# Effect of Metformin on Endometrial Proteome of Diet-induced Obese Mice

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## Abstract

Obesity is known to have detrimental effects on female fertility, influencing both ovarian and endometrial functions. There is evidence that the endometrial function is altered in obese and/or insulin resistant women. Metformin, an insulin-sensitizing drug, has shown potential in treating metabolic and reproductive disorders, including polycystic ovary syndrome (PCOS) and may enhance fertility outcomes by improving endometrial dysfunction. Using a mouse model, this study aimed to investigate how a high-fat diet impacts endometrial-specific protein expression and whether metformin can mitigate these effects. C57BL/6N mice were fed a standard or a high-fat diet and either received metformin treatment or did not. Proteomic analyses revealed significant alterations in endometrial protein expression due to the high-fat diet, while metformin administration appeared to restore many of these changes to normal levels. Metformin's impact was evident through alterations in specific proteins associated with reproductive health and metabolic functions, such calcium-independent phospholipase $A_2\gamma$, ATP-binding cassette sub-family D member 1, RAC-beta serine/threonine-protein kinase, acyl-CoA:lysophosphatidylglycerol acyltransferase 1, O-GlcNAcase, scavenger receptor class A member 3, protein kinase C beta type, sortilin, beta-2-microglobulin and apolipoprotein C-III. These results suggest a potential therapeutic role for metformin in normalizing endometrial protein expression, providing insights into how this drug could improve fertility outcomes in obese or insulin-resistant females, besides normalising ovulation patterns. Overall, this study enhances our understanding of the relationship between obesity, endometrial function, and metformin's therapeutic potential, offering a foundation for further research into reproductive health and metabolic disorders.

# Introduction

The clinical impacts of obesity on female reproduction are well established (Mintziori et al., 2020, Practice Committee of the American Society for Reproductive Medicine, 2021), affecting fertility through multiple mechanisms, including effects on ovarian and endometrial functions (Broughton and Moley, 2017, Gambineri et al., 2019). Alterations in endometrial epithelial receptivity can result from various factors, including leptin, free fatty acids, and cytokines produced by adipose tissue, and peripheral insulin resistance (IR) (Gambineri et al., 2019). Molecularly, endometrial tissue from obese women often shows increased steroid receptor staining and altered expression of genes associated with implantation and unexplained infertility (Practice Committee of the American Society for Reproductive Medicine, 2021, Argenta et al., 2014, Bellver et al., 2011). Furthermore, numerous genes involved in lipid homeostasis have been identified, and endometrial transcriptomic analysis has emerged as a diagnostic tool for dating the window of implantation (Yang et al., 2022).

Glucose and lipid metabolism are intricately linked, interacting in ways that remain incompletely understood (Parhofer, 2015). Dysfunctional adipose tissue in long-term obesity and overnutrition plays a pivotal role in the development of IR and type 2 diabetes (Ahmed et al., 2021, Tzanavari et al., 2010). Additionally, alterations in lipid metabolism timing during pregnancy have been observed in gestational diabetes mellitus (GDM) pregnancies, suggesting that changes in the lipidome may contribute to the development of GDM (Furse et al., 2021).

Metformin, an insulin-sensitizing drug used to treat type 2 diabetes (Flory and Lipska, 2019, Practice Committee of the American Society for Reproductive Medicine, 2017, Palomba et al., 2009, Zeng et al., 2020), has shown promise in managing polycystic ovary syndrome (PCOS) by increasing ovulation rates, reducing androgen levels, improving insulin sensitivity, and modifying lipoprotein patterns (Palomba et al., 2009, Practice Committee of the American Society for Reproductive Medicine, 2017, Zeng et al., 2020). Moreover, metformin has

demonstrated efficacy in weight reduction among both insulin-sensitive and insulin-resistant overweight and obese patients (Yerevanian and Soukas, 2019). Notably, pre-pregnancy administration of metformin has been shown to enhance fertility (Morin-Papunen et al., 2012, Kjotrod et al., 2011, Palomba et al., 2021), impacting the endometrium by enhancing vascularity and blood flow and potentially improving receptivity in women with PCOS (Shao et al., 2014, Jakubowicz et al., 2001, Palomba et al., 2006). Recent studies have shown direct effects of metformin on endometrial gene and protein expression (Germeyer et al., 2011, Jung et al., 2015, Lange et al., 2021), and suggest that metformin rescues the disruption of the implantation process due to IR by improving the endometrial insulin signalling, e.g. by increasing endometrial GLUT4 expression in women with PCOS (Zhai et al., 2012, Palomba et al., 2021, Carvajal et al., 2013). Furthermore, a recent study demonstrated that metformin can positively affect endometrial function by modulating gene expression and DNA methylation. For instance, the study on women with PCOS found that metformin, combined with a carbohydrate-controlled diet, improved endometrial receptivity by increasing the expression of key genes like *HOXA10*, *GAB1*, *SLC2A4*, and *ESR1* (Garcia-Gomez et al., 2023).

