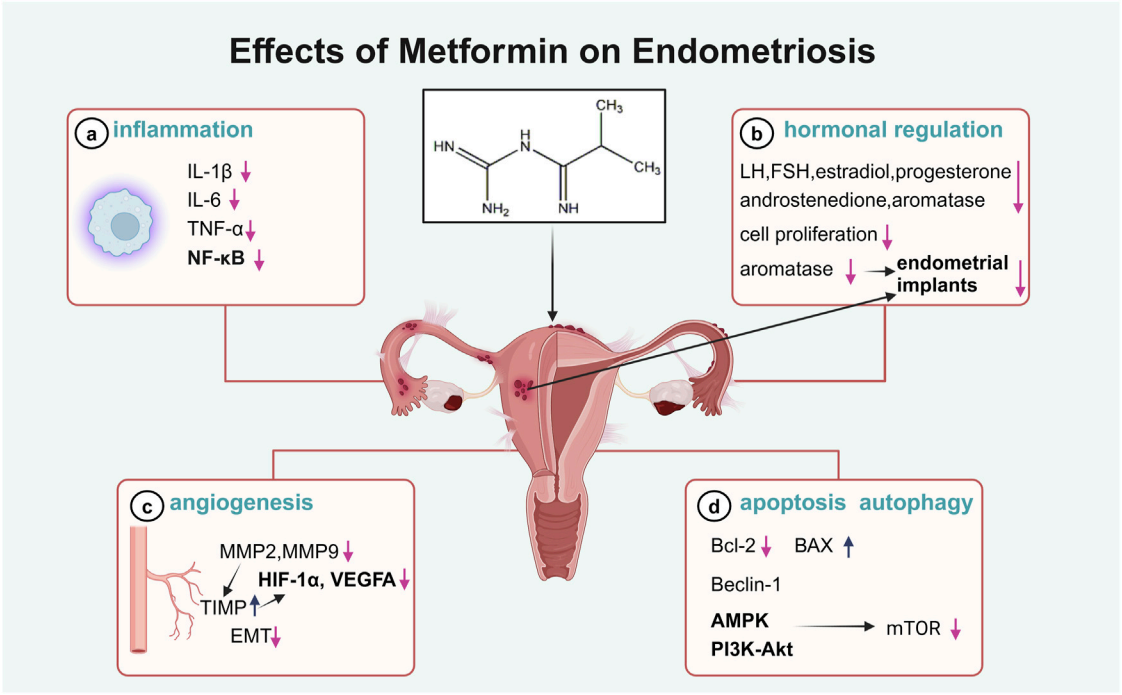

FIGURE 2
Illustration of the effects of metformin in endometriosis.

3.3 Effects of metformin on angiogenesis in endometriosis

Angiogenesis is fundamental to the growth of endometriotic tissue, which starts with the destabilization of the pre-existing vasculature, and is driven by the release of pro-angiogenic factors and the degradation of the extracellular matrix (ECM). Matrix metalloproteinases (MMPs), regulated by their endogenous inhibitors—tissue inhibitors of metalloproteinases (TIMPs) (Ruth, Day et al.) (Ruth, Day et al.)—are critical for ECM breakdown and the promotion of angiogenesis (Zafrakas et al., 2020). Higher levels of vascular endothelial growth factor (VEGF), MMPs, and reduced TIMPs have been observed in endometriotic lesions, serum, and peritoneal fluid from endometriosis patients (Zafrakas et al., 2020; Li et al., 2021) and in animal models (Lu et al., 2006; Maoga et al., 2023). Suppression of MMPs has been reported to inhibit the establishment of ectopic lesions derived from human endometrium in nude mice (Bruner et al., 1997). The potential effect of metformin on angiogenesis in endometriosis may be mediated through the regulation of MMP activity. Metformin reduces the expression of MMP2 and MMP9 and enhances TIMP expression, accompanied by increased expression of angiogenesis-related genes, such as *HIF-1α* and *VEGFA*, in human ectopic endometrial cells (Yari et al., 2021) and endometriotic implants of endometriosis rat models (Yilmaz et al., 2010; Cheng et al., 2022).

Furthermore, vascular dysfunction is associated with the upregulation of endothelin-1 (ET-1) and reduced endothelial nitric oxide synthase (eNOS) production and contributes to endothelial damage in endometriosis (Wu et al., 2003). In a mouse model of endometriosis, daily metformin for 3 months led to a significant reduction in ET-1 expression, an increase in eNOS expression, and diminished endometriosis-associated endothelial dysfunction (Martins et al., 2022; Yang et al., 2024). Studies involving metformin administration in women with endometriosis are scarce. However, it has been demonstrated that metformin treatment has an impact on the angiogenic, inflammatory, and ECM-related genes, suggesting its potential role in the regulation of angiogenesis in endometriosis.

3.4 Effects of metformin on apoptosis and autophagy in endometriosis

Endometriosis is characterized by enhanced proliferation and diminished apoptosis of endometrial cells. Endometriotic endometrial cells show an overexpression of anti-apoptotic genes such as *Bcl-2* and insufficient expression of pro-apoptotic factors such as Bax (Braun et al., 2007), and an apoptosis-inducing agent is considered to be a promising therapeutic strategy for endometriosis. Apoptosis is controlled by NF-κB transcription factors in a wide range of cell types, including endometrial cells. Metformin increases the apoptotic index by modulating the Bcl-2/Bax ratio in endometrial cells via NF-κB activation (Sapmaz et al., 2022; Wang et al., 2022).

Autophagy has been considered an efficient regulator of apoptosis (Kobayashi et al., 2024). Beclin-1 is an important autophagic factor and can interact with Bcl-2. The levels of the Beclin-1 and Bcl-2 proteins in endometriotic lesions suggest the existence of an autophagy and apoptosis dysfunction in

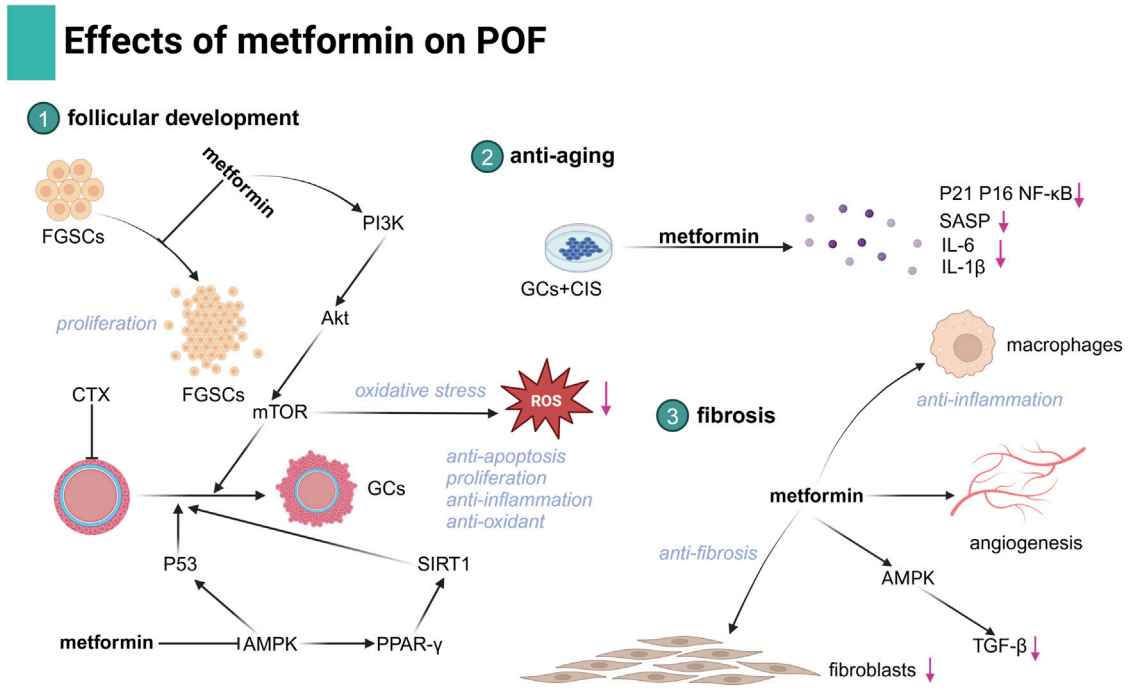

FIGURE 3
Illustration of the effects of metformin in POF.

endometriosis in humans (Li et al., 2021) and induced rodent models (Li et al., 2023). The autophagy process is associated with the PI3K/AKT/mTOR signaling pathway and downstream target molecules, including autophagy-related proteins (Kobayashi et al., 2024). mTOR is the main suppressor of autophagy (Kim and Guan, 2015; Jamali et al., 2021), and metformin has a strong inhibitory effect on mTOR by stimulating AMPK or NF-κB (Lu et al., 2021; Saber and El-Kader, 2021). In *in vitro* cultured human endometrial stromal cells (HESCs), overexpression of HIF-1α results in enhanced cell migration, invasion ability, and autophagy, and Beclin-1 and light chain 3 (LC3) are upregulated (Liu et al., 2017; Zhang et al., 2024). In endometriotic lesions of mice, the PI3K/AKT/mTOR pathway was activated, and metformin administration was associated with the enhanced autophagy process through mTOR inhibition (Jamali et al., 2021). These observations suggest an anti-endometriotic effect of metformin through autophagy-related pathways. To date, clinical studies on metformin's effects on autophagy have been conducted in patients with T2DM, PCOS, and cancer (Lu et al., 2021). Further investigation is needed to determine the pharmacological effects of metformin on endometriosis via autophagy.

4 Effects of metformin on POF

POF is an important cause of infertility characterized by the functional decline of the ovary. POF affects 3%–7% of reproductive-aged women, particularly those undergoing chemotherapy (Golezar et al., 2019). During folliculogenesis, GCs play a crucial role in supporting oocyte growth and maturation. Dysfunction of GCs is a key factor in the development of POF (Wu et al., 2023). The etiologies of POF are heterogeneous and multifactorial, and traditional therapies have limited effectiveness, benefiting only a small proportion of patients. Metformin, with its low cost and established safety profile, has demonstrated promising effects in improving ovarian function. It exerts anti-inflammatory, antioxidant, and anti-apoptotic effects, which contribute to the restoration of ovarian hormonal function. These effects suggest that metformin could be a potential therapeutic option for fertility preservation in POF, offering a novel approach for managing this condition in the near future. Despite promising results, further research is needed to optimize metformin's efficacy in POF treatment (Figure 3).

4.1 Effects of metformin on follicular development in POF

POF occurs due to the exhaustion of a number of ovarian follicle-associated endocrine dysfunctions (Wu et al., 2023). Female germline stem cells (FGSCs) are stem cells that can differentiate into mature oocytes and remodel ovarian function. Some studies have shown that replenishing exhausted FGSCs is a promising approach to restore ovarian function (Hong et al., 2022). It has been demonstrated that metformin treatment can promote FGSC proliferation and facilitate follicular maturation, thus enhancing ovarian reserve capabilities (Chen et al., 2023).

There exists abnormal GC damage in the ovary of POF patients (Lin et al., 2022). GCs play a crucial role in follicle initiation and development, and abnormal GC proliferation or apoptosis is an important factor causing POF. Steroid hormone secretion is one of the key functions of GCs. *In vitro* studies using cultured ovarian cells

have demonstrated a direct metformin effect on ovarian steroidogenesis (Kurzthaler et al., 2014). Moreover, cyclophosphamide (CTX) induces increased apoptosis and reduced cellular proliferation of GCs, and metformin can exert anti-apoptotic and cell proliferative effects against chemotoxicity via the AMPK-dependent p53 signaling pathway (Huang et al., 2021).

Recent studies using a chemotherapy- and D-galactose-induced POF mouse model have reinforced the beneficial effects of metformin in enhancing ovarian function (Du et al., 2022; Ellibishy et al., 2024). Cisplatin exposure directly induces follicle loss, while metformin treatment encourages the resumption of follicular growth and development in the ovaries, showing that primordial follicles and growing follicles are largely restored and atretic follicles are significantly reduced in cisplatin-treated ovaries. In the CTX- and D-galactose-exposed mouse model, the use of metformin is associated with improved serum hormonal levels and follicle numbers, exhibiting mTOR-inhibitory and anti-apoptotic effects (Huang et al., 2021). Additionally, the effect of metformin may also be achieved through an improvement in anti-inflammatory and antioxidant properties on GCs, and the activation of the PI3K/AKT/mTOR or AMPK/PPAR-γ/SIRT1 signaling pathway is involved (Ellibishy et al., 2024; Yang et al., 2024).

In addition to the hormonal and metabolic dysfunctions commonly associated with POF, genetic factors also play a significant role in its pathogenesis. Genetic mutations, such as in the *FMR1* gene (fragile X messenger ribonucleoprotein 1), have been linked to genetically induced POF (Ojavee et al., 2023). There is also some evidence that metformin may be effective in improving aberrant behavior and correcting electrophysiological abnormalities in patients with fragile X syndrome, but no clinical trials have tested its effects on ovary function in these patients (Proteau-Lemieux et al., 2021). Further research is needed to evaluate the therapeutic limitations and potential effects of metformin in patients with genetically induced POF. Furthermore, genetic mutations causing POF may impair follicular development and function independently of insulin or metabolic regulation, and metformin may aid in managing secondary metabolic complications.

4.2 Metformin as an anti-aging agent in POF

Metformin has been identified as an anti-aging agent in a clinical trial-TAME (targeting aging by metformin) and can relieve age-associated pathologies (Kulkarni et al., 2020). Beyond its use in cardiovascular diseases, cancer, osteoarthritis, Alzheimer's disease, and obesity, metformin could alleviate aging and age-related phenotypes by suppressing senescence through mechanisms such as redox balance, autophagy, and immune and inflammation response in the ovary (Qin et al., 2019; Lu et al., 2021; Landry et al., 2022; Yang et al., 2024).

In vitro cultures of cisplatin-treated GCs to which metformin was added have displayed a significant reduction in the expression of senescence markers (p21, p16, and NF-κB) and senescence-associated secretory phenotype (SASP) markers, IL-6 and IL-1β, and in a number of senescence-associated beta-galactosidase (SAβ-gal)-staining cells, and ultimately, improved functional and structural changes occurred in cisplatin-induced POF mice (Du et al., 2022). Oxidative stress promoting aging in GCs was considered the primary pathogenesis of POF (Lin et al., 2022). Metformin could attenuate H_2O_2-induced oxidative stress and further decrease excessive autophagy caused by oxidative stress via the PI3K/AKT/mTOR pathway in rat GCs (Xu et al., 2022). Additionally, metformin significantly reduces macrophage-induced ROS accumulation and senescence in primary GCs by inducing the expression of the AMPK pathway and protects against CTX-induced POF (Yang et al., 2024).

