CHARLES UNIVERSITY

Faculty of Pharmacy in Hradec Králové

Department of Pharmacology and Toxicology

Targeting placental inflammation: Investigating the effects of glucose, metformin, and LPS on the NLRP3 inflammasome

Diploma thesis

Diploma thesis supervisor: PharmDr. Rona Karahoda, Ph.D.

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ABSTRACT

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Gestational diabetes mellitus (GDM) is one of the most common obstetric complications defined as glucose tolerance first detected during pregnancy. Recent research indicates that GDM is associated with chronic low-grade inflammation, a phenomenon observed within the placenta as well. Importantly, the human placenta expresses high levels of NLRP3-inflammasome-associated molecules and secretes large amounts of proinflammatory cytokines IL-1β and IL-18. Elevated circulating levels of these cytokines, including IL-1β, have been linked to GDM. Moreover, metformin, a hypoglycemic agent used in pregnancy, has gained attention for its potential to modulate inflammasome activation. Thus, our study aimed to investigate the inflammatory response of human placental tissue to high glucose levels and LPS exposure and explore the potential anti-inflammatory effects of metformin. The study was conducted in villous placental explants isolated from human term placenta. qPCR and ELISA were used to evaluate the gene expression and cytokine release, respectively. We show a strong and modest effect of LPS and high glucose, respectively, on the placental NLRP3 pathway. This aligns with the evidence linking hyperglycemia with a low-grade inflammatory state. Surprisingly, we observed a proinflammatory effect of metformin in the presence of LPS, challenging the conventional understanding of its anti-inflammatory properties. These findings highlight the complexity in the modulation of inflammatory responses in the placenta. Further research is necessary to identify mechanisms underlying these effects on placental NLRP3 inflammasome regulation.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

A	absorbance
ADM	adipose tissue macrophages
ADA	American Diabetes Association
AMPK	adenosine monophosphate protein kinase
API	aqua pro injection
ATP	adenosine triphosphate
ASC	apoptosis-associated speck-like protein
BS	basal membrane
CARD	caspase recruitment domain
CD	cluster of differentiation
CTB	cytotrophoblast
DAMP	damage-associated molecular pattern
DHA	docosahexaenoic acid
DM	diabetes mellitus
eEVTs	endovascular trophoblast
ELISA	Enzyme-linked Immunosorbent Assay
FCS	foetal calf serum
FPG	fasting plasma glucose
GDM	gestational diabetes mellitus
GLUT	glucose transporter
GS	giant cell
GSDMD	gasdermin D
hCG	human chorionic gonadotropin
HPL	human placental lactogen
HGH	human growth hormone
HRP	horseradish peroxidase
IDF	International Diabetes Federation
iEVTs	interstitial trophoblast
IL	interleukin
ICM	inner cell mass
IgG	immunoglobulin G
LGA	large for gestational age

LPS	Lipopolysaccharide
mTOR	mammalian target of rapamycin
MVM	microvillous membrane
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells (κB)
NICE	National Institute for Health and Care Excellence
NLR	NOD-like receptor
NLRP3	Nucleotide-binding oligomerization domain (NOD), leucine-rich repeat-(LRR), and pyrin domain-containing 3
OCT	organic cation transporters
oGTT	oral glucose tolerant test
PAMP	pathogen-associated molecular pattern
PRR	pattern recognition receptor
PS	primitive syncytium
PYD	pyrin domain
qPCR	quantitative polymerase chain reaction
RAGE	receptor for advanced glycation end products
ROS	reactive oxygen species
SIRT1	sirtuin 1
SLC	solute carrier family
SmPC	summary of product characteristics
STB	syncytiotrophoblast
T2DM	type 2 diabetes mellitus
TE	trophectoderm
TGF- β	transforming growth factor β
TLR	Toll-like receptor
TMB	tetramethylbenzidine
TNF	tumour necrosis factor
TRI	triazole
Trx1	thioredoxin-1
TXNIP	thioredoxin interacting protein

INTRODUCTION

Gestational diabetes mellitus (GDM) is a significant health issue during pregnancy, affecting approximately 14% of women worldwide. It is characterized by glucose intolerance, which is first recognized during gestation. (1) The prevalence of maternal obesity, which is associated with a higher risk of GDM, is an increasing global health problem. (2) GDM poses serious consequences for both the mother and offspring, including an increased risk of cardiovascular disease and type 2 diabetes mellitus for the mother, and complications such as macrosomia, birth difficulties, and potential long-term consequences like obesity, type 2 diabetes mellitus, and cardiovascular disease for the offspring. (3)