Due to the genetic homology between mouse and human, the knowledge gained from mouse models has been carried over into human patients (Islam and Loots du, 2009, Peltonen and McKusick, 2001). Mouse models have proven invaluable in the basic science of obesity and type II diabetes mellitus. Mice treated with a high-fat diet are commonly utilized as experimental animal models for studying hyperlipidemia and hyperglycemia (Li et al., 2020).

This study investigates how a high-fat diet affects endometrium-specific protein expression and evaluates whether metformin can partially reverse these changes in a mouse model.

# Material/Methods

### *Animal model, Metformin treatment and tissue preparation*

All animal procedures for this project were conducted in accordance with German regulations and legal requirements and were approved by the local government authorities (Bezirksregierung Koeln; LANUV NRW; 84-02.04.2016.A046). C57BL/6N mice (Janvier Labs, Le Genest-Saint-Isle, France) were kept at the animal facility at the University of Cologne. Further details on animal housing are available in our previous studies (Kretschmer et al., 2020a, Kretschmer et al., 2020b, Nusken et al., 2019, Schmitz et al., 2022). At the age of three weeks, female animals were randomly assigned to one of two diet groups. The first group received a standard diet (SD; R/M-H, ssniff®; Soest, Germany), the second group a high-fat diet (HFD; C1057; Altromin, Lage, Germany). Both diets were provided ad libitum and maintained continuously until the end of the experiment.

At the age of 14-17 weeks (adolescence stage; defined as the ‘start of experiment’), the metformin (MF) treatment was started in a subgroup of the HFD group, thereby generating the third test group HFD-MF. Mice from the HFD-MF group received MF via their drinking water. Before starting the experiments, daily water intake of the HFD group was measured (data not shown) to calculate the required dose per ml water. We aimed for a daily MF intake of 380 mg/kg body weight which corresponds to a human equivalent dose of 30 mg/kg body weight daily. Water intake was monitored at random during the experiments to ensure adequate daily MF dosage. MF was applied for 14-28 days until sacrifice (defined as the ‘end of experiment’). Mean age at death was 19.0 wk (SD), 19.2 wk (HFD) and 18.2 wk (HFD-MF).

Body weight was monitored at the start and end of the experiment over a period of 14-28 days. At the end of the experiment, we also dissected the perigonadal white adipose tissue (pgWAT) fat pad and determined its weight.

Some animals were analysed in a µCT screen (see below) at the end of the experiment to determine total and visceral fat mass.

During dissection, both uterus horns were collected. The right uterus horn was quick frozen in TissueTek® (Sakura Finetek Germany GmbH, Umkirch, Germany) for LMD analysis. The left uterus horn was opened up with a scissor and endometrium was scratched out using a metal spatula. Endometrial tissue was added to 100 µl sterile distilled water and quick frozen on dry ice. Prior to further use for BCA or Mass Spectrometry analysis (see sections below), the tissue was sonicated for 60 sec to physically lyse the cells.

***Metformin and proline quantification by Mass Spectrometry***

Metformin levels in serum and endometrium were measured by a liquid chromatography mass spectrometry (LC-MS) method (Schommers et al., 2017). Serum samples were diluted 1:100 with acetonitrile, while endometrium samples were diluted 1:5 in acetonitrile. 10 µL of each diluted sample was examined via LC-MS. Results were compared to a calibration series of 0–50 ng/mL metformin. Additionally, the amino acid proline was detected in endometrium samples for normalization. Proline was chosen because the peak area followed dilution steps best. Briefly, proline was applied (A 0.1 % formic acid, B 0.1 % formic acid in acetonitrile; isocratic flow 0.3 ml/min, 40 % B, stop at 4 min) to a SeQuant ZIC-HILIC column (5 µm, 2.1 x 100 mm; Dichrom, Marl, Germany) in an SLC-20AD Prominence HPLC instrument (Shimadzu, Kyoto, Japan) and then transferred via positive electrospray to a 4000 QTRAP mass spectrometer (AB Sciex, Darmstadt, Germany). For selected reaction monitoring, the parent ion was set to m/z 116, the fragment ion to m/z 70, and the collision energy to 22 V. The peak area was obtained from integrating intensity above background vs. time in the proper elution time interval.