4.3 Effects of metformin on fibrosis in POF

POF is associated with significant changes in the structural organization of collagen, characterized by excessive deposition of the ECM, resulting in ovarian fibrosis (Pellicer et al., 2023). Metformin has been demonstrated to effectively treat and prevent fibrosis in various organs, including the lungs (Cheng et al., 2021), kidneys (Wang et al., 2016), liver (Pinyopornpanish et al., 2021), and heart (Li et al., 2023). In the fibrotic ovary, several studies have demonstrated that metformin attenuates ovarian fibrosis through the modulation of immune cells, fibroblasts, and angiogenesis, and pro-fibrotic and inflammation signaling pathways were involved, particularly in aged or high-fat diet-induce mouse models (McCloskey et al., 2020; Landry et al., 2022; Velazquez et al., 2023).

Fibrosis is associated with a pro-inflammatory cascade characterized by enhanced M1-like macrophages, reduced M2-like macrophage infiltration, and elevated pro-inflammatory chemokines, which are believed to damage GCs in aged and POF ovaries (McCloskey et al., 2020; Yang et al., 2024). Transforming growth factor-β (TGF-β) is known to promote fibrosis and contribute to POF (Persani et al., 2011), and collagen content and fibroblast proliferation are significantly reduced, which is related to AMPK-mediated suppression of TGF-β production after metformin administration (McCloskey et al., 2020). In addition, mitochondrial dysfunction in stromal cells is the key causal factor triggering fibrosis-induced ovarian decline, and metformin's reversal of ovarian fibrosis converges on related mitochondrial metabolic pathways that are upstream of oxidative stress and inflammation (Umehara et al., 2022). Ovarian fibrosis has been established in human chemotherapy-induced POF, but the effect of metformin on POF-associated murine ovarian fibrosis has yet to be extended to human POF (Meirow et al., 2007).

5 Effects of metformin on uterine fibroids

Uterine leiomyomata or fibroids are extremely common benign gynecological tumors that occur in 80% of reproductive-aged women, and they are commonly associated with heavy menstrual bleeding (Moravek and Bulun, 2015; George, 2023). Recent studies have suggested that the drug metformin might exert a beneficial effect on the management of uterine fibroids through mechanisms including anti-proliferative actions, promotion of apoptosis, and angiogenesis inhibition (Li et al., 2013; Tadakawa et al., 2015).

A population-based retrospective cohort study supports a reduced risk of uterine fibroids associated with metformin use in Taiwanese female patients with T2DM (Tseng, 2019). *In vitro*, metformin has anti-tumor properties, inhibiting proliferation and inducing apoptosis on uterine leiomyoma cells via the activation of AMPK, followed by the inhibition of the mTOR pathway (Li et al., 2013; Tadakawa et al., 2015). Metformin has also been shown to inhibit the proliferation of leiomyoma cells to reduce tumor size in clinical trials (Wang et al., 2019).

Additionally, fibroid growth is primarily dependent on the levels of circulating estrogen and the regulation of estrogen signaling (Borahay et al., 2017). 17β-hydroxysteroid dehydrogenase (17β-HSD) and aromatase are the major enzymes catalyzing the conversion of androstenedione to estrone and are found to be overexpressed in fibroid tissue than in normal myometrium, and this suggests that fibroids convert circulating androstenedione into estrone *in situ* (Sumitani et al., 2000). In contrast, metformin could suppress the expression of HSD and aromatase (P450 arom) in uterine fibroid cells and reduce estrogen synthesis, thereby inhibiting the growth of fibroids (Wang et al., 2019).

Angiogenesis and vascularization have been regarded as crucial factors controlling the growth of tumors. Multiple angiogenic factors, such as VEGF and its receptors, are overexpressed in leiomyoma tissue compared to the adjacent myometrium (Di Tommaso et al., 2013). In addition, metformin suppresses VEGF expression through the mTORC1/HIF-1α pathway, further indicating its anti-angiogenic properties in uterine fibroids (Tadakawa et al., 2015).

6 Effects of metformin on gynecologic malignancies

6.1 Ovarian cancer

Based on population-based retrospective studies and meta-analysis, there is a markedly reduced risk of ovarian cancer incidence associated with metformin use by patients with T2DM (Tseng, 2015; Shi et al., 2019). Additionally, in clinical trials, metformin treatment enhances the survival of ovarian cancer patients (Kumar et al., 2013). The anti-tumor effects of metformin on ovarian cancer are attributed primarily to its ability to inhibit cancer cell proliferation, enhance the sensitivity of cancer cells to chemotherapeutic agents, modulate the cell cycle, and promote apoptosis in cancerous cells.

Extensive research has demonstrated that metformin treatment can suppress the proliferation, chemoresistance, and metastasis of ovarian cancer cells (Rattan et al., 2011; Min et al., 2020). The anti-proliferation effect of metformin may involve p53 protein ubiquitination or the NF-κB signaling pathway (Min et al., 2020; Zheng et al., 2022). Moreover, metformin induces cell cycle arrest and apoptosis through the Bcl-2-family of proteins in primary human ovarian carcinoma cells, isolated from ascitic fluid or omental metastases (Liu et al., 2018). In addition, metformin significantly inhibits angiogenesis through downregulated VEGF activity induced by the IL-6/STAT3 or AMPK/mTOR signaling pathways (Rattan et al., 2011; Yang et al., 2021).

Furthermore, metformin enhances the anti-tumor efficacy by diminishing tumor resistance *in vivo* and *in vitro*. Several studies suggest that metformin induces growth inhibition and shows synergistic effects in overcoming chemoresistance, especially in cisplatin-resistant ovarian cancer cells (Lee et al., 2019), and the chemosensitizing effect of metformin seems to be dependent on p53 function (Han et al., 2019). Increased Bcl-2-protein family-dependent apoptosis was also linked to metformin's chemosensitizing effects in ovarian cancer (Yasmeen et al., 2011). In cisplatin-resistant ovarian cancer patient-derived xenograft models, *in vivo* treatment with metformin partially reversed platinum resistance (Ricci et al., 2019). Furthermore, metformin sensitization of drug-resistant ovarian cancer to chemotherapeutic agents is possibly through the induction of autophagy (Yang et al., 2019). These clinical and laboratory studies support the essential role of metformin in the development and growth of ovarian cancer, highlighting its potential as a therapeutic agent against ovarian cancer.

6.2 Cervical cancer

Retrospective cohort studies demonstrated that metformin use in patients with diagnosed T2DM was associated with a lower risk of cervical cancer (Kim et al., 2022). Although there is one study demonstrating no association between metformin use and the survival outcome of women with cervical cancer (Takiuchi et al., 2017), several studies revealed that metformin treatment decreased cervical cancer-specific and overall mortality in older women with T2DM (Han et al., 2016) and reduced the recurrence rate in patients with T2DM (Hanprasertpong et al., 2017).

It has been reported that metformin inhibits cell viability, migration, and metastasis and induces cell-cycle arrest, autophagy, and apoptosis in cervical cancer cell lines and cervical cancer xenografts (Cheng and Hao, 2016; Xia et al., 2018). These actions of metformin are mediated via various mechanisms, such as PI3K/AKT/mTOR inhibition and AMPK pathway activation (Xia et al., 2020; Chen et al., 2021). Metformin has been reported to inhibit heme oxygenase-1 (HO-1) expression in cervical cancer HeLa cells, contributing to the regulation of angiogenesis and cell proliferation (Do et al., 2013).

By targeting the PI3K/AKT and p53 pathways, metformin could also enhance NK cell cytotoxicity and may be used as an immunopotentiator for combination therapy with immunotherapy (Xia et al., 2020). By targeting mitochondria, metformin suppresses the malignant phenotype and metastatic potential of cervical cancer cells due to the modulation of mitochondrial respiration and glucose metabolism through the citric acid cycle (Tyszka-Czochara et al., 2017). Furthermore, metformin is found to amplify chemotherapy-induced AMPK activation and sensitize cervical cancer cells to the action of cisplatin (Tyszka-Czochara et al., 2017). These results suggest that metformin exerted a synergistic action in chemotherapy-based strategies for cervical cancer treatment.

6.3 Endometrial carcinoma

The incidence of endometrial cancer is increasing in parallel with the rising prevalence of metabolic syndrome, obesity, and T2DM (Drevinskaite et al., 2023). Metformin is used as a first-

line therapy to target risk factors that contribute to endometrial cancer, such as PCOS and obesity, due to its effects on IR and glucose metabolism. Although epidemiologic studies evaluating the relationship between metformin use and endometrial cancer risk are conflicting (Tang et al., 2017; Drevinskaite et al., 2023), other studies demonstrated that metformin could improve endometrial cancer outcomes, reducing mortality risk and prolonging overall survival of endometrial cancer patients (Xie et al., 2024).

Endometrial cancers are traditionally classified as type 1 (estrogen-dependent) and type 2 (estrogen-independent) categories, and type 1 cancers, which are usually endometrioid in histology, account for ~90% of all endometrial cancer cases . Endometrioid endometrial cancer is driven by obesity and IR (Kitson et al., 2019). Metformin improves IR, and evidence has suggested that its use is associated with improved glucose metabolism in women with endometrial cancer (Davis et al., 2018). In endometrial cancer cell cultures, metformin treatment reduces the secretion of insulin-like growth factor (IGF-1) and downregulates the expression of the insulin receptor and IGF-1R (Sarfstein et al., 2013). In addition to IGF pathways, anti-tumor effects on PI3K/AKT/mTOR and MAPK/ERK pathways with metformin exposure were observed, leading to the upregulation of markers of cell cycle arrest, apoptosis, and autophagy and downregulation of markers associated with senescence and the inhibition of cell migration (Hanna et al., 2012; Lee et al., 2018; Cao et al., 2019; Ruiz-Mitjana et al., 2023).

More importantly, metformin exhibits properties of sensitizing cancer cell to chemotherapy and hormone therapies. Progesterone is a key hormone in the endometrium that opposes estrogen-driven growth, and insufficient progesterone action strikingly increases the risk of endometrial cancer (Alhujaily et al., 2021). Glyoxalase I (GLOI) and ten–eleven translocation 1 (TET1), along with 5-hydroxymethylcytosine (5-hmC), contribute to progestin resistance and chemotherapeutic resistance in endometrial cancer (Lv et al., 2017; Alhujaily et al., 2021; Liu et al., 2021). It has been reported that metformin treatment increased the sensitivity of endometrial cells to cisplatin and paclitaxel, an effect that was associated with reduced levels of GLOI expression (Hanna et al., 2012). Additionally, metformin sensitizes progestin and exerts anti-estrogen capacity in endometrial cancer through the TET1-5hmC-GLOI signaling pathway (Jiang et al., 2019). Isocitrate dehydrogenase 1 (IDH1) is an enzyme that catalyzes isocitrate to produce α-ketoglutarate (α-KG), a substrate of TET1, which controls TET1-mediated progestin- and chemotherapy-resistant genes in endometrial cancer (Li et al., 2024). Metformin could mediate the induction of chemosensitivity resulting from the downregulation of Nrf2, leading to the inhibition of IDH1-α-KG-TET1-Nrf2 signaling (Bai et al., 2018; Jiang et al., 2019). Overall, the efficacy of metformin use in endometrial cancer is promising. However, in one multi-center, randomized phase-III trial, short-term treatment with metformin did not reduce tumor proliferation in women with endometrioid endometrial cancer (Kitson et al., 2019); therefore, clinical trials to confirm the effects of metformin on endometrial cancer are warranted (Figure 4).

7 Conclusion

Many *in vitro*, *in vivo*, and pre-clinical studies have demonstrated various effects of metformin, such as anti-diabetic, antioxidant, anti-inflammatory, anti-tumor, and cardioprotective effects in gynecological disorders, which are mainly due to the effects on the IGF system and several intracellular signaling transduction pathways, such as AMPK, PI3K/AKT/mTOR, and MAPK/ERK pathways. Although clinical studies confirm the efficacy of metformin in treating conditions such as PCOS, the

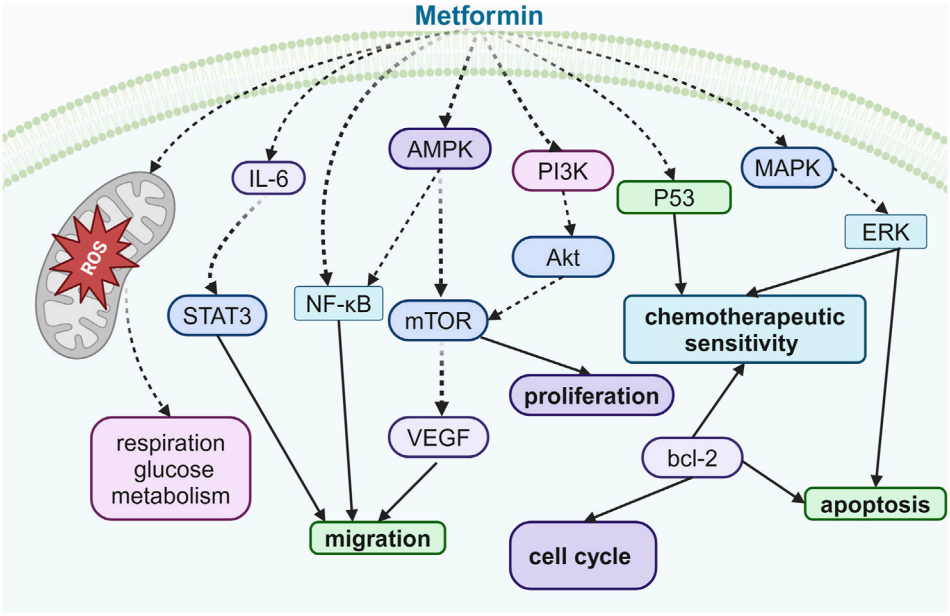

FIGURE 4
Illustration of the signaling pathway by which metformin affects ovarian, cervical, and endometrial cancers.

evidence for its role in endometriosis, POF, myomas, and gynecological cancers is limited. In terms of gynecological cancers, while some clinical trials have shown mixed results regarding metformin's anti-proliferative activity, there is emerging evidence suggesting its role in enhancing chemotherapy sensitivity and improving insulin sensitivity in cancer patients. However, the specific contribution of metformin in the treatment of endometrial cancer remains unclear, and further research is needed to clarify its potential as an adjunct to existing cancer therapies. In conclusion, while metformin has shown promise in several gynecological disorders, more targeted clinical trials are necessary to establish its role in endometriosis, POF, myomas, and gynecological malignancies.