The placenta, a transient organ, primarily supplies the fetus with oxygen and nutrients, eliminates waste products, produces hormones, and acts as an immunological barrier. (4) The development and functions of the placenta are shaped by the mother´s environment, potentially resulting in effects on the fetus's development and programming. (5) In GDM, the placenta plays a crucial role, as it is exposed to the mother's environment and can be affected by the chronic, low-grade inflammation that occurs in these pregnancies. (2) This phenomenon, also called sterile inflammation, is associated with damage-associated molecular patterns (DAMPs), which are released from damaged and necrotic cells and activate the inflammasome assembly process. Inflammasome is a multiprotein complex that causes the release of proinflammatory cytokines. (6) The NLRP3 inflammasome is the most researched, secreting interleukin 1β (IL-1β) and interleukin 18 (IL-18). (7)

The treatment of GDM typically begins with dietary changes, but if glycemic levels do not decrease, pharmacological treatment is considered. Insulin and off-label metformin are common treatments, with metformin having an advantage in its minimal risk of hypoglycemia. (1) Despite concerns about the lack of long-term safety data, metformin has been prescribed for decades. It has recently attracted interest because of its potential impact on NLRP3 inflammasome and sterile inflammation, which may broaden its therapeutic applications.

1 THEORETICAL BACKGROUND

1.1 Gestational diabetes mellitus – a frequent gestational disorder

Gestational diabetes mellitus (GDM) is defined as a glucose metabolism disorder that first appears during pregnancy and resolves spontaneously within a few weeks after delivery. (8-10) Maternal obesity is one of the most significant risk factors for GDM, with its prevalence significantly increasing in the past years. (8) Another risk factor for this disease is advanced maternal age - in a study in the USA (mainly white ethnicity), pregnant women aged 40+ had two times higher risk of GDM compared to women under 30 years old. (11) The variability in prevalence in countries with multi-ethnic populations is also very interesting, as notable differences between ethnicities have been observed. For example, in northern California, the highest prevalence was between the Philippines and Asians. (11) Other risk factors for GDM include GDM in previous pregnancies, family history of type 2 diabetes mellitus, cigarette smoking, lack of physical activity, and unhealthy diet. (11)

Diagnosis differs in various parts of the world. (9) In the Czech Republic, GDM screening is a two-phase process. In the first phase, which takes place until the 14th week of pregnancy, venous blood is taken on an empty stomach. All pregnant women undergo this process, apart from women already diagnosed with a pre-gestational glucose metabolic disorder. If the glycemia is less than 5,1 mmol/l, it is normal, and the woman proceeds to the second phase of the screening. In case of equal or higher glycemia than 5,1 mmol/l, the woman is sent to a diabetes specialist with suspicion of GDM or preexisting diabetes mellitus (DM). (10)

The oral glucose tolerance test (oGTT) is performed in the second phase. (11) Screening usually takes place at 24-28 weeks of pregnancy because in the second trimester, insulin resistance increases, and lack of insulin causes higher glucose levels. (9) To determine GDM, 75g glucose in 200-300 ml of water is given to a pregnant woman after a minimum of 8 hours of fasting. The fasting plasma glucose (FPG) is initially measured, followed by subsequent measurements at time intervals after glucose administration. (9) If one or more of these values are equal to or higher than the values in Table 1, GDM is diagnosed. (12)

Table 1 Criteria for diagnosing GDM using the oral glucose tolerance test.

Glucose measure	Glucose concentration threshold*		Above threshold (%)
	mg/dl	mmol/l	
FPG	92	5.1	8.3
1-h Plasma glucose	180	10.0	14.0
2-h Plasma glucose	153	8.5	16.1

*One or more of these values from a 75-g OGTT must be equalled or exceeded for the diagnosis of GDM.