### *BCA analysis for total protein detection*

Total protein in endometrium samples (in distilled water) was measured via BCA method. Briefly, samples were diluted 1:5 in distilled water and a dilution series with bovine serum albumin as reference protein was prepared. The prepared BCA working reagent of a Pierce™ BCA Protein Assay (#23225; Thermo Fisher, Waltham, MA, USA, reagent A + reagent B 50:1) was added to the diluted endometrium samples and BSA standards on a 96-well plate. After incubating the plate at 37°C for 30 min the optical density was measured at a wavelength of 562nm with an absorbance reader (Tecan, Infinite M200 Pro; Männedorf, Switzerland).

### *µCT for fat analysis*

Whole mice were scanned post mortem with a µCT scanner (SkyScan 1176, Bruker, Belgium) with an isotropic voxel size of 35 $\mu m^3$. The x-ray settings for each scan were 45 kV and 475 µA using a 0.5 mm aluminum filter. All scans were performed over 360 degrees with a rotation step of 0.6 degrees and frame averaging of 2. Images were reconstructed, analyzed and visualized using NRecon, CTAn and CTVox software, respectively (Bruker, Belgium). Images were segmented based on tissue density for both total volume and fat volume. Total fat volume was further segmented into visceral and subcutaneous fat using the abdominal muscular wall as orientation.

### *Estrous cycle monitoring*

Vaginal smear samples were taken via cotton swab post mortem and applied on microscope slides (VWR, Radnor, PA, USA). Samples were air-dried and stained with a toluidine blue staining solution (Sigma #89640, 1% w/v in water) for 1 min, followed by a washing step in distilled water for 1 min. Samples were mounted in aequous mounting medium (Sigma, St. Louis, MO, USA) and visually monitored at a light microscope (Olympus BX43F; Olympus,

Hamburg, Germany). Estrous cycle monitoring was performed using the criteria described by Byers et al. and Cora et al. (Byers et al., 2012, Cora et al., 2015).

### *Laser dissection microscopy of endometrium, protein extraction and Mass Spectrometry*

Consecutive 16 µm cryo sections from uteri embedded in TissueTek® were prepared on a microtome (Leica CM3050 S) and attached to PEN membrane slides (Leica No. 11600288, Herborn, Germany). Only uteri from mice in the diestrus phase at the time of death were used. Sections were fixed in 70% ethanol containing cOmplete® protease inhibitor (Roche, Basel, Switzerland) for 1 minute, quickly rinsed in distilled water and then stained with a toluidine blue staining solution (1% w/v in water). After a washing step with 70% ethanol, samples were air-dried before extracting the endometrial tissue via laser dissection microscopy (LMD6000B, Leica Microsystems; Leica, Wetzlar, Germany). Dissected tissue pieces were collected in a tube with 10% SDS in PBS buffer and proteins were extracted as described previously. Briefly, samples were incubated at 95°C and then processed with a Bioruptur to degrade chromatin. After supplementation with dithiothreitol (DTT, 5 mM final concentration) and chloroacetamide (CAA, 40 mM final concentration), samples were digested with trypsin for subsequent LC-MS analysis. Subsequently, staff of the Proteomics Facility (Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases) performed the Mass Spectrometry and proteomics data analysis. The method has been described in detail in (Nusken et al., 2019). Statistical significance of altered proteins was determined with p-values corrected for multiple testing by Benjamini-Hochberg (q-values < 0.05). Principal component analysis (PCA) and volcano plots were generated with GraphPad Prism 9.5.0. FunRich 3.1.4 was used for generating Venn diagrams (Fonseka et al., 2021, Pathan et al., 2017).

### *Literature Review and Enrichment Analysis*

To identify relevant proteins associated with endometrial function, a systematic literature search was conducted. Using the 88 overlapping proteins from the comparisons HFD vs. SD and HFD-MF vs. HFD, we searched for associations with key terms, including *endometrium, implantation, miscarriage, pregnancy, obesity/lipid metabolism, insulin resistance/DMII/GDM,* and *metformin*. This approach allowed us to select proteins with known or potential relevance to endometrial physiology.