Author contributions

PN: writing – original draft. MW: conceptualization, validation, and writing – original draft. YM: writing – original draft and formal analysis. HZ: methodology and writing – original draft. QZ: data curation and writing – original draft. GL: supervision and writing – review and editing. PLi: conceptualization, funding acquisition, and writing – original draft.

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Conflict of interest

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

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

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Randomized, double-blind, placebo-controlled pilot study of metformin as an adjunctive therapy in Parkinson's disease

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Background: Parkinson's disease (PD) is caused by the progressive loss of dopaminergic neurons in the substantia nigra. Neuroinflammation is considered a key factor contributing to the pathophysiology of PD. Current gold-standard therapies for PD provide only symptomatic relief without slowing disease progression, highlighting the need to develop new disease-modifying treatments. Metformin has been demonstrated to exert a neuroprotective role in several neurodegenerative disorders including PD.

Aim: This study aimed to clarify the role of metformin as adjuvant therapy in patients with PD.

Methods: Sixty patients with PD were divided into 2 groups (n = 30). Patients in group 1 received levodopa/carbidopa (250/25 mg) three times daily for 3 months plus placebo (Control group), while those in group 2 received levodopa/carbidopa (250/25 mg) three times daily and 500 mg metformin two times daily (Metformin group). Patients were assessed via Unified Parkinson's Disease

Rating Scale (UPDRS). The serum concentrations of toll like receptor 4 (TLR-4), α-synuclein, brain derived neurotropic factor (BDNF), and high mobility group box 1 (HMGB-1) were measured before and after treatment.

Primary outcome: The improvement in UPDRS from baseline to 3 months.

Secondary outcome: Change in the level of biological markers.

Results: The control group did not show significant difference in UPDRS when compared to their baseline value by Wilcoxon test ($P > 0.05$), meanwhile the metformin group showed significant difference when compared to before treatment by Wilcoxon test ($P < 0.05$). There were no significant differences between the two groups in UPDRS after treatment ($P > 0.05$) by Man Whitney test. However, the metformin group showed a significant decrease in TLR-4, HMGB-1, and α-synuclein along with a statistically significant increase in BDNF ($P < 0.05$) when compared to its baseline and control group. The control group did not show any significant changes in all markers when compared to their baseline.

Conclusion: While no significant differences in UPDRS scores were observed between the metformin and control groups, trends in biomarker changes suggest a potential impact of adjunctive metformin use on the underlying pathophysiology of PD. Further studies are needed to assess its effects on motor symptoms over a longer duration.

Clinical Trial Registration: identifier NCT05781711.

KEYWORDS

Parkinson disease, metformin, neuro-inflammation, α-synuclein, TLR-4

1 Introduction

Parkinson's disease (PD) is the second most common and and fastest-growing neurodegenerative disorder worldwide (Dorsey et al., 2018; Badawoud et al., 2024). The primary clinical manifestations of PD are motor symptoms, which have been attributed to the selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Cherian and Divya, 2020; Al-Kuraishy et al., 2024). The formation of intracellular proteinaceous aggregates, known as Lewy bodies—primarily composed of α-synuclein (α-Syn) —in surviving neurons is another hallmark of PD. Studies have shown that α-synuclein aggregates can induce neuronal toxicity, leading to neuronal death through various mechanisms (Cherian and Divya, 2020; Alrouji et al., 2024a; Alrouji et al., 2024b). The aggregation of α-Syn plays a pivotal role in the pathogenesis of PD and other synucleinopathies. α-Syn is a lysine-rich, soluble, and amphipathic protein that is predominantly expressed in neurons (Serratos et al., 2022; Turkistani et al., 2024). Pathogenic mechanisms affecting the structural and functional stability of α-Syn—including endoplasmic reticulum stress, Golgi complex fragmentation, dysfunctional protein degradation systems, aberrant interactions with mitochondrial membranes and nuclear DNA, altered cytoskeleton dynamics, disruption of the neuronal plasma membrane, impaired vesicular transport, and the formation of extracellular toxic aggregates—contribute to the progression of PD and other synucleinopathies (Serratos et al., 2022; Turkistani et al., 2024).

Both genetic and environmental factors play significant roles in PD risk, with genetic factors accounting for approximately 10%–15% of cases and 5%–10% have a monogenic form of the disease with Mendelian inheritance (Bogers et al., 2023; Deng et al., 2018). Neuroinflammation is considered a key factor significantly influencing the pathophysiology of PD. High mobility group box-1 (HMGB1) protein has been identified as a potential inflammatory biomarker in PD (Gan et al., 2020). Targeting key cell receptors, including advanced glycation end products (AGE) and Toll-like receptor 4 (TLR-4), mediates immune responses primarily through the stimulation of endothelial cells and macrophages (Cross et al., 2024). HMGB1 in the nucleus is translocated to extracellular target cells via passive and active release, where it interacts with the receptor for AGE. (Yuan et al., 2024). AGE is expressed on endothelial cells, monocytes, macrophages, and other cells surfaces. After combining with HMGB1, it mediated the activation of nuclear factor kappa-B (NF-kB), janus kinases (JAK), signal transducer and activator of transcription factor (STAT), and mitogen activated protein kinase (MAPK) family (Herold et al., 2007). The main receptors for HMGB1 on the surface of macrophages are TLR-2 and TLR-4, with TLR-4 playing a crucial role in neurodegenerative diseases (Paudel et al., 2020a). HMGB1 expression promotes the activation of astrocyte AGE–mitogen activated protein kinase (MAPK) signaling, which in turn promotes the expression of chemokines, cyclooxygenase 2 (COX-2), matrix metalloproteinase 9, and many other bioactive molecules especially those involved in neuroinflammation (Karuppagounder et al., 2014). A previous study showed that HMGB1 induced the expression of interleukin (IL) and other inflammatory cytokines in brain tissues (Tang et al., 2022). Expression of this neuroinflammatory cytokine promotes neuron apoptosis and increases the development and progression of neurodegenerative disease in the central nervous system (Zhang et al., 2023). HMGB1 also regulated the release of excitatory

neurotransmitters (Lin et al., 2020). It was suggested that HMGB1 also promotes the release of endogenous glutamic acid and D-aspartic acid *in vitro* from glial cells (Dai et al., 2021). In-depth research on HMGB1 has shown that HMGB1 is associated with TLR-4-mediated inflammatory response and a variety of diseases, such as sepsis, gliomas, and PD (Yang et al., 2018a). The HMGB1–TLR-4 axis is key to the inflammatory response; damaged cells and activated macrophages actively or passively release HMGB1, which induces the secretion of tumor necrosis factor-α (TNF-α), IL-6, and other inflammatory cytokines through signaling pathways. Early proinflammatory factors and HMGB1 itself promote the release of HMGB1 to form a loop, which amplifies the inflammatory response (Wang et al., 2022).

Metformin, a member of the biguanide family commonly used to treat type 2 diabetes, appears to both reduce hepatic glucose production and enhance insulin sensitivity in the liver and peripheral tissues (Alrouji et al., 2023). Metformin is widely recognized as an adenosine monophosphate-activated protein kinase (AMPK) stimulator, potentially accelerating AMPK phosphorylation at the Thr172 residue (Alrouji et al., 2023). Notably, metformin is an effective treatment for PD, significantly reducing dopaminergic neuron death and enhancing antioxidant activity (Ordovich-Clarkson et al., 2024). The neuroprotective potential of metformin has been investigated based on emerging evidence from preclinical and clinical studies (Paudel et al., 2020b; Roberts et al., 2024; Vassal et al., 2024). Regarding the underlying molecular mechanisms, metformin has been shown to inhibit α-syn phosphorylation and aggregation, prevent mitochondrial dysfunction, attenuate oxidative stress, modulate autophagy primarily via AMPK activation, and prevent neurodegeneration and neuroinflammation (Paudel et al., 2020b). Several preclinical studies have been conducted to investigate the effects of metformin in PD models (Patil et al., 2014; Lu et al., 2016; Tayara et al., 2018; Katila et al., 2017). For instance, metformin reduced dopaminergic neuronal loss and motor deficits in methylphenidate tetrahydropyridine (MPTP)-induced mouse models of PD, and attenuated α-synuclein accumulation and mitochondrial dysfunction in rotenone-treated rats (Lu et al., 2016; Katila et al., 2017). These studies highlight metformin's ability to modulate key pathological processes such as neuroinflammation, oxidative stress, and autophagic dysfunction, supporting its potential as a disease-modifying agent in PD.

In light of these findings, the present study aimed to investigate the possible protective role of metformin as added on therapy in PD based on these previous investigations.

2 Patients and methods

The study was conducted from June 2023 to August 2024 at the Neuro-Psychiatry Department of Tanta University's Faculty of Medicine. Sixty participants from the Outpatient Clinic who met the inclusion criteria were included in the study. The National Research Ethics Committee of Tanta University Faculty of Medicine approved the study under license code (36264PR198/5/23). The study design and methodology adhered to the principles of the Helsinki Declaration and its 1964 revisions. Participants were informed that they could withdraw from the study at any time.

2.1 Inclusion criteria

Participants who were 50 years of age or older, male or female, had a diagnosis of PD, and receiving Levodopa/Carbidopa medication were eligible. Patients were diagnosed according to the Movement Disorder Society Clinical Diagnostic Criteria for Parkinson's Disease (Postuma et al., 2015), which outline the key motor and non-motor symptoms required for a diagnosis, as well as exclusion criteria.

Regarding the age criterion, 50 years was selected based on epidemiological data (Mehanna et al., 2022; Ben-Shlomo et al., 2024) or the study's objectives.

1. Epidemiological Justification: PD is predominantly diagnosed in individuals over 50 years old, with the incidence increasing significantly with age. This criterion aligns with the typical age of onset in most patient populations.
2. Study Design Considerations: By focusing on individuals 50 years and older, we aimed to reduce variability in disease onset and progression often observed in younger-onset cases, which may have distinct genetic and clinical profiles. This approach also allowed for a more homogenous participant pool, enhancing the reliability of our findings.

2.2 Exclusion criteria

Exclusion criteria included secondary parkinsonism, diabetes, cardiovascular diseases, atypical parkinsonian syndromes, prior stereotactic surgery for PD, senile tremors, Wilson's disease, current cancer, as well as patients taking anti-inflammatory drugs. Pregnant and lactating females, individuals with a history of alcohol and/or drug addiction, and those with known allergies to the studied medications were also excluded.

2.3 Study design

This was a prospective, randomized, double-blind pilot clinical study aimed at determining the safety and efficacy of metformin in PD patients. The trial was registered on ClinicalTrials.gov with the identifier NCT05781711. Participants were randomly assigned to two groups (n = 30 each), as depicted in the CONSORT flow diagram in Figure 1. Metformin (El-Haggar et al., 2024) and levodopa/carbidopa (Khrieba et al., 2024) doses were based on earlier research. The dose of metformin used in our study (e.g., 1,000 mg/day in divided doses) was chosen based on both safety considerations and translational relevance, referencing prior clinical studies in non-diabetic neurological conditions (Abdelgaied et al., 2024; Halabitska et al., 2025). The 3-month follow-up period was selected based on several considerations. First, previous preclinical and clinical studies have shown that metformin exerts measurable effects on neuroinflammatory and neuroprotective markers—including TLR4, HMGB1, α-synuclein, and BDNF—within a similar or shorter duration. For example, in rodent PD models, metformin significantly modulated inflammatory pathways and mitochondrial markers within 4–8 weeks (Lu et al., 2016; Katila et al., 2017). Additionally, in

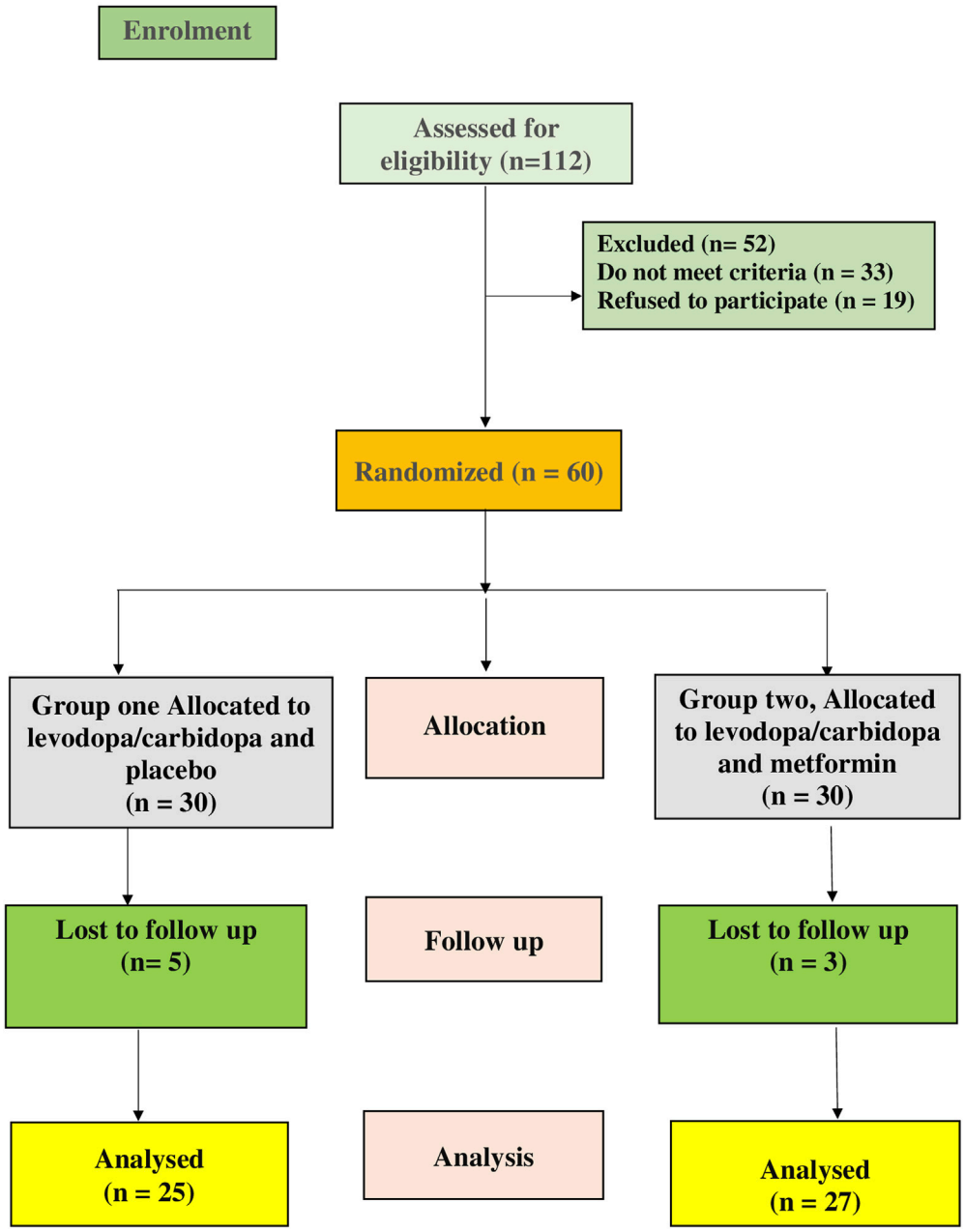

FIGURE 1
CONSORT diagram showing the flow of patients during the study.

human studies, 12 weeks of metformin treatment has been associated with significant changes in circulating cytokines (Halabitska et al., 2025; Banaszewska et al., 2011). From a clinical standpoint, a 3-month duration provides a practical balance between capturing early biological responses and maintaining high patient compliance in an elderly population that is often burdened by complex medication regimens and comorbidities.