Source: (12)

GDM is the most common complication in pregnancy. Owing to a lack of uniformity in the screening of GDM, the prevalence of GDM varies in different countries worldwide, but the International Diabetes Federation (IDF) estimates that GDM affects circa 14% of pregnancies worldwide (data from 2017). (3, 9, 11)

1.2 Pathophysiology of GDM

The pathophysiology of GDM involves complex factors and pathological alterations, the most important of which are beta cell dysfunction, increased insulin resistance, and inflammatory changes. (13)

A woman´s healthy pregnancy is associated with many physiological changes in the body, including changes in insulin sensitivity. In the first stage of pregnancy, insulin sensitivity increases, leading to glucose storage in adipose tissue for energy in the later stages. At the end of the first trimester, placental hormones such as estrogen, progesterone, leptin, cortisol, placental growth hormone, and placental lactogen are released, which cause, among others, an increase in insulin resistance with the peak insulin resistance occurring in the third trimester. (13) In women without a predisposition to diabetes, the reduced effect of insulin is compensated by its adequately elevated secretion. However, this compensation mechanism fails in women with GDM. Another aspect is that in GDM pregnancies, insulin resistance is increased compared to physiological insulin resistance. (14) This pathological insulin resistance causes hypertrophy and hyperplasia of pancreatic cells, and dysfunction of the pancreatic beta cells occurs. (13)

Subclinical inflammation is one of the contributing factors to insulin resistance in GDM pregnancies. (14) The significance of low-grade inflammation in the pathogenesis of GDM has recently become an aim of studies and research on adipose tissues has shown that GDM is linked to elevated concentrations of resident adipose tissue macrophages (ATM), which release proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin 6 (IL-6), and interleukin 1-beta (IL-1β). These proinflammatory cytokines disrupt insulin release from pancreatic cells and its signaling. (3) Another characteristic of GDM is hypertrophy of adipocytes. These cells become less differentiated, which, in combination with insulin resistance, leads to glucose and lipid toxicity in other organs. (13)

1.3 Placenta development and functions

The placenta is a temporary organ that interfaces the mother and fetus and plays a crucial role during the nine months of gestation. During this period, the fetus is entirely reliant on the placenta and its functions, such as supplying oxygen and nutrients, waste product excretion, production of hormones, and last but not least, immunological function. (15) Although the placenta is critical for the health of both the mother and offspring, it is still, to this date, the least understood human organ. (16)

1.3.1 Human placental structure

The placenta, as shown in Figure 1, is discoid-shaped, approximately 22 cm in diameter, 2.5 cm thick in the middle, and weighs around five hundred grams. We can identify the chorionic plate, which connects to the umbilical cord on one side (towards the fetus), and on the other side (towards the mother), the basal plate interfaces with the maternal endometrium. In the middle is intervillous space, where villous trees represent independent exchange units for maternal and fetal blood. (15)

Maternal

Maternal artery
Brings oxygenated blood from the mother to the placenta.

Intervillous space
Nutrient, gas, and waste exhange occurs.

Maternal vein
Collects deoxygenated blood from placenta and returns to mother.

Chorionic villus
Increase surface area for exchange of materials.

Fetal vein
Collects oxygenated blood from the placenta and returns to fetus.

Umbilical cord
Connects fetus to placenta, supplies oxygen and nutrients to fetus, and removes waste products.

Fetal artery
Brings deoxygenated blood from the fetus to the placenta.

Fetal

Source: (17)

Figure 1 Placental structure.

1.3.2 Human placenta development

Approximately 4-5 days after fertilization, the trophectoderm (TE) is formed, from which the trophoblast cells subsequently differentiate. TE creates a line of cells, delimiting the blastocyst, inside which we find the inner cell mass (ICM), which forms the basis of the future fetus. (18) 6-7 days after intercourse, the blastocyst implants in the endometrium of the uterus. Then, the TE cells differentiate into the first trophoblast cells, specifically the cytotrophoblast (CTB) or the primitive syncytium (PS). PS helps the blastocyst to invade deeper into the maternal endometrium. Around the tenth day, the first signs of villi development occur - CTBs expand syncytial cells and form villi. (18)

CTBs and PS proliferate and differentiate until multinucleated syncytiotrophoblast (STB) is formed, with the mother-facing microvillous membrane (MVM) on one side and the fetus-facing basal membrane (BS) on the other side. (19) CTBs also differentiate into i) interstitial trophoblasts (iEVTs), which help to establish interactions with uterine cells for better attachment and immunological acceptance, and ii) endovascular trophoblasts (eEVTs), which help remodel the maternal spiral arteries. (20) iEVTS fuse into giant cells (GS). (19, 20)