Furthermore, an enrichment analysis was performed using Enrichr-KG to identify significantly enriched biological processes and pathways (Evangelista et al., 2023). Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were applied to uncover molecular functions and signalling pathways related to lipid metabolism, insulin signalling, and other metabolic processes in the endometrium. These combined analyses provided a comprehensive framework for selecting and discussing key proteins in the context of the current study.

### *Statistical analysis*

GraphPad prism 9.5.0 was used for statistical analyses on body weight and body fat data. To test for normal distribution, a D'Agostino & Pearson test was used. Since at least one data set for each comparison was not normally distributed, a Mann-Whitney test was used to compare two groups (SD vs HFD; HFD vs HFD-MF). All data are presented as mean $\pm$ SEM in the graphs. Mean $\pm$ SEM data for MF dosage and serum/tissue levels were also calculated with GraphPad prism 9.5.0.

# Results

### *Daily metformin dosage and metformin serum/tissue levels*

To simulate a realistic clinical setting, we considered the average MF dosage for adult humans and aimed for a dose of 380mg/kg/d for our mice, calculated using the „human equivalent dose“ (based on Nair and Jacob, 2016) (Nair and Jacob, 2016). After measuring water intake and body weight of the mice, the mean calculated daily MF intake in the HFD-MF group was slightly higher, with 451.1 mg ± 11 MF/kg body weight per day (n=17). MF serum levels were 1.15 ± 0.14 µg MF/ml (n=9). In the endometrial samples, we detected 15 ± 3 µg MF/µg total protein (n=6) or 3.7 ± 0.6 µg MF/µg proline (n=8), respectively. No detectable levels of MF were found in samples from the SD and HFD groups.

### *Body weight and body fat – phenotypic data of the mouse cohort*

At the start of the experiment, body weight of female mice was measured, showing that HFD mice were heavier than those in the control (SD) group (Fig. 1a). The HFD group was then randomly split to generate the subgroup HFD-MF, wherein the female mice received MF via drinking water while kept on a HFD. 14-28 days after the start of the experiment, mice were sacrificed (end of experiment) and body weight was again recorded. As can be seen in Fig. 1b, HFD-MF mice lost weight during this time period, while SD and HFD mice either maintained or slightly increased their body weight. This is also represented by body weight data at the end of experiment (Fig. 1c). At this time point, HFD mice remained heavier than SD mice, but were also heavier than HFD-MF mice. Additionally, the dissected pgWAT weight at the time of sacrifice was highest in HFD mice, while both SD and HFD-MF mice displayed lower pgWAT weight compared to HFD mice (Fig. 1d). µCT analysis of mice from the three test groups corroborated findings. Both the percentage of total and visceral fat in HFD mice at the end of the experiment were higher compared to SD and HFD-MF mice (Fig. 1e and f).

### *Proteomic analysis of endometrium*

Cryo sections of uteri in the diestrus cycle phase were laser-dissected to collect endometrial tissue. A proteome screen was performed to compare the global proteome of the SD, HFD, HFD-MF groups. A total of 393 proteins was detected. The three groups displayed a clear clustering when plotted in a PCA plot (Fig. 2a). Data were also displayed as volcano plots to visualize significantly (q-value < 0.05 and fold change > +/-1.5) down- and up-regulated proteins in the pairwise group comparisons (Fig. 2b). To identify significantly and relevantly altered proteins when comparing HFD vs SD and HFD-MF vs HFD, we set the following filters: q-value < 0.05 and fold change > +/-1.5. We moreover added proteins that did not fit the q-value and fold change criteria but displayed valid values of 5 vs 0 or 0 vs 5, respectively. Using these criteria, we identified 165 significantly and relevantly altered proteins when comparing HFD vs SD, and 287 significantly and relevantly altered proteins when comparing HFD-MF vs HFD (Fig. 2c).