Randomization was performed using random permuted blocks and a computer-generated random number sequence. Patients were required to discontinue all unnecessary medications, except for Levodopa/Carbidopa, for at least 2 weeks prior to trial participation.

Group 1: Control group (Levo-dopa group, n = 30) who received placebo and levodopa/carbidopa (250/25 mg) three times daily for 3 months (Sinemet^{R} tablets, Merck, Germany).
Group 2: Metformin group (n = 30) who received levodopa/carbidopa (250/25 mg) three times daily plus metformin 500 mg twice daily for 3 months (Glucophage^{R} tablets, Mina Pharm, Egypt).

2.4 Sample size calculation

No previous studies were available to estimate the actual effect size of metformin use on change in unified Parkinson disease rating

scale (UPDRS). This study was constructed as a pilot study, as recommended by Sim and Lewis (2012), who proposed a sample size of at least 55 to adequately identify small to medium effect sizes and minimizing variability. The study used a randomised sample size of 30 patients per group, with an α-error of 0.05 (2-tailed) and a power of 0.80, with an adjustment for a 10% dropout rate.

2.5 Therapeutic assessment

2.5.1 Primary outcome

The improvement in the Unified Parkinson's Disease Rating Scale (UPDRS) was the primary outcome. The UPDRS was first introduced in 1987 at the "Recent Developments in Parkinson's Disease" conference by a group of professionals in the field (Fahn, 1987). The UPDRS is designed to assess the signs and symptoms of Parkinson's disease (PD). It can be administered across multiple patient encounters to track PD progression over time. The scale consists of 42 questions, some of which have multiple parts, as well as the Hoehn and Yahr Stage and the Schwab and England Activities of Daily Living Scale. It includes subscores for the following sections: "Mentation, Behavior, and Mood," "Activities of Daily Living," "Motor Examination," and "Complications of Therapy," along with an overall UPDRS score. Both the overall score and subscores are calculated by summing the numerical responses in the respective sections. The maximum possible UPDRS score is 199, reflecting the most severe level of disability due to PD, while the lowest score is 0, indicating the absence of PD signs and symptoms (Fahn, 1987).

2.5.2 Secondary outcomes

Serum levels of biomarkers such as TLR-4, brain derived neurotropic factor (BDNF), HMGB1, and α-syn were evaluated as a secondary outcome measure to assess the therapeutic effects of drugs.

2.6 Study protocol

A neurologist evaluated the patients at baseline and 3 months after they started the medication. Patients were also questioned about drug adherence and potential adverse effects. Every 2 weeks, patients were contacted by phone to monitor their adherence to the study medication and report any side effects. All medications were administered orally. Both the type of treatment and the randomization process were kept blinded from both the patients and medical professionals. To assess patient adherence, the number of tablets remaining in each medication supply was counted. An unblinded pharmacist, who was not involved in outcome assessment, provided the study drugs to participants to ensure accurate therapy assignment. Blinding would only be broken by the responsible neurologist in the event of an emergency requiring knowledge of the current treatment. Once the blinding was broken, the patient would be withdrawn from the trial. Participants were also withdrawn if they discontinued the trial medication for seven consecutive days.

2.7 Sample collection

Ten milliliters (10 mL) of venous blood were drawn from the antecubital vein before the study began and 3 months after the intervention. The blood was carefully placed into test tubes, allowed to clot, and then centrifuged for 10 min at 4,500 g (Hettich Zentrifugen EBA 20). The serum was divided into two portions: the first was used for routine tests, and the second was stored at −80°C for biomarker analysis.

2.8 Biochemical analysis

A spectrophotometric kinetic approach was used to quantify the hepatic enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Glycated hemoglobin (HbA1c) was measured for each patient before starting the trial. Measurements of serum creatinine (SCr) levels, a marker of renal function, were performed using the Jaffé reaction.

According to the instructions of the manufacturer (Sunred, Shanghai, China), commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to measure serum levels of BDNF (catalogue no: 201-12-1303), TLR-4 (catalogue no: 201-12-0347), and α-syn (catalogue no: 201-12-1314), and HMGB-1 (catalogue no: 201-12-1636).

2.9 Statistical analysis

Prism 9 (GraphPad software, Inc., San Diego, CA, United States) was used to conduct the statistical analyses. The normal distribution of continuous variables was examined using the Shapiro-Wilk test. Significant differences within the group before and after therapy were found using Wilcoxon test for nonparametric data. To find significant variations between groups before and after therapy, unpaired Student's t-tests and Man Whitny test were performed for parametric and nonparametric data respectively. In terms of numbers, median, interquartile range and percentages, qualitative variables were provided, while quantitative values were expressed as mean and SD. On categorical data, the Chi-square test and fisher exact test were applied. All p values were two-tailed, with $p < 0.05$ considered statistically significant.

3 Results

3.1 Clinical and demographic characteristics

There were no statistically significant differences in baseline demographic data between the control and metformin groups as followed; age ($p = 0.260$), sex ($p = 0.795$), body mass index ($p = 0.805$), ALT ($p = 0.827$), AST ($p = 0.905$), SCr ($p = 0.474$), smoking ($p = 0.273$), duration of the disease ($p = 0.526$), and HbA1C ($p = 0.276$) (Table 1. Five patients were withdrawn from the control group because they developed progressive symptoms and required amantadine as an added-on therapy. Three patients were withdrawn from the metformin group because two of them were shifted to combined treatment and the remaining one did not attend to the

TABLE 1 Clinical and demographic data in the two study groups.

Parameter	Control group (n = 30)	Metformin group (n = 30)	*p*-value
Age (year)	64.80 ± 6.63	66.97 ± 8.054	0.260
Sex (M/F)	15/15	14/16	0.795
BMI (kg/m^2)	23.73 ± 1.081	22.99 ± 1.683	0.805
ALT (U/L)	21.83 ± 6.909	21.30 ± 5.459	0.741
AST (U/L)	25.37 ± 6.599	23.90 ± 7.331	0.418
SCr (mg/dL)	0.978 ± 0.166	0.948 ± 0.152	0.474
Smoking (n, %)	8 (26.6%)	12 (40%)	0.273
Duration of disease (years)	2.253 ± 0.924	2.413 ± 1.016	0.526
HA1c (%)	5.376 ± 0.246	5.287 ± 0.370	0.276

Data are expressed as mean ± SD, percentage and numbers, M, male; F, female; BMI, body mass index; ALT, Alanine amino-transferase; AST, Aspartate amino-transferase; SCr, Serum creatinine; HA1c, glycated haemoglobin. Significance at ($p < 0.05$). Differences between groups for each characteristic were tested for significance using unpaired t-test for continuous data and Chi square test for categorial data.

TABLE 2 Analysis of unified Parkinson's disease rating scale and its subscale in the two study groups.

Character	Control group (n = 25)			Metformin group (n = 27)			[b]*p* value	Effect size
	Before therapy	After therapy	[a]*p* value	Before therapy	After therapy	[a]*p* value	After therapy	Rank-biserial correlation coefficient (r)
Mentation, Behavior and Mood	12 (10-14.5)	11 (10-12.5)	0.603	13 (12-14)	10 (9-12)	0.005	0.195	0.208
Activities of Daily Living	42 (38.5-44)	39 (35-41)	0.215	41 (38-45)	36 (30-44)	0.039	0.441	0.125
Motor Examination	88 (52-96)	84 (38-90.5)	0.537	90 (63-94)	72 (57-86)	0.011	0.426	0.130
Complications of Therapy	16 (9.5-18)	13 (6.5-17)	0.144	17 (14-20)	12 (7-16)	0.007	0.705	0.062
UPDRS total score	141 (115-164)	139 (101-151.5)	0.278	152 (129-166)	128 (98-151)	0.001	0.515	0.106
Schwab and England Activities of Daily Living Scale	60 (50-70)	70 (30-90)	0.393	50 (40-60)	80 (50-90)	0.0002	0.137	0.238
Modified Hoehn and Yahr Staging	3 (2-3)	2 (1.5-3)	0.263	3 (2.5-4)	1 (1-3)	0.003	0.082	0.278

Data are expressed median, and interquartile range. Control group: patients received levodopa/carbidopa and placebo for three months, Metformin group: patients received levodopa/carbidopa for three months plus metformin for three months. UPDRS, Unified Parkinson's Disease Rating Scale. (a) within group comparison using Wilcoxon test, (b) between group comparison using Man Whitney test, Significance at ($p < 0.05$).

hospital, accordingly the statistical analysis was performed per protocol to evaluate the biological and causal effects of the treatment as shown in (Figure 1).

3.2 Analysis of unified Parkinson's disease rating scale and its subscale in the two study groups

Table 2 demonstrated no significant difference in baseline values between the two groups using Man Whitney test ($p > 0.05$).

Regarding control group, within group comparison, Wilcoxon test showed that there was no significant decrease in the median value for the following parameters when compared to baseline as followed: Mentation, Behaviour and Mood [12 (10–14.5) versus 11 (10–12.5), $p = 0.603$], Activities of Daily Living [42 (38.5–44) versus 39 (35–41), $p = 0.215$], Motor Examination [88 (52–96) versus 84 (38–90.5), $p = 0.537$], Complications of Therapy [16 (9.5–18) versus 13 (6.5–17), $p = 0.144$], and UPDRS total score [141 (115–164) versus 139 (101–151.5), $p = 0.278$]. Additionally, Wilcoxon test showed that there was no significant change in median value of the following parameters: Schwab and England Activities of Daily Living Scale [60 (50–70) versus 70 (30–90), $p = 0.393$], and Modified Hoehn and Yahr Staging [3 (2–3) versus 2 (1.5–3), $p = 0.263$] (Table 2).

Regarding metformin group, within group comparison, Table 2 revealed that the following parameters were significantly reduced by

TABLE 3 Analysis of serum biomarkers in the two study groups.

Character	Control group (n=25)			Metformin group (n=27)			[b]p value	Effect size
	Before therapy	After therapy	[a]p value	Before therapy	After therapy	[a]p value	After therapy	Rank-biserial correlation coefficient (r)
α-synuclein (ng/ml)	70 (60.35-90)	68.7 (35.40-84.59)	0.107	73 (60-81)	54.8 (26.5-66)	0.0005	0.03	0.331
BDNF (ng/ml)	4.7 (3.4-6.355)	5.2 (4.24- 8.91)	0.074	4.16 (3.28-4.7)	8.28 (5.25- 9.62)	0.003	0.02	0.367
HMGB-1 (ng/ml)	155 (143-173.5)	150 (143-173.5)	0.610	151 (103-176)	120 (75-160)	0.001	0.03	0.336
TLR4 (ng/ml)	10.48 (9.27-11.22)	9.5 (6.7-10.67)	0.140	9.6 (8.47-10.96)	5.36 (3.62-10.62)	0.0008	0.04	0.322

Data are expressed as median and interquartile range, Significance at ($p < 0.05$). Control group: patients received levodopa/carbidopa and placebo for three months, Metformin group: patients received levodopa/carbidopa for three months plus metformin for three months. Brain derived neurotropic factor (BDNF), toll like receptor 4 (TLR-4), high mobility group box protein 1(HMGB-1). (a) within group comparison using Wilcoxon test, (b) between group comparison using Man Whitney test, Significance at ($p < 0.05$).

using Wilcoxon test when compared to their baseline values as followed: Mentation, Behavior and Mood [13 (12–14) versus 10 (9–12), $p = 0.005$], Activities of Daily Living [41 (38–45) versus 36 (30–44), $p = 0.039$], Motor Examination [90 (63–94) versus 72 (57–86), $p = 0.011$], Complications of Therapy [17 (14–20) versus 12 (7–16), $p = 0.007$], and UPDRS total score [152 (129–166) versus 128 (98–151), $p = 0.001$]. Also, Wilcoxon test showed that there was a significant change in the following parameters: Schwab and England Activities of Daily Living Scale [50 (40–60) versus 80 (50–90), $p = 0.0002$], and Modified Hoehn and Yahr Staging [3 (2.5–4) versus 1 (1–3), $p = 0.003$] (Table 2).

Between group comparison, Man Whitney test showed that there were no statistically significant changes in UPDRS and its subscale after 3 months of intervention between the two groups, as followed: Mentation, Behaviour and Mood ($p = 0.195$), Activities of Daily Living ($p = 0.441$), Motor Examination (p = 0.426), Complications of Therapy ($p = 0.705$), UPDRS total score ($p = 0.515$), Schwab and England Activities of Daily Living Scale ($p = 0.137$), and Modified Hoehn and Yahr Staging ($p = 0.08$) (Table 2).

3.3 Analysis of serum biomarkers in the two study groups

Table 3 demonstrated no statistically significant difference in baseline values between the two groups using Man Whitney test ($p > 0.05$).

Regarding control group, within group comparison, Wilcoxon test demonstrated that there was no significant change in median value of the following parameters when compared to baseline as followed: α-syn [70 (60.35–90) versus 68.7 (35.40–84.59), $p = 0.107$], HMGB-1 [155 (143–173.5) versus 150 (143–173.5), $p = 0.610$], TLR-4 [10.48 (9.27–11.22) versus 9.5 (6.7–10.67), $p = 0.140$], and BDNF [4.7 (3.4–6.355) versus 5.2 (4.24–8.91), $p = 0.074$].

Regarding metformin group, within group comparison by Wilcoxon test, Table 3 revealed that the following parameters were significantly reduced when compared with their baseline values as followed: α-syn [73 (60–81) versus 54.8 (26.5–66), $p = 0.0005$], HMGB-1 (151 (103–176) versus 120 (75–160), $p = 0.001$], and TLR-4 [9.6 (8.47–10.96) versus 5.36 (3.62–10.62), $p = 0.0008$], as well as a significant increase in BDNF [4.16 (3.28–4.7) versus 8.28 (5.25–9.62), $p = 0.0003$].

Between group comparison, Man Whitney test showed that there were statistically significant changes in all studied markers after 3 months of intervention, as followed: α-syn ($p = 0.03$), TLR-4 ($p = 0.04$), BDNF ($p = 0.02$), and HMGB-1 ($p = 0.03$).

3.4 Analysis of drug-related adverse effects between the groups

Table 4 showed that there were no significant differences between the studied groups in terms of side effects as followed: nausea ($p = 0.844$), vomiting ($p = 0.705$), diarrhea ($p = 0.669$), hypotension ($p = 0.278$), delusions ($p = 0.705$), and abdominal pain ($p = 0.423$).

4 Discussion

To our knowledge, this is the first clinical research to investigate the neuroprotective role of metformin in PD and explore its mechanistic pathways in this neurodegenerative disorder.

Drug repurposing, also known as drug repositioning, is a promising approach for identifying new therapeutic uses for already approved medications. This strategy has demonstrated success in managing various conditions, such as PD, depression, non-alcoholic fatty liver disease, ulcerative colitis, breast cancer, inflammatory disorders, and colorectal cancer (Jarada et al., 2020; Pushpakom et al., 2019; Aldossary et al., 2024; Alarfaj et al., 2024; Alarfaj et al., 2023; Shawky et al., 2022; El-Haggar et al., 2022).

In the current study, the metformin group significantly decreased UPDRS and its subscale when compared to their baseline values. Levodopa/carbidopa is the cornerstone in the management of patients with PD. Adding metformin to the standard therapy reduced UPDRS scores; however, the change

TABLE 4 Comparison of drug-related adverse effects between the groups.

Side effect	Control group (n = 25)	Metformin group (n = 27)	*p* value
Nausea	5	6	0.844
Diarrhoea	2	4	0.669
Vomiting	3	5	0.705
Hypotension	5	9	0.278
Delusions	3	5	0.705
Abdominal pain	5	8	0.423

Control group: patients received levodopa/carbidopa for 3 months, metformin group: patients received levodopa/carbidopa for 3 months plus metformin for 3 months. Data were presented as numbers. Significance at ($p < 0.05$) using Chi square or fisher exact test as appropriate.

was not statistically significant compared to monotherapy. Although the metformin group showed significant changes in biomarkers, the differences in total UPDRS scores between the two groups did not reach statistical significance. Potential reasons for non-significant changes in UPDRS may include short follow-up period as the 3-month duration of the study may have been insufficient to observe significant clinical symptom improvements, as motor symptoms often progress slowly and may require a longer time to respond to interventions. The UPDRS, while widely used, may not be sensitive enough to detect subtle or early improvements in motor symptoms, particularly over a short duration. This limitation could obscure potential clinical benefits associated with biomarker changes. Also, individual variability such as differences in disease severity, progression rates, and response to treatment among participants could contribute to variability in UPDRS outcomes, potentially diluting the statistical significance. The disconnection between biomarkers and clinical symptom changes may also due to biomarker lag effect as improvements in biomarkers may precede observable clinical symptom changes, reflecting underlying disease-modifying effects that require more time to translate into functional improvements. Finally, complex pathophysiology of PD as PD involves multifactorial mechanisms, and changes in specific biomarkers may not directly correspond to symptomatic relief due to compensatory or unrelated pathways affecting clinical outcomes.

The current study demonstrated that the metformin group significantly reduced serum α-synuclein levels compared to both baseline and the control group. These findings are consistent with other research (Katila et al., 2017; Saewanee et al., 2021). According to Pérez-Revuelta et al., metformin reduces levels of Ser-129 phosphorylated α-syn by activating mammalian target of rapamycin (mTOR)-dependent protein phosphatase 2A (Pérez-Revuelta et al., 2014). By reducing lipid peroxidation, Ozbey et al. demonstrated that metformin decreased the levels of α-syn in rotenone-induced dopaminergic neurotoxicity (Ozbey et al., 2020). Metformin, acting independently of the pro-survival kinase and without stimulating the autophagic response, restored AMPK activity and reduced the *in vitro* neurotoxicity associated with α-synuclein overexpression (Dulovic et al., 2014). AMPK-dependent protection against extracellular α-syn was also demonstrated in the rat neuron-like pheochromocytoma cell line (PC12) (Dulovic et al., 2014; Jardim et al., 2018).

Several studies have established a substantial association between changes in blood biomarkers and clinical outcomes in PD, giving compelling evidence for their potential involvement in disease progression monitoring. Stewart et al. found a strong correlation between α-syn levels and the UPDRS. They also observed that cerebrospinal fluid (CSF) α-syn levels increased over approximately 2 years of disease progression in the Deprenyl and Tocopherol Antioxidative Therapy of Parkinsonism (DATATOP) cohort. These findings suggest that α-synuclein levels are associated with disease severity and clinical outcomes (Stewart et al., 2015; Stewart et al., 2014).

Ju-Hee Kang and colleagues developed a multivariate logistic regression (MLGR) model to examine the association between CSF biomarkers and PD diagnosis. As an initial step, they conducted a bivariate analysis of each CSF biomarker and PD clinical features, adjusting for confounders such as age, sex, and education. This analysis revealed significant associations between CSF T-tau ($P = 0.02$), P-tau-181 ($P = 0.005$), and α-syn ($P = 0.04$) with PD diagnosis (Kang et al., 2013). Also, Longitudinal changes in α-syn species reflect PD progression (Majbour et al., 2016).

The current study also revealed a significant decrease in the serum levels of TLR-4 and HMGB-1 in the metformin group compared to both their baseline values and the control group. These findings are consistent with previous studies (Qu and Qu, 2019; Alomar et al., 2021; Liu et al., 2024). It has also been reported that HMGB1 and TLR-4 expression levels were higher in the peripheral blood of patients with PD compared to healthy volunteers. PD patients with poor treatment outcomes exhibited significantly higher levels of HMGB1 and TLR-4 expression than those with stable treatment outcomes. Elevated HMGB1 and TLR-4 expression levels were observed in patients at more advanced stages of PD, and patients with a disease duration longer than 4 years showed significantly higher expression levels of HMGB1 and TLR-4 than those with a disease duration of less than 4 years (Yang et al., 2018b). High expression of the HMGB1–TLR-4 axis is crucial for the diagnosis and treatment of PD and is strongly associated with the onset, progression, treatment efficacy, staging, and duration of the disease (Yang et al., 2018b).

Alomar et al. reported that metformin suppresses TLR-4/NF-κB expression and glutamate excitotoxicity (Alomar et al., 2021). Metformin has also been shown to inhibit acute neutrophil activation and recruitment through an AMPK-dependent mechanism (Ashayeri Ahmadabad et al., 2024). Furthermore, it has been shown that intercellular adhesion molecule 1 (ICAM-1) expression is regulated by NF-κB, and metformin reduces ICAM-1 expression, which in turn reduces TLR-4 (Liu et al., 2021). Co-treatment with the natural HMGB1 inhibitor Glycyrrhizin exerts neuroprotection and reverses PD-like pathology (Ren et al., 2022). Metformin directly binds the alarmin HMGB1 and inhibits its proinflammatory activity (Horiuchi et al., 2017). Metformin also alleviates HMGB1-mediated oxidative stress through the mTOR pathway in experimental periodontitis (Sun et al., 2023). Metformin ameliorates doxorubicin-induced cardiotoxicity by targeting the HMGB1/TLR4/NLRP3 signaling pathway in mice (Alzokaky et al., 2023).

The current study demonstrated that metformin combined with levodopa/carbidopa therapy significantly increased BDNF compared to both the baseline value and the control group. These findings are consistent with previous studies (Katila et al., 2017; Houshmand et al., 2019). According to research by Katila et al., metformin boosts neurotrophic factor levels in the methylphenidate-tetrahydropyridine (MPTP) animal model of PD (Katila et al., 2017). According to Miyoshi et al., significant behavioral improvements were observed following the administration of a neurotrophic factor, when comparing the levodopa dose-response before and after therapy (Miyoshi et al., 1997). Additionally, parkinsonian animals treated with levodopa/carbidopa alone experienced side effects as dystonias, dykinesias, vomiting, and stereotypical movements (Miyoshi et al., 1997). These levodopa-induced side effects were greatly decreased by the administration of neurotrophic factor along with levodopa/carbidopa, with a >90% reduction in adverse reactions observed at the mid-levodopa/carbidopa dose level (250 mg levodopa-25 mg carbidopa) (Miyoshi et al., 1997). Thus, combining metformin, a neurotrophic factor upregulator, with levodopa/carbidopa treatment may be therapeutically beneficial in treating parkinsonism by improving functional response and reducing adverse effects of levodopa/carbidopa. Additionally, by increasing BDNF and generating neurotrophic factors, metformin-induced AMPK activation promotes remyelination. According to studies by Paintlia et al., metformin treatment enhanced the production of BDNF in rats with experimental autoimmune encephalomyelitis (EAE) (Paintlia et al., 2013). A previous research regarding metformin suggests that metformin enhances neurogenesis by stimulating an atypical Protein kinase C-CREB-binding protein (PKC-CBP) pathway (Wang et al., 2012), which play fundamental role in neurodevelopment, neuroprotection, and synaptic plasticity (Sakamoto et al., 2011).

Since each drug is metabolized by a distinct isoenzyme, it is noteworthy that no pharmacokinetic interactions between metformin and levodopa/carbidopa have been documented (Gong et al., 2012; Contin and Martinelli, 2010). Furthermore, there were no significant differences in the baseline clinical data between the patients. Since these differences cannot explain the variations in therapeutic responses between the groups, the therapeutic benefits are most likely due to the effects of the combined treatments.

Despite the promising results of the current study, some studies on type 2 diabetes mellites patients with high doses and long-term metformin therapy reported that metformin may increase the risk of PD by inducing hyperhomocysteinemia and deficiency of folate and vitamin B12 (Infante et al., 2021; Alrouji et al., 2024c; Tiwari et al., 2023). Long-term metformin use has been associated with reduced vitamin B12 absorption, potentially leading to deficiency (Infante et al., 2021). This condition can cause neurological and hematological complications, which are particularly concerning in populations already at risk for neurodegenerative diseases. We recommend routine monitoring of vitamin B12 levels in long-term users to mitigate these risks. Thus, further studies are required to validate these conflicting results. A secondary effect of vitamin B12 deficiency is the elevation of homocysteine levels, which may contribute to vascular complications (Mohan et al., 2023). While the clinical relevance of this in the context of metformin use is still under investigation, we acknowledge this as a potential risk and suggest that future studies should explore its implications more thoroughly.

Furthermore, the variability in metabolic rates among participants could affect biomarker levels, particularly those associated with energy expenditure and metabolic processes. While we did not measure basal metabolic rates directly, we acknowledge this limitation and suggest it as a point for further investigation in future studies. Also, Differences in dietary intake, including macronutrient composition and caloric consumption, could influence certain biomarkers. Dietary habits that may influence biomarkers in Parkinson's disease (PD) include a generally healthy diet, the protein-restricted diet (PRD), the ketogenic diet (KD), the Mediterranean diet (MD), and the Mediterranean-DASH Intervention for Neurodegenerative Delay (MIND) diet (Knight et al., 2022). Although dietary habits were not controlled in this study, we have emphasized the need to account for these variables in subsequent research to reduce their confounding effects.

Moreover, there were some limitations that included short duration period, small sample size, and lack of different doses of metformin to determine the optimum dose. We recognize that certain effects of metformin, particularly those involving neuroprotective mechanisms or disease-modifying properties, may take longer to manifest. A longer follow-up would provide a more comprehensive evaluation of treatment outcomes and help assess sustained effects on PD progression. To address this limitation, we have emphasized the need for future studies to incorporate follow-up periods extending beyond 6 months to 1 year or more. Such studies could provide a more detailed understanding of metformin's long-term impact on PD motor symptoms, biomarkers, and overall disease trajectory.

While our current study focused on key biomarkers related to inflammation (TLR-4, HMGB-1), neurotrophic support (BDNF), and protein aggregation (α-synuclein), we recognize the value of broadening this panel. Future studies will consider markers of oxidative stress, mitochondrial function, and synaptic integrity to provide a more comprehensive understanding of metformin's mechanisms in PD.

The present study is a monocentric study performed on a Middle East population. Accordingly, the benefit observed in this study should be verified in multicenter studies and in other ethnic groups, we recommend large scale and different doses clinical trials to validate these results. It would have been advisable to assess lipid profile, vitamin B12, and blood glucose also at the end of the study. Furthermore, conducting longer-term studies to assess whether biomarker improvements eventually result in clinical symptom changes. Investigating additional factors, such as participant heterogeneity and interaction between biomarkers and clinical features, to better understand the biomarker-symptoms relationship.

5 Conclusion

While no significant differences in UPDRS scores were observed between the metformin and control groups, trends in biomarker changes suggest a potential impact of adjunctive metformin use on

the underlying pathophysiology of PD. Metformin could alleviate inflammatory and oxidative stress biomarkers by modulation of HMGB-1/TLR-4, and α-syn signaling pathways. Further clinical trials are required to confirm the benefits and safety profile of metformin in PD.

Data availability statement

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

Ethics statement

The studies involving humans were approved by the Tanta University, Faculty of Medicine, Tanta 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

HA: Conceptualization, Funding acquisition, Software, Writing – review and editing. MB: Funding acquisition, Methodology, Supervision, Writing – original draft. TE: Data curation, Funding acquisition, Methodology, Writing – review and editing. EE: Data curation, Funding acquisition, Methodology, Resources, Writing – original draft. FK: Conceptualization, Funding acquisition, Investigation, Supervision, Writing – original draft. RE: Funding acquisition, Investigation, Methodology, Project administration, Software, Writing – review and editing. YE: Funding acquisition, Software, Supervision, Writing – review and editing. MK: Funding acquisition, Methodology, Project administration, Writing – review and editing. WN: Funding acquisition, Investigation, Resources, Writing – review and editing. AH: Data curation, Formal Analysis, Funding acquisition, Writing – review and editing. KA: Conceptualization, Funding acquisition, Software, Writing – review and editing. MS: Formal Analysis, Funding acquisition, Methodology, Writing – original draft. MY: Data curation, Funding acquisition, Software, Visualization, Writing – original draft. ME: Data curation, Funding acquisition, Investigation, Project administration, Writing – review and editing. MH: Data curation, Investigation, Methodology, Writing – review and editing. NE: Funding acquisition, Investigation, Software, Supervision, Validation, Writing – original draft. MEE: Software, writing – review and editing. MA: Funding acquisition, Software, Visualization, Writing – original draft, Writing – review and editing.