A Venn diagram revealed 88 overlapping proteins within the comparisons HFD vs SD and HFD-MF vs HFD (Fig. 2c, Table 1). Notably, all proteins altered by the high-fat diet were regulated in the opposite direction when metformin was administrated, potentially normalizing their expression. Enrichment analysis revealed biological pathways (GO and KEGG) associated with lipid metabolism, fatty acid oxidation, triglyceride homeostasis, carbohydrate digestion and absorption, and insulin resistance and were selected for further discussion: *APOC3*, *SORT1*, *PNPLA8*, *ABCD1*, *AKT2*, *LPGAT1*, and *PRKCB*. These pathways are particularly relevant due to their significant roles in metabolic processes that might be influenced by the treatments or conditions studied, such as metformin treatment or dietary interventions. None of the genes in our study were directly associated with GO or KEGG terms related to the endometrium, pregnancy, or miscarriage. Sortilin (SORT1), apolipoprotein C-III (APOC3), and protein kinase C beta type (PKCβ) are further highlighted in the literature, where they have been shown to play significant roles in regulating metabolic and endometrial processes, particularly in the context of obesity, insulin resistance, and reproductive health. In

addition to these, other proteins such as O-GlcNAcase (OGA), beta-2-microglobulin (β2M), and scavenger receptor class A member 3 (SCARA3) also play crucial roles in these processes, as identified through systematic literature research and were chosen for discussion. An overview of the proteins and their functions can be seen in Table 2.

# Discussion

Even though metformin is a widely used medication, it remains unclear which serum level is believed to be the perfect therapeutic level. Values between 0.1 and 4 µg/mL have been described in the literature (Kajbaf et al., 2016). Metformin serum levels in HFD-MF mice of this study were about 1.15 µg/mL, which is within a physiological setting. Furthermore, metformin was detected in the endometrium of HFD-MF, but not in HFD and SD mice, indicating that metformin is indeed enriched in the endometrium, potentially affecting it.

While insulin resistance (IR) was not directly assessed in this study, previous studies suggest a link between HFD and IR. The study by Janoschek et al. demonstrated that, prior to pregnancy, nonpregnant HFD female mice exhibited significant metabolic alterations, including increased body weight, elevated fasting blood glucose levels, impaired glucose tolerance, and higher insulin and leptin levels (Janoschek et al., 2016).

Endometrial proteomic analysis revealed intriguing results. While the feeding of a high-fat diet led to changes in the endometrial protein expression, as expected, the addition of metformin in HFD mice seemed to result in a normalization of the protein expression changes. This is the first analysis that shows a potential benefit of metformin in vivo application in an animal model on the local environment of the endometrium. As metformin is used off-label in anovulatory women with PCOS (Practice Committee of the American Society for Reproductive Medicine, 2021), who are often obese, this study provides initial evidence of a local endometrial effect, which could potentially influence the implantation potential, in addition to its known role in normalization of ovarian function. It's also important to address the limitations of this study, including the transferability of the results to humans and the use of artificial diets.

In the following sections, we discuss specific proteins identified through enrichment analysis and/or systematic literature research, interpreting our findings in the context of the current literature.

**Calcium-independent phospholipase $A_2\gamma$**

Calcium-independent phospholipase $A_2\gamma$ ($iPLA_2\gamma$) is a membrane-bound, calcium-independent phospholipase A2 that plays a crucial role in lipid homeostasis, lipid mediator synthesis, and cytoprotection. It has been implicated in the development of various diseases (Hara et al., 2019). *PNPLA8*, which encodes $iPLA_2\gamma$ is involved in several biological processes related to lipid metabolism. Studies have shown that genetic ablation of $iPLA_2\gamma$ can prevent obesity and insulin resistance during high-fat feeding by promoting mitochondrial uncoupling and increasing adipocyte fatty acid oxidation (Mancuso et al., 2010). Furthermore, a high-fat diet has been found to activate hepatic $iPLA_2\gamma$, generating eicosanoids that mediate metabolic stress (Moon et al., 2021).

In our study, we observed that $iPLA_2\gamma$ was downregulated in the endometrium following high-fat diet exposure. This finding contrasts with reports from other tissues, such as the liver, where iPLA2γ is activated during metabolic stress. These differences suggest that the regulation of iPLA2γ may vary depending on the tissue and metabolic context. Our results highlight the potential for endometrium-specific roles of $iPLA_2\gamma$ in lipid homeostasis and metabolic processes, under conditions of metabolic stress like high-fat diet. Further research will be necessary to fully elucidate the role of $iPLA_2\gamma$ in different tissues and its implications for metabolic and reproductive health.