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Conflict of interest

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

Publisher's note

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Systematic review and meta-analysis of current guidelines, and their evidence base, on risk of renal function after administration of contrast medium for diabetic patients receiving metformin

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Purpose: Our study aimed to determine through a meta-analysis whether continuing metformin use in diabetic patients receiving contrast agents would increase the risk of renal impairment and metabolic abnormalities.

Methods: We searched the PubMed, EBSCO, Medline, and the Cochrane Central Register of Controlled Trials from the inception dates to March 2024. The included studies comparing metformin users and non-users during contrast agent administration in diabetic patients. Outcome measures included contrast-induced acute kidney injury (CI-AKI), serum creatinine, estimated glomerular filtration rate (eGFR), lactate level, and incidence of metabolic acidosis. We used odds ratio (OR) for dichotomous outcomes and weighted or standardized mean difference (WMD or SMD) for continuous outcomes, depending on scale consistency across studies.

Results: Analysis involved 2 randomized controlled trials and 5 retrospective cohorts comprising 2020 patients. There were no significant differences between the metformin and non-metformin groups in CI-AKI incidence (OR: 0.87, 95% CI: 0.63–1.20), changes in renal function (serum creatinine: SMD: −0.15, 95% CI: −0.64–0.35; eGFR: WMD: 3.35, 95% CI: −1.60–8.29), incidence of metabolic acidosis (OR: 0.90, 95% CI: 0.57–1.43), and lactate levels (SMD: 0.29, 95% CI: −0.53–1.11). Sensitivity analysis excluding one study revealed a significant reduction in creatinine with metformin. Logistic regression meta-analysis showed that metformin use was not significantly associated with CI-AKI or metabolic acidosis, while contrast volume was the only consistent predictor of CI-AKI. Lower baseline CO_2 was independently associated with increased risk of metabolic acidosis.

Conclusions: Our analysis indicates that continuing metformin during contrast agent administration does not increase the risk of CI-AKI, acidosis, or

eGFR compared to discontinuation or non-use of metformin. Additionally, continuation of metformin may be associated with a modest reduction in serum creatinine levels after contrast exposure. However, the limited quality of included studies may weaken the strength of these conclusions.

Systematic review registration: https://www.crd.york.ac.uk/PROSPERO/view/CRD42023459602, identifier: CRD42023459602.

KEYWORDS

contrast agent, diabetic, renal function, CI-AKI, lactate

Introduction

Type 2 diabetes mellitus (T2DM) represents one of the most significant health challenges of the twenty-first century, with a global prevalence on the rise. Data from the IDF Global Diabetes Map indicates that in 2025, the global adult diabetes population reached 589 million (11.1%) (1). T2DM can directly or indirectly lead to other more severe conditions, with ∼30–40% of diabetic patients progressing to chronic kidney disease (2). Diabetic kidney disease stands as one of the leading causes of mortality among T2DM patients and is a primary contributor to end-stage renal disease worldwide, posing immense risks (3). Metformin serves as a frontline therapeutic agent for T2DM. In patients with preserved renal function, metformin does not exert direct nephrotoxic effects; however, in the presence of renal impairment, metformin can elevate blood lactate levels by inhibiting lactate metabolism, thereby resulting in metformin-associated lactic acidosis, with mortality rates reaching 30–50% (4). Additionally, patients seemingly with normal renal function but at risk of acute kidney injury (AKI), such as those with volume depletion, heart failure, sepsis, or exposure to nephrotoxic agents, also face an increased likelihood of developing metformin-associated lactic acidosis. Metformin increases lactate concentration primarily through its inhibitory effect on mitochondrial respiratory chain complex I, leading to reduced oxidative phosphorylation and a shift toward anaerobic glycolysis, thereby promoting lactate production (5). Additionally, metformin suppresses hepatic gluconeogenesis, particularly through inhibition of mitochondrial glycerophosphate dehydrogenase, leading to decreased hepatic lactate uptake and clearance (6). These effects are generally well-tolerated in patients with normal renal and hepatic function. However, in individuals with impaired renal function or tissue hypoxia, the reduced clearance and increased production of lactate can synergistically increase the risk of metformin-associated lactic acidosis (7). With the widespread use of contrast media (CM) in diagnostic and interventional procedures, contrast-induced acute kidney injury (CI-AKI) is becoming increasingly prevalent. While CI-AKI incidence ranges from 12 to 27% in the general population, the rate increases to 50% or more in patients with multiple risk factors (8). Consequently, early expert consensus and guidelines strongly recommend discontinuing metformin prior to CM use in T2DM patients (9, 10). However, emerging research suggests that the role of metformin in lactic acidosis may be overemphasized, with most complications related to T2DM serving as the primary culprits (11–13). In recent years, several guidelines regarding metformin have been revised. For instance, the American College of Radiology (ACR) revised its 2015 version recommendations for patients with an estimated glomerular filtration rate (eGFR) of 30–60 mL/min/1.73 m^2. Previous versions advised discontinuation within 48 h post-surgery, while the new version (2023 edition) suggests continuation without cessation (9, 10, 14). While these updates are promising, they lack a strong evidence base, often relying on limited or outdated studies. This gap highlights the need for a comprehensive and up-to-date evaluation of the available evidence. Therefore, we conducted this study to rigorously assess whether discontinuing metformin prior to imaging in diabetic patients provides direct evidence on how to manage metformin use for T2DM patients undergoing CM administration. Our study aims to fill the gaps in the existing guidelines and provide more definitive evidence to guide clinical practice.

Methods

The study conforms to the principles outlined in the Handbook of the Cochrane Collaboration (15), along with the guidelines established by the PRISMA statement (16). The protocol for this meta-analysis was registered on PROSPERO (Registration No: CRD 42023459602).

Inclusion criteria

(1) Study compared patients with diabetes who were using metformin vs. those who were not using metformin during the administration of contrast agents;
(2) Study focused on relevant outcome;
(3) Diabetic patients (including T1DM & T2DM).

Exclusion criteria

(1) Studies if they were letters, case reports, reviews, animal trials, or republished studies;
(2) Articles with missing data;
(3) Non-diabetic patient;
(4) Immunological diseases.

Outcomes

The primary outcome was CI-AKI after the recent contrast medium injection. Secondary outcomes were serum creatinine,

eGFR, lactate level, and incidence of metabolic acidosis after contrast medium used.

Search strategy

Two of the authors performed the search in PubMed, EBSCO, Medline, and the Cochrane Central Register of Controlled Trials from the inception dates to March 2024, using the keywords "metformin" and "contrast medium" and "diabetes" and "(renal function OR serum creatinine OR eGFR OR lactate level OR metabolic acidosis)".

Data collection process

Two investigators used a standard data extraction form to extract all related data from selected studies independently. Data extracted included the first author's name, year of publication, country, type of study, sample size, age, and outcomes. Disagreements were resolved by consensus.

Assessment of risk of bias and quality of evidence

Two researchers independently assessed the quality of all included studies based on Cochrane risk-of-bias criteria or the Newcastle–Ottawa scale (NOS) (17, 18).

Data synthesis

The meta-analysis used Stata (version 17). Heterogeneity was assessed by the Q-test and I^2-value. A random effects model was applied. Odds ratios (OR) with 95% CI were used for dichotomous outcomes. For continuous outcomes, weighted mean differences (WMDs) were used when measurement scales were consistent across studies, and standardized mean differences (SMDs) were applied when different scales were used. Statistical significance was set at $P < 0.05$.

Results

A total of 232 potentially relevant articles were retrieved. After excluding 102 duplicate articles, a review of titles and abstracts of the remaining 130 articles led to the exclusion of 120 articles. Upon full-text reading of the remaining 10 articles, 4 articles were further excluded (2 clinical trials with no results, 1 article not using CM, and 1 article lacking a control group). Additionally, 1 previously conducted systematic review article was included, resulting in a total of 7 studies meeting our eligibility criteria (19–25). These studies comprised 2020 diabetic patients, among whom 893 continued metformin use during CM examinations, while 1,127 did not. The included 7 studies consisted of 2 RCTs and 5 retrospective cohort studies, with basic information about the included studies detailed in Table 1. Results indicated generally high quality across the included studies. The study flow diagram is depicted in Figure 1.

CI-AKI

In the 7 studies, 6 studies described the incidence rate of CI-AKI (19–21, 23–25). Their results consistently found that the risk of developing CI-AKI after CM administration was not associated with the use of metformin. The pooled analysis showed that there was no statistically significant increase in the risk of CI-AKI among patients continuing metformin use (OR: 0.87, 95% CI: 0.63–1.20, $I^2 = 20.9\%$, $P = 0.404$; Figure 2).

Serum creatinine

A total of 5 studies reported serum creatinine levels (19, 22–25) among which 3 studies found that patients continuing metformin use showed decrease serum creatinine levels compared to the non-metformin group after receiving CM (19, 22, 25), while 1 studies found no difference between the two groups (23). In contrast, one study reported that continuing metformin was associated with increased serum creatinine levels (24). The pooled analysis indicated that there was no difference in creatinine levels between diabetic patients continuing metformin use and those not using metformin after CM (SMD: −0.15, 95% CI: −0.64–0.35, $I^2 = 95.3\%$, $P = 0.562$; Figure 3A). However, when the study by Yu et al. (24) was excluded in sensitivity analysis due to its methodological heterogeneity and large contribution to between-study variance, the results changed notably. The updated pooled analysis based on the remaining four studies showed a statistically significant reduction in serum creatinine levels in the metformin group (SMD: −0.38, 95% CI: −0.58 to −0.18; $I^2 = 63.5\%$, $P < 0.001$; Figure 3B).

eGFR

Four studies reported on the eGFR situation (22–25). Among them, three studies found that the eGFR values in the metformin group were higher than those in the control group. However, Zeller et al.'s (25) study found that the eGFR values in the metformin group were lower than those in the group that discontinued metformin after receiving contrast agents. The pooled analysis showed that there was no statistically significant difference in eGFR change between patients continuing metformin use and those discontinuing metformin before and after contrast imaging (WMD: 3.35, 95% CI: −1.60–8.29, $I^2 = 86.6\%$, $P = 0.184$; Figure 4).

Metabolic acidosis

Among the 7 studies, 2 studies reported the results of metabolic acidosis (19, 21). Both studies found no significant correlation between continuing metformin use during iodine contrast agent administration and metabolic acidosis. The pooled analysis indicated that there was no statistically significant increase in the risk of metabolic acidosis among patients continuing metformin use (OR: 0.90, 95% CI: 0.57–1.43, $I^2 = 40.6\%$, $P = 0.661$; Figure 5). To better understand patient-related risk factors for lactic acidosis, we further summarized baseline data from the included studies in Table 2. Key variables such as liver cirrhosis

TABLE 1 Characteristics of included studies.

Study	Design	Country	Participants	Duration	Treatment group	Control group	No. of subjects		Age		Outcomes	Risk of bias assessment tool (RCTs) or NOS (observational)
							Metformin	Non-metformin	Metformin	Non-metformin		
Jung et al. (19)	RCS	Korea	T2DM	2015–2017	Metformin	Other oral hypoglycaemic agents	157	217	72.9 ± 9.1	72.5 ± 10.0	CI-AKI, LA, Scr, eGFR, metabolic acidosis	6
Kalkan et al. (20)	RCS	Turkey	Diabetic patients	2014–2019	Metformin	Non-metformin	148	195	61.3 ± 11.9	63.7 ± 12.5	CI-AKI	7
Kim et al. (21)	RCS	Korea	Diabetic patients	2012.01–2012.12	Metformin	Other oral hypoglycaemic agents	105	112	67.9 ± 10.6	65.3 ± 12.5	CI-AKI, Scr, metabolic acidosis	7
Namazi et al. (22)	RCT	Iran	Diabetic patients	2012.02–2012.11	Metformin	Non-metformin	83	79	61.5	60.1	LA, Scr, eGFR	High
Oktay et al. (23)	RCT	Turkey	T2DM	2016.01–2016.12	Metformin	Non-metformin	134	134	59.4 ± 7.7	61.4 ± 6.5	CI-AKI, LA, Scr, eGFR	High
Yu et al. (24)	RCS	China	T2DM	2008–2018	Metformin	Non-metformin	119	165	NA	NA	CI-AKI, Scr, eGFR	8
Zeller et al. (25)	RCS	France	T2DM	2001–2010	Metformin	Non-metformin	147	225	61 ± 11	65 ± 13	CI-AKI, Scr, eGFR	7

RCS, retrospective cohort study; RCT, Randomized Controlled Trial; NOS, Newcastle–Ottawa scale; LA, lactate; CI-AKI, contrast-induced acute kidney injury; eGFR, estimated glomerular filtration rate; Scr, serum creatinine; T2DM, Type 2 diabetes mellitus; NA, not applicable.

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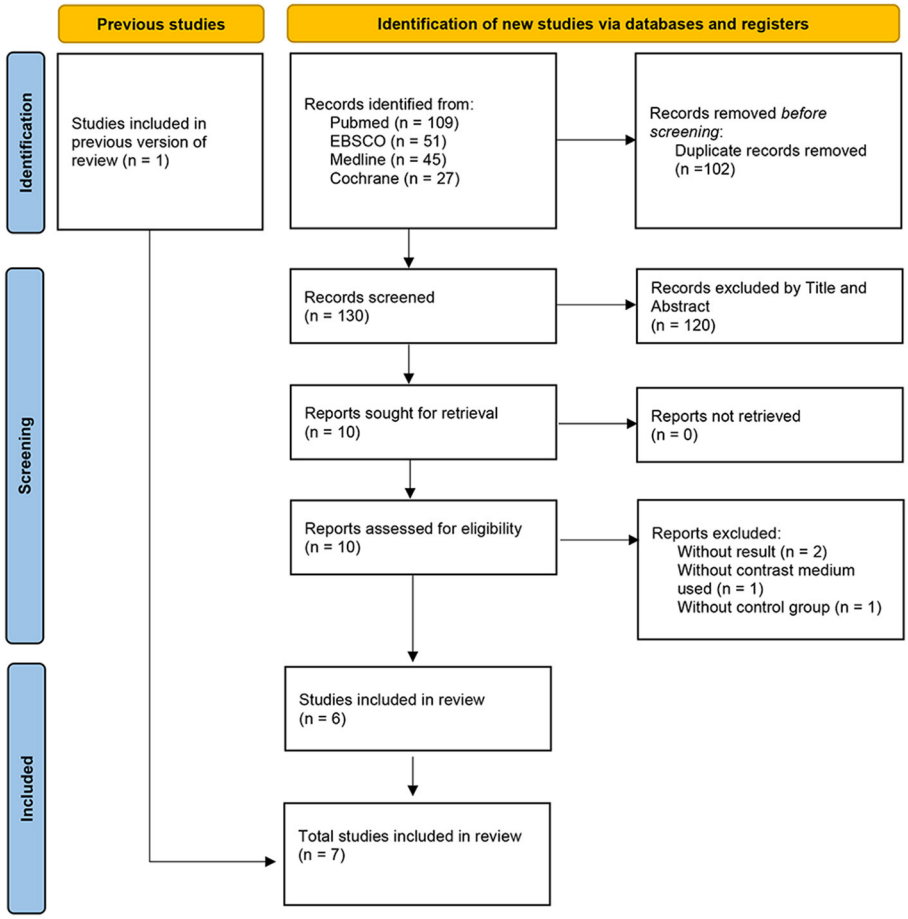

FIGURE 1
Flow diagram for search and selection of included studies.

status, hemoglobin A1c (HbA1c), glucose, serum creatinine, eGFR, and CO_2 levels were extracted when available.

Lactate level

Two studies reported on post-examination blood lactate levels (22, 23). Among them, Namazi et al. (22) found that continued use of metformin was associated with an increase in lactate levels, whereas Oktay et al. (23) reported no significant difference between the metformin and non-metformin groups. The pooled analysis indicated that there was no statistically significant difference in blood lactate levels after CM administration between patients continuing metformin use (SMD: 0.29, 95% CI: −0.53–1.11, $I^2 = 94.1\%$; $P = 0.489$, Figure 6).

Meta-analysis of logistic regression of CI-AKI

Meta-analysis of logistic regression results from the included studies revealed that the use of metformin was not significantly associated with an increased risk of CI-AKI. The pooled adjusted OR was 0.66 (95% CI: 0.40–0.92; Figure 7A), suggesting a potential protective effect. However, sensitivity analysis showed that after excluding the study by Zeller et al. (25), the association was no longer statistically significant (pooled OR: 0.78, 95% CI: 0.43–1.13; Figure 7B). This result warrants careful interpretation. The study by Zeller et al. (25) did not adjust for baseline eGFR as a continuous variable in their regression model; instead, they used a binary variable (eGFR <30 mL/min/1.73 m^2). Notably, the baseline eGFR was significantly higher in the metformin group compared to the control group. This imbalance might have artificially exaggerated the decline in renal function post-contrast in the metformin group, contributing to a biased protective effect. Therefore, the apparent significance of metformin in the initial pooled model is likely driven by this methodological limitation. In addition to metformin, we also analyzed other covariates that were included in at least two studies. Contrast volume was identified as a consistent and statistically significant independent risk factor for CI-AKI (pooled OR: 1.01, 95% CI: 1.003–1.02; Figure 8A), underscoring the importance of minimizing contrast exposure. Although baseline creatinine is a clinically important variable, only two studies included it as a covariate in their multivariable models, and the pooled analysis showed that its effect

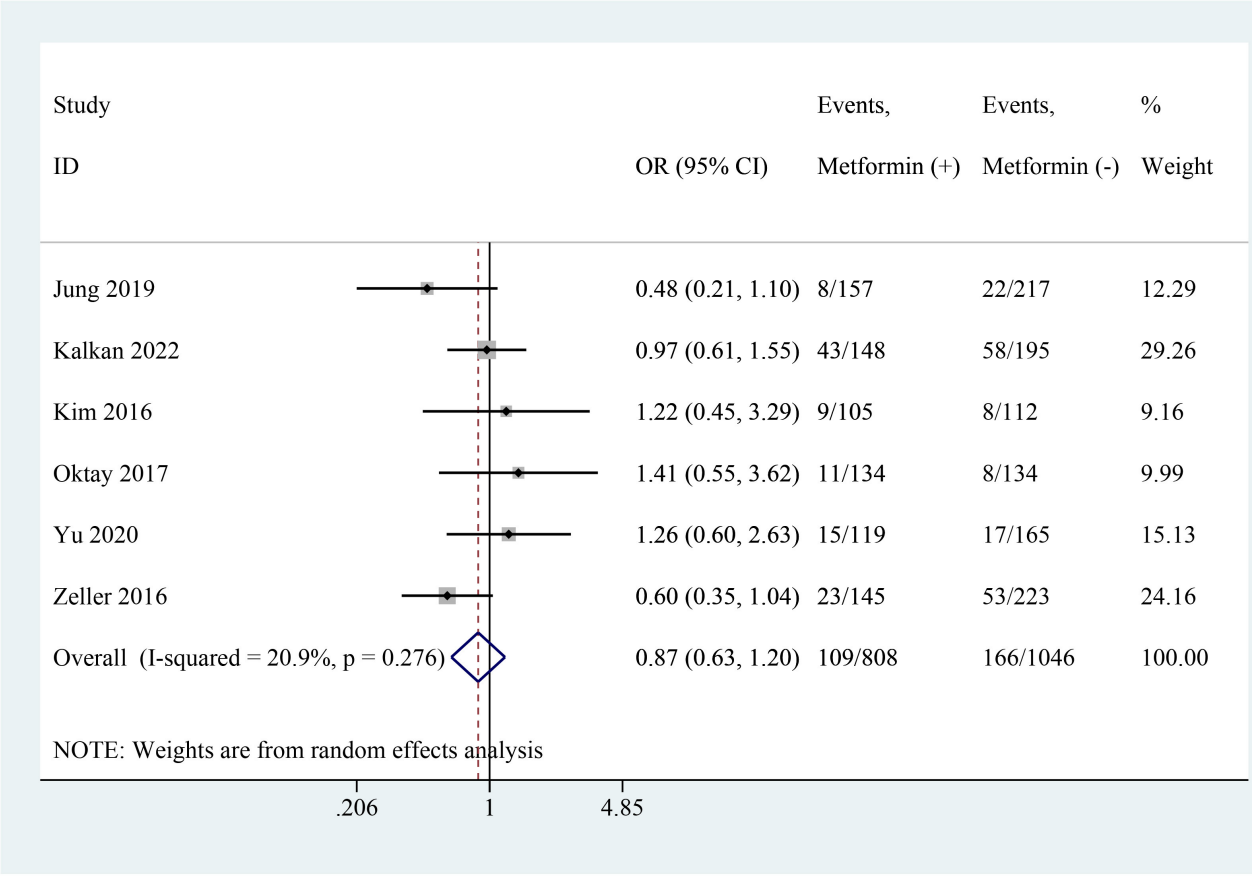

FIGURE 2
Pooled analysis of CI-AKI between the metformin and non-metformin.

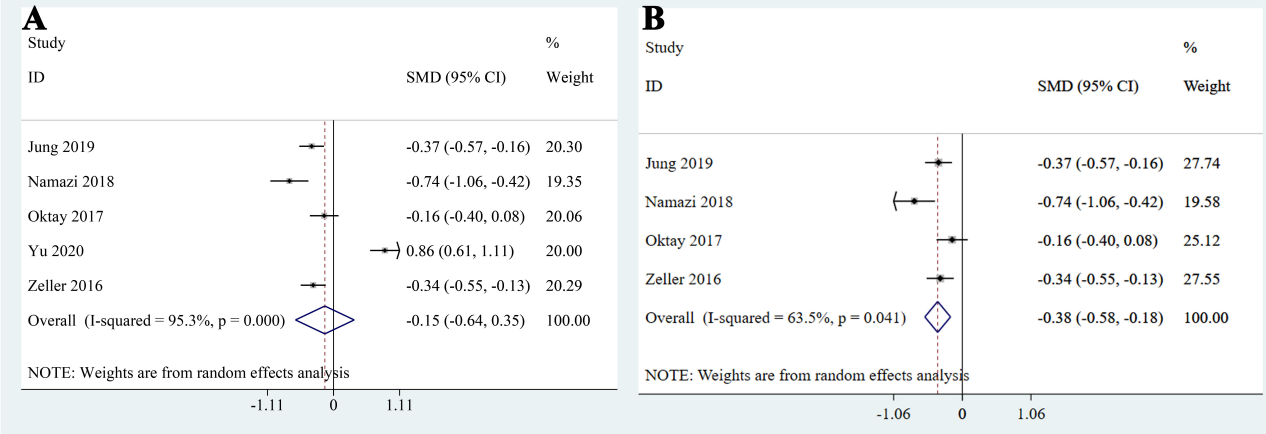

FIGURE 3
Pooled analysis of serum creatinine between the metformin and non-metformin **(A)** Overall pooled analysis; **(B)** Sensitivity analysis excluding the study by Yu et al. due to high heterogeneity.

was not significant (pooled OR: 1.19, 95% CI: 0.74–1.65; Figure 8B), likely due to inconsistent adjustment methods and definitions. Other variables such as age (pooled OR: 1.01, 95% CI: 0.99–1.02; Figure 8C), glycated hemoglobin (pooled OR: 1.06, 95% CI: 0.94–1.19; Figure 8D), and hemoglobin level (pooled OR: 1.01, 95% CI: 0.89–1.13; Figure 8E) were also not significantly associated

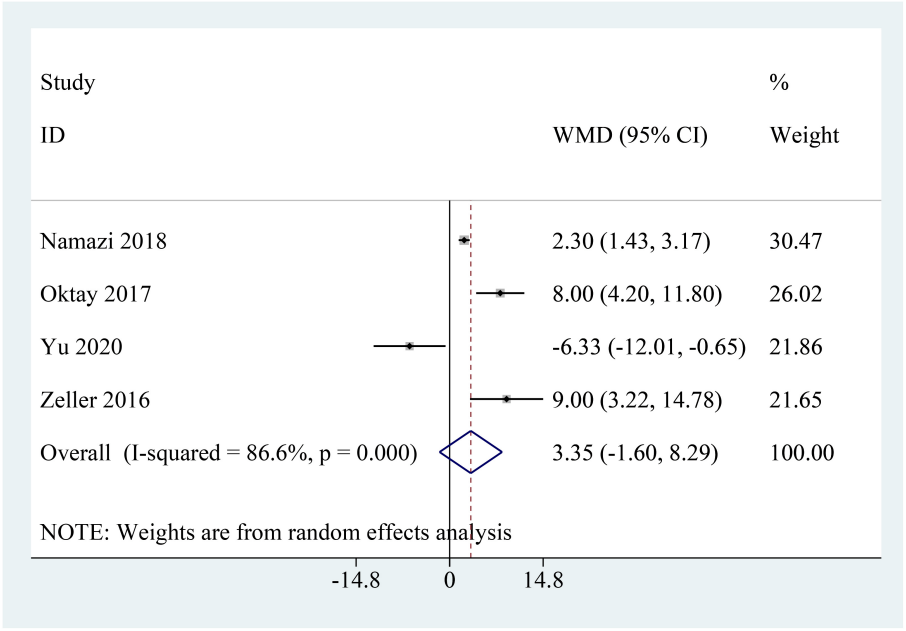

FIGURE 4
Pooled analysis of eGFR between the metformin and non-metformin.

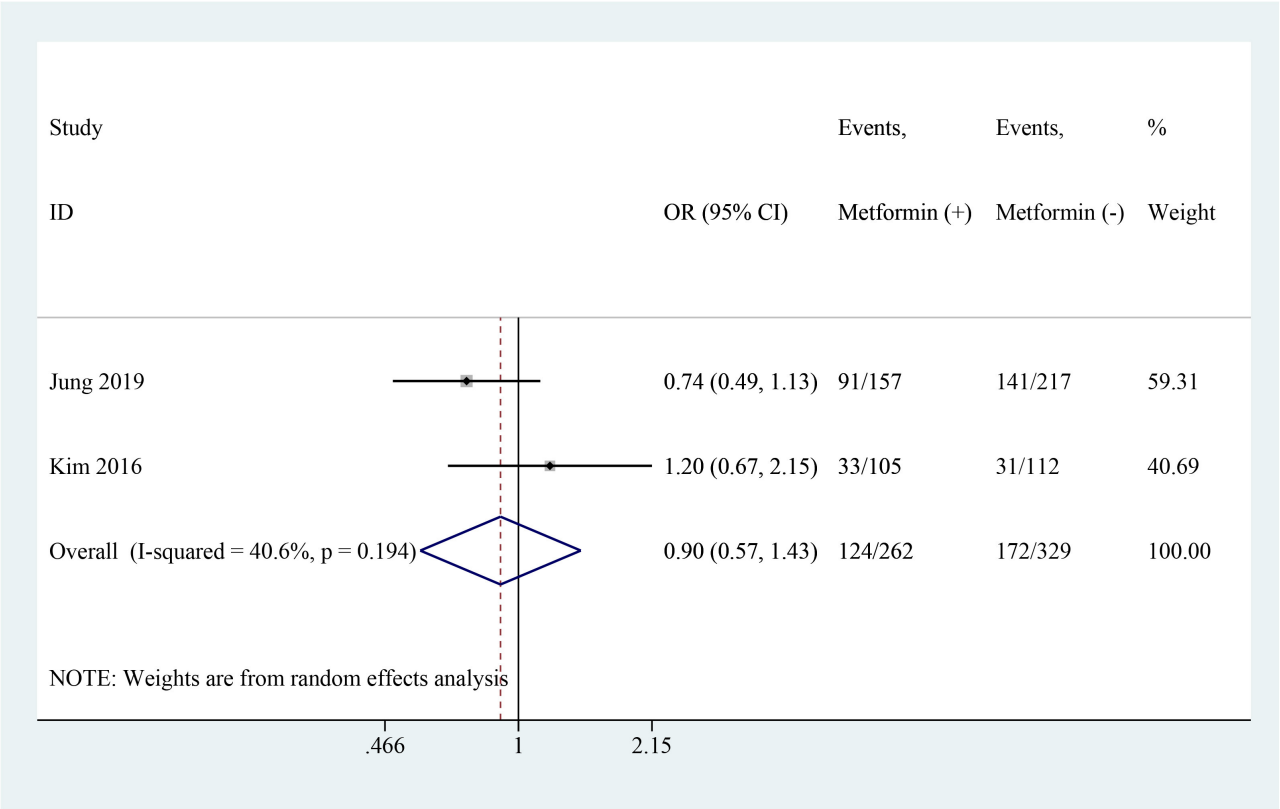

FIGURE 5
Pooled analysis of metabolic acidosis between the metformin and non-metformin.

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TABLE 2 Baseline clinical characteristics and lactic acidosis–related risk factors of patients in included studies.