**ATP-binding cassette sub-family D member 1**

ATP-binding cassette sub-family D member 1 (ABCD1) is a peroxisomal membrane protein that plays an essential role in the transport of very long-chain fatty acids (VLCFAs) into peroxisomes for beta-oxidation. Mutations in the *ABCD1* gene lead to X-adrenoleukodystrophy (X-ALD), a disorder characterized by impaired peroxisomal beta-oxidation and the accumulation of VLCFAs in various tissues, such as the nervous system, adrenal cortex, and testes (Ferrer et al., 2010). While *ABCD1*'s role in VLCFA metabolism and its involvement in X-ALD are well-documented, there is limited information regarding its function in the endometrium.

Our findings show that ABCD1 expression was downregulated after high-fat diet (HFD) and upregulated after metformin treatment, suggesting its potential modulation in response to metabolic changes in the endometrium. Metformin appears to restore the expression of ABCD1, which may contribute to improving lipid metabolism in the endometrium.

**RAC-beta serine/threonine-protein kinase**

RAC-beta serine/threonine-protein kinase (AKT2) is a member of the AKT family of kinases, which are involved in various cellular processes, including metabolism, cell survival, and growth (Fabi and Asselin, 2014). AKT2 has been shown to play a significant role in glucose metabolism and reproduction (Fabi and Asselin, 2014). *Akt2*-deficient mice develop a diabetes-like syndrome, with slightly reduced body mass, but no major birth defects (Cho et al., 2001, Garofalo et al., 2003). Further, *Akt2*-deficient mice exhibit impaired glucose metabolism, reduced body mass, and reduced testis and brain weight (Dummler et al., 2006). Studies suggests that AKT is necessary for blastocysts' basic glucose metabolism, making it crucial for proper implantation in the maternal endometrium (Riley et al., 2005).

In our study, AKT2 expression was found to be downregulated following a high-fat diet and upregulated after metformin treatment, providing insight into how AKT2 may respond to metabolic changes in the endometrium under conditions of metabolic stress. These findings suggest that AKT2 is potentially involved in the adaptive mechanisms of lipid and glucose metabolism in the endometrium, potentially affecting the implantation process.

**Acyl-CoA:lysophosphatidylglycerol acyltransferase 1**

Acyl-CoA:lysophosphatidylglycerol acyltransferase 1 (LPGAT1) plays a key role in lipid metabolism by converting lysophosphatidylethanolamine (LPE) into phosphatidylethanolamine (PE). It regulates stearate/palmitate homeostasis and the metabolites of the PE methylation pathway (Xu et al., 2022). LPGAT1 is crucial for lipid biosynthesis and impacts body fat content and longevity (Xu et al., 2022). LPGAT1 deficiency protects mice from diet-induced obesity,

but leads to hepatopathy, insulin resistance, and non-alcoholic fatty liver disease due to oxidative stress and mitochondrial dysfunction (Zhang et al., 2019).

Our study shows that LPGAT1's expression was reduced in response to high-fat diet (HFD) and restored after metformin treatment, further implicating it in the adaptive mechanisms of the endometrium to metabolic changes. Further investigations are required to understand the exact role of LPGAT1 in the endometrium, particularly in the context of insulin resistance, lipid dysregulation, and reproductive health.

**O-GlcNAcase**

O-GlcNAcylation, the modification catalyzed by O-GlcNAcase (OGA), plays important regulatory roles in various cellular processes that are crucial for pregnancy, including cell signalling, gene expression, and metabolism. O-GlcNAc modification appears to be pivotal in regulating endometrial receptivity and embryo implantation (Han et al., 2019), and is essential for blastocyst formation (Ruane et al., 2020). Two recent studies have suggested a potential link between decreased O-GlcNAcylation and placental dysfunction during hypertensive pregnancies and emphasized its relevance in hyperglycemia-associated pregnancies (Ning and Yang, 2021, Dos Passos Junior et al., 2022).

The current study demonstrates that a high-fat diet leads to downregulation of OGA in the endometrium. Although we did not directly assess insulin resistance, this observation is consistent with existing literature suggesting a connection between obesity, insulin signalling, and altered O-GlcNAc cycling (Keembiyehetty et al., 2015, Li et al., 2019). While the precise mechanism remains to be elucidated, it may be related to metformin’s known effects on glucose metabolism, AMPK activation, or improved insulin sensitivity. Furthermore, metformin treatment led to an upregulation of OGA expression, suggesting a potential therapeutic effect. While the precise mechanism remains to be elucidated, it may be related to metformin’s known effects on glucose metabolism, AMPK activation, or improved insulin sensitivity (Foretz et al., 2014). This compensation of OGA expression may therefore contribute to a potential

improvement in implantation, as well as embryogenesis, potentially even leading to a better placental function in hyperglycemia-associated pregnancies.

**Scavenger receptor class A member 3**

Scavenger receptors plays a key role in lipid metabolism, homeostasis and immune response (Cheng et al., 2021, Canton et al., 2013). Some SR-A family members are involved at the clearance of modified lipoproteins, such as the oxidized forms of low-density lipoproteins (LDLs), which are key to the pathogenesis of atherosclerosis (Cheng et al., 2021, Canton et al., 2013). Scavenger receptor class A member 3 (SCARA3) has been suggested to be associated with several metabolic disorders, such as weight loss, weight gain, glucose intolerance, and insulin resistance (Peng et al., 2020).

In our study, we observed a decreased expression of SCARA3 in the endometrium under high-fat diet conditions, which was reversed following metformin treatment. This suggests that SCARA3 could be involved in endometrial function and may contribute to the molecular mechanisms that are impacted by metabolic disturbances. The upregulation of SCARA3 after metformin administration may indicate a regulatory benefit, potentially helping to restore normal cellular functions and improve reproductive outcomes under obese conditions.

**Protein kinase C beta type**

Protein kinase C (PKC) is a family of enzymes involved in various cellular processes, including cell proliferation, differentiation, apoptosis, and metabolism. There are several isoforms of PKC, including the beta isoforms, PKCβ1 and PKCβ2, both encoded by the *PRKCB* gene but differing slightly in their structure and tissue distribution (Webb et al., 2000, Jubaidi et al., 2022). Dysregulation of PKCβ1 has been associated with various diseases, including cancer, cardiovascular disease, and diabetes. It may contribute to their disease pathogenesis through its effects on cell proliferation, apoptosis, and metabolism (Jubaidi et al., 2022). In addition, PKCβ2 has been linked to the development of insulin resistance and diabetic complications. It may contribute to an impaired insulin signalling and glucose metabolism in insulin-sensitive

tissues such as muscle and adipose tissue (Turban and Hajduch, 2011, Martin and Ramos, 2021). Hyperglycemia determines the vascular complications of diabetes through different mechanisms, one of which is excessive activation of the isoform PKCβ2. In endothelial cells, metformin inhibits hyperglycemia-induced PKCβ2 translocation due to a direct antioxidant effect (Gallo et al., 2005).

In our study, contrary to the expected regulation direction, the HFD group exhibited reduced expression of the PKCβ type compared to the SD group, with expression levels increasing following metformin intervention (HFD-MF vs. HFD). This observation, to the best of our knowledge, is the first report of such an effect in the endometrium of a mouse model, which may reflect a complex, tissue-specific response to a high-fat diet and metformin treatment. Further research is needed to better understand the mechanisms underlying this result.

**Sortilin**

Sortilin (SORT1) plays an important role in lipid metabolism and has garnered increasing research interest in relation to obesity and diabetes (Gao et al., 2017, Bi et al., 2013, Chen et al., 2019, Ai et al., 2012). Interestingly, hepatic SORT1 was down-regulated in diabetic mice, which was partially restored after the administration of the insulin sensitizer metformin (Li et al., 2015). Another study, showed that hepatic SORT1 protein, was markedly lower in Western diet-fed mice. Knockdown of hepatic SORT1 increased plasma triglyceride in mice. Feeding mice a fish oil-enriched diet completely restored hepatic SORT1 levels in Western diet-fed mice. (Li et al., 2014). Regadring pregnancy, elevated maternal serum SORT1 levels were associated with intrahepatic cholestasis of pregnancy (ICP) (Ertas et al., 2022).

Our results reveal, for the first time, a similar effect in the endometrium in a mouse model, showing a downregulation of SORT1 expression in high-fat diet mice, which was restored after metformin administration, suggesting an endometrial metabolic regulation by SORT1.

**Beta-2-microglobulin**

Beta-2-microglobulin (β2M) is a small protein, presented in nearly all nucleated cells and most biological fluids (Li et al., 2016). It has been shown, that there is an association between higher β2M expression and decidualization of human endometrial stromal cells (Komatsu et al., 1998), which is essential for the establishment of a successful pregnancy (Okada et al., 2018). Furthermore, cytokine modulation of β2M, may constitute one mechanism for local control of trophoblast and endometrial proliferation (Modric et al., 2000). Moreover, major histocompatibility complex class I-deficient nonobese diabetic mice, with inactivated β2M loci, are diabetes and insulitis resistant (Serreze et al., 1994, Wicker et al., 1994, Sumida et al., 1994). Those findings underline the role of β2M in endometrium and insulin metabolism.