Study	Liver cirrhosis		HbA1c (%)		Glucose (mg/dL)		Creatinine (mg/dL)		eGFR (mL/min)		CO_2 (mmol/L)	
	Metformin	Non-metformin	Metformin	Non-metformin	Metformin	Non-metformin	Metformin	Non-metformin	Metformin	Non-metformin	Metformin	Non-metformin
Jung et al. (19)	10.20%	10.60%	7.5 ± 1.7	7.4 ± 1.9	173 (129–247)[‡]	193 (135.6–269)[‡]	1.33 (1.18–1.48)[‡*]	1.43 (1.22–1.64)[‡]	48.6 (40.3–54.3)[‡*]	44.3 (37.8–50.8)[‡]	20.8 ± 4.2	20.6 ± 4.3
Kalkan et al. (20)	NS	NS	NS	NS	177 ± 97	170.3 ± 116	1.01 ± 0.74	1.03 ± 0.5	NS	NS	NS	NS
Kim et al. (21)	12.40%	10.70%	NS	NS	7.6 (5.7–11.7)[‡§]	7.8 (5.1–12.2)[‡§]	69.8 (54.8–87.5)[¶]	74.3 (60.1–95.5)[¶]	66.2 (50.9–87.9)[‡]	63.6 (47.8–92.4)[‡]	23.7 ± 4.1	23.7 ± 3.1
Namazi et al. (22)	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Oktay et al. (23)	NS	NS	7 (5.6–13)[‡]	7.3 (5.5–12.9)[‡]	131 (87–271)[‡]	150 (90–328)[‡]	0.84 ± 0.18 mg/dL	0.84 ± 0.13	86 ± 18	81 ± 9	NS	NS
Yu et al. (24)	NS	NS	NS	NS	NS	NS	76 (66–86)[¶]	73 (61–84)[¶]	89 (73–104)[‡]	94 (72–113)[‡]	NS	NS
Zeller et al. (25)	NS	NS	7.5 (6.7–8.6)[‡]	7.4 (6.6–8.6)[‡]	13.7 (9.54–16.88)[‡§]	12.98 (9.18–16.58)[‡§]	87 (73–105)[‡*¶]	94 (77–120)[‡¶]	80 ± 26*	71 ± 29	NS	NS

HbA1c, Hemoglobin A1c; eGFR, estimated glomerular filtration rate; NS, not specified.
*Significant difference between the two groups.
‡Median (IQR).
§Unit: mmol/L.
¶Unit: μmol/L.

with CI-AKI. These findings suggest that, among the available covariates, contrast volume remains the most consistent predictor, while the role of other factors remains uncertain due to limited and heterogeneous reporting.

Meta-analysis of logistic regression of metabolic acidosis

Our pooled analysis showed that neither age (Figure 9A) nor continuation of metformin (Figure 9B) use was significantly associated with the occurrence of metabolic acidosis. In contrast, baseline CO_2 levels demonstrated a significant inverse association with metabolic acidosis risk. Specifically, the pooled logistic regression analysis indicated that lower CO_2 levels were independently associated with a higher risk of metabolic acidosis (OR: 0.71, 95% CI: 0.65–0.76; Figure 9C), highlighting CO_2 as a potential protective factor.

Publication bias

With fewer than 10 trials, publication bias was not assessed using funnel plots.

Discussion

The study found no significant differences in the occurrence of CI-AKI, lactate levels, incidence of acidosis, and alterations in renal function parameters between diabetic patients who are using metformin and those who are not. However, sensitivity analysis showed that after excluding the study by Yu et al., which introduced methodological heterogeneity, the pooled results indicated a statistically significant reduction in serum creatinine levels among patients who continued metformin. In addition, meta-analysis of logistic regression results revealed that metformin use was not independently associated with an increased risk of CI-AKI. The pooled adjusted OR suggested a potential protective effect, though this association became no significant after excluding the study by Zeller et al. (25), which did not adjust for baseline eGFR as a continuous variable. Among other covariates, contrast volume consistently emerged as a statistically significant independent risk factor for CI-AKI, while age, baseline creatinine, HbA1c, and hemoglobin were not associated. For metabolic acidosis, logistic regression meta-analysis demonstrated that neither age nor metformin continuation was associated with increased risk. In contrast, lower baseline CO_2 levels were significantly associated with a higher risk of metabolic acidosis, highlighting CO_2 as a potential independent predictor. Previous meta-analyses have also examined the relationship between metformin use and the occurrence of CI-AKI in patients undergoing iodine contrast agent examinations (26–28). Their conclusions indicated that continued use of metformin was not associated with CI-AKI. However, these studies primarily focused on patients taking metformin, whereas our meta-analysis specifically compared diabetic patients taking metformin with those who were not. Metformin is best known for its role in lowering blood glucose levels in

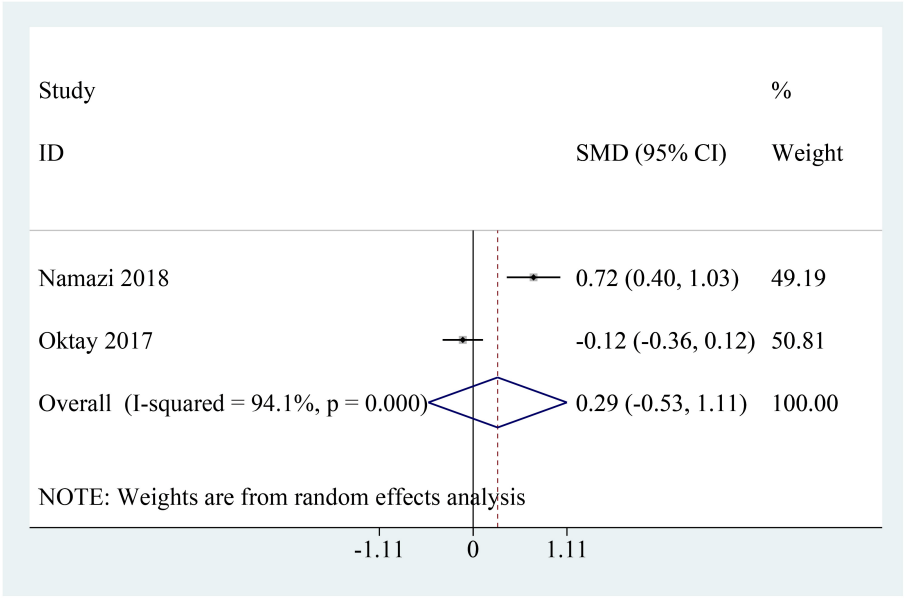

FIGURE 6
Pooled analysis of lactate level between the metformin and non-metformin.

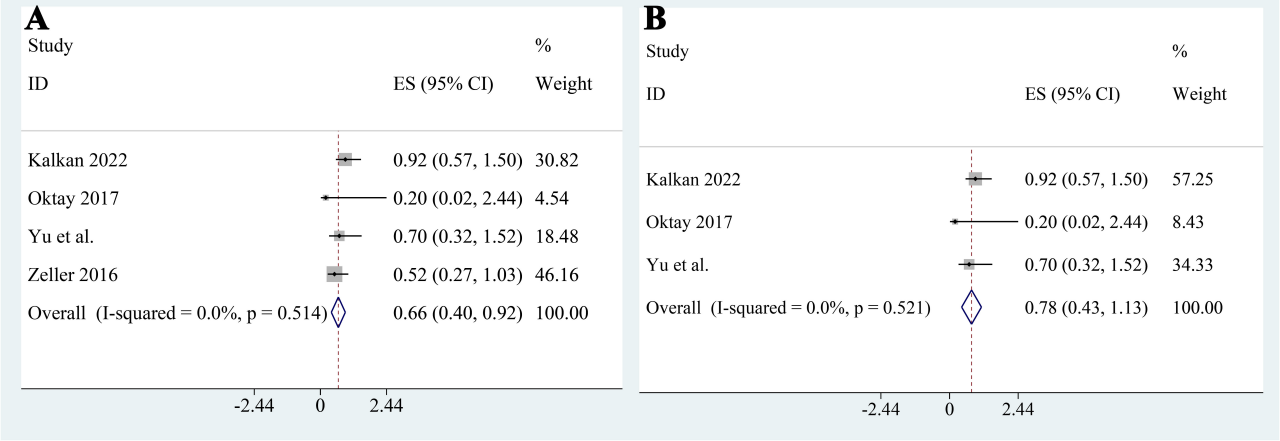

FIGURE 7
Pooled logistic regression analysis of the association between metformin use and CI-AKI. **(A)** Combined adjusted OR from all included studies; **(B)** Sensitivity analysis excluding Zeller et al.

diabetic patients (29). As research advances, investigators have discovered significant therapeutic effects of metformin in reducing cardiovascular events and combating obesity, among other benefits (30–32). Furthermore, there is increasing evidence suggesting potential benefits of metformin in conditions such as cancer, neurodegenerative diseases, metabolic syndrome, polycystic ovary syndrome, aging, and COVID-19 (33–39).

Diabetic patients have a higher susceptibility to developing lactic acidosis and renal function impairment compared to non-diabetic individuals (40). Poor glycemic control and abnormal energy metabolism contribute to increased lactate production, while impaired kidney function reduces lactate clearance. Additionally, diabetic patients may experience both ketoacidosis and lactic acidosis simultaneously, leading to more severe accumulation of acidic substances in the body (41). However, the population solely taking metformin includes non-diabetic individuals as well. Therefore, when Qiao et al. combined data from diabetic and non-diabetic patients for analysis, the study exhibited considerable heterogeneity, which undermined the credibility of its conclusions (28).

The belief that metformin should be discontinued in patients undergoing contrast agent treatment arises from the association of metformin with lactic acidosis and CI-AKI. This perspective stems from phenformin, a drug related to metformin, which was found to increase hepatic lactate production, leading to lactic acidosis and subsequently withdrawn from the market starting

FIGURE 8
Pooled logistic regression results for additional covariates potentially associated with CI-AKI: **(A)** contrast volume; **(B)** baseline serum creatinine; **(C)** age; **(D)** glycated hemoglobin; **(E)** hemoglobin level.

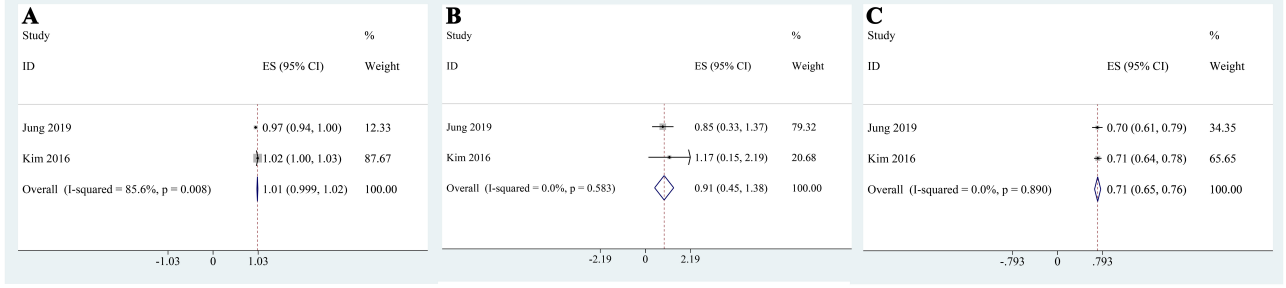

FIGURE 9
Pooled logistic regression results for predictors of metabolic acidosis: **(A)** age; **(B)** metformin use; **(C)** baseline CO_2 level.

in 1978. Despite the similarity in their names, the chemical structure of metformin is significantly different. Metformin can inhibit hepatic gluconeogenesis without altering lactate turnover (42). A community-based cohort study involving around 1 million diabetic patients in the United States also indicates that metformin use is associated with acidosis only when the eGFR is below 30 mL/min/1.73 m^2 (27, 43). Furthermore, emerging evidence indicates that in diabetic patients receiving metformin treatment, the majority of cases of lactic acidosis cannot be attributed to metformin toxicity (12, 13). Lactic acidosis can occur in non-diabetic patients in various conditions such as sepsis, hepatic failure, and renal failure. In fact, almost all reported cases of metformin-associated lactic acidosis occur in patients with comorbidities. Our study's results also confirm that metformin is not associated with the occurrence of lactic acidosis. The Korean Diabetes Association have reached a consensus on the use of metformin in type 2 diabetes complicated by renal insufficiency, particularly when these patients undergo imaging studies with CM. Renal function should be assessed before any CM-related procedure (44). Metformin is safe with eGFR ≥45; use ≤1,000 mg daily if eGFR is 30–44. It is contraindicated if eGFR <30 mL/min/1.73 m^2. As the included studies did not specifically specify populations based on glomerular filtration rate, with Oktay et al. and Zeller et al. analyzing populations with eGFR <60 mL/min/1.73 m^2 (23, 25), there were no reports on populations with glomerular filtration rates <30 mL/min/1.73 m^2, so subgroup analysis based on different eGFR levels could not be performed.

Limitations

This study has several limitations. First, apart from the 2 RCT studies, the remaining 5 studies were retrospective cohort studies. While their NOS scores were all >6 points, retrospective cohort studies are subject to risks of information bias and recall bias compared to RCT. Due to the inability to control confounding

factors, their internal validity is lower, which may lead to biased results. Second, the included populations exhibited significant heterogeneity. In studies by Kalkan et al. (20) and Kim et al. (21), the creatinine levels in the non-metformin group were higher than those in the metformin group, reducing the accuracy and credibility of the combined effects. Third, since most diabetic patients often have multiple comorbidities, including hypertension, and may require antihypertensive medications, these drugs may also influence the study results (20). Forth, different trails may have treated the control group differently. Some studies suspended metformin intake before imaging, while in other studies, the control group took other antidiabetic medications. Fifth, subgroup analyses based on baseline eGFR and metformin dosage were not feasible due to inconsistent and incomplete reporting across studies. We acknowledge the value of such stratification and recommend future studies provide standardized data to enhance clinical applicability.

Conclusion

No evidence suggests that continuing metformin during contrast medium administration increases the risk of CI-AKI, lactic acidosis, or worsening eGFR compared to those who discontinue or do not use metformin. In contrast, continuation of metformin may be associated with a modest reduction in serum creatinine following contrast exposure. For patients with eGFR <30 mL/min/1.73 m^2, safety data is insufficient, requiring further research. More large-scale RCTs are needed to confirm these findings.

Data availability statement

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

Author contributions

QX: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. WH: Data curation, Investigation, Methodology, Validation, Writing – original draft. QL: Investigation, Methodology, Project administration, Validation, Writing – original draft. TB: Data curation, Methodology, Writing – original draft. HL: Data curation, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing. XL: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

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