Our results demonstrate, for the first time, a significantly higher expression of β2M in HFD compared to SD mice, and a significantly lower expression in HFD-MF compared to HFD, proposing a role in alterations of the endometrium in obesity and diabetes. Metformin appears to exert a regulatory/neutralizing effect. Interestingly, the regulation of β2M in endometrium contrasts with expected results from the above-mentioned study, which may be tissue-specific or influenced by other factors such as the duration of treatment or the metabolic environment.

**Apolipoprotein C-III**

Apolipoprotein C-III (APOC3) is a protein primarily synthesized in the liver and, to a lesser extent, in the intestine. APOC3 has been linked to insulin resistance and hypertriglyceridemia, which can have systemic effects on the metabolic health (Bard et al., 2001). Studies have shown that women who subsequently develop GDM have significantly higher plasma levels of APOC3 than controls (Kim et al., 2012).

In this study we showed that APOC3 is present in the endometrial tissue and is upregulated due to high-fat diet and downregulated when metformin is administered. Since the endometrium is a metabolically active tissue, alterations in lipid metabolism and insulin signalling may impact endometrial function and reproductive outcomes. Dysregulation of lipid metabolism and insulin resistance in GDM may have implications for endometrial receptivity

and implantation, although direct evidence linking APOC3 to endometrial function in GDM is limited so far.

Our study identified several proteins whose differential expression may play critical roles in the regulation of endometrial function, particularly in the context of implantation and endometrial receptivity. These proteins are involved in key pathways such as lipid metabolism, inflammation, and cellular stress responses, which are essential for maintaining a healthy endometrial environment conducive to embryo implantation.

The relationship between metabolic diseases, such as PCOS, and endometrial dysfunction is well established. Women with PCOS often experience reduced endometrial receptivity, which is believed to be a result of chronic hyperandrogenism and insulin resistance. Our findings suggest that some of the proteins we identified, such as those involved in lipid metabolism and insulin signalling, may be relevant to this process. In particular, the modulation of these proteins in response to metabolic interventions like metformin could offer insights into potential therapeutic strategies for improving endometrial receptivity in women with PCOS or other metabolic disorders.

In summary, proteins with (potential) significance for endometrial function become dysregulated due to high-fat diets (HFD) but can be partially normalized by metformin. These potentially interesting proteins should now be studied further to understand their mechanisms. Additionally, validating these findings in human endometrium through procedures such as endometrial biopsy could be valuable. While our findings provide significant insights into the potential effects of metformin on endometrial protein expression, we acknowledge that the lack of functional experiments represents a limitation of the current study. Future studies should aim to include such experiments to further validate and expand upon the results presented here.

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## Author Contributions

| Term | Definition |
|---|---|
| Conceptualization | Ideas; formulation or evolution of overarching research goals and aims |
| Methodology | Development or design of methodology; creation of models |
| Software | Programming, software development; designing computer programs; implementation of the computer code and supporting algorithms; testing of existing code components |
| Validation | Verification, whether as a part of the activity or separate, of the overall replication/ reproducibility of results/experiments and other research outputs |
| Formal analysis | Application of statistical, mathematical, computational, or other formal techniques to analyze or synthesize study data |

| Term | Definition |
|---|---|
| Investigation | Conducting a research and investigation process, specifically performing the experiments, or data/evidence collection |
| Resources | Provision of study materials, reagents, materials, patients, laboratory samples, animals, instrumentation, computing resources, or other analysis tools |
| Data Curation | Management activities to annotate (produce metadata), scrub data and maintain research data (including software code, where it is necessary for interpreting the data itself) for initial use and later reuse |
| Writing - Original Draft | Preparation, creation and/or presentation of the published work, specifically writing the initial draft (including substantive translation) |
| Writing - Review & Editing | Preparation, creation and/or presentation of the published work by those from the original research group, specifically critical review, commentary or revision – including pre-or postpublication stages |
| Visualization | Preparation, creation and/or presentation of the published work, specifically visualization/ data presentation |
| Supervision | Oversight and leadership responsibility for the research activity planning and execution, including mentorship external to the core team |
| Project administration | Management and coordination responsibility for the research activity planning and execution |
| Funding acquisition | Acquisition of the financial support for the project leading to this publication |

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## Conflicts of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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