15

1.3.3 Transport function

Placental villi are the main transport unit in the human placenta – their primary function is to supply the fetus with nutrients and oxygen and remove waste products (21). These villi undergo significant morphological changes during the gestational period, developing into efficient and highly vascularised structures. (18)

Nutrients are mainly hydrophilic, and transport is typically provided by the solute carrier family (SLC). (22) Placental glucose transport is facilitated by glucose transporters (GLUTs), primarily GLUT1 (SLC2A1). (23) GLUTs are predominantly present on the MVM and significantly less on the BM. The BM serves as the rate-limiting step in the transport. (19) The fetus and the placenta rely on glucose as their main energy source, so insulin is not required for transport. Nevertheless, the placenta retains the insulin receptor, and insulin can influence the glucose metabolism of the fetus. (3)

1.3.4 Hormones production

In addition to nutrient transport, the placenta is a highly endocrine organ. (18) The main source of human placental hormones is the STB, which synthesizes, among others, human chorionic gonadotropin (hCG). The function of hCG is to stimulate the corpus luteum, produce progesterone, and maintain the pregnancy. (24) Because hCG is secreted exclusively during pregnancy (except for some tumor cells) and it is detectable in maternal blood and urine, it is used as a pregnancy test. (25)

As discussed, progesterone is a crucial hormone for the maintenance of pregnancy. In the first weeks, the corpus luteum is the main producer, but then the STB takes over this function. (25) Progesterone prevents further ovulation and relaxes smooth muscle - preventing premature contractions of the uterus. (26, 27) In addition, estrogen production increases threefold during pregnancy, primarily influencing the growth of the placenta. Consequently, the placenta becomes the primary source of estrogen during pregnancy. (26)

The STB cells of the placenta also produce human placental lactogen (hPL), which shows a lactogenic effect, enhances the impact of hCG on the corpus luteum (luteotropic effect),

and amplifies the effect of human growth hormone (hGH), ensuring a positive nitrogen balance is maintained throughout pregnancy. (26, 28)

1.4 Alteration of placental formation and function in response to GDM

In pregnancies affected by GDM, notable alterations in the placental environment occur. Changes in placental development have been observed, with placentas from GDM mothers often exhibiting larger size, increased syncytiotrophoblast surface, and hypervascularization of villi. These pathological modifications result in an expanded fetoplacental endothelial surface, impacting the transport of nutrients. (29)

Research has indicated heightened expression and activity of glucose transporters, leading to elevated fetal glucose levels. (29) Elevated glucose levels trigger insulin release, promoting accelerated fetal growth, with excess glucose being stored as fat, potentially influencing future health outcomes. Moreover, the transport of amino acids and fatty acids is also enhanced in GDM pregnancies. (29) Interestingly, deficiencies in Docosahexaenoic acid (DHA) transfer have been noted in GDM, an omega-3 fatty acid crucial for fetal brain development. (29)

In addition, changes in buffering capacity in GDM placentas are observed as adaptive responses to the hyperglycemic environment. The placenta adapts its structure and function by increasing vascular resistance and buffering excess maternal glucose to regulate glucose transfer to the fetus, ensuring fetal growth remains within a normal range to prevent overgrowth. However, excessive fetal growth may occur if the glucose level exceeds the placenta´s capacity to respond adequately. (30)

Probably due to placental dysfunction in GDM, a significant alteration in placental perfusion is also observed. Reduced fetal oxygenation levels (oxygen saturation and content) and elevated lactate concentrations were detected in GDM-affected placentas. (31) Lastly, studies have indicated that GDM is linked to chronic low-grade inflammation that is also evident in the placenta. (2)

1.5   Complications of untreated GDM

GDM increases the risk of complications for both the mother and fetus. Women experiencing GDM face an increased likelihood of premature delivery, developing pre-eclampsia, and the need for cesarean section. Except for those short-term complications, women diagnosed with GDM are more susceptible to the development of type 2 DM and cardiovascular disease. (32)

In addition to the effects on the mother, a high glucose level in the maternal blood causes the release of fatty and amino acids, which pass along with glucose through the placenta to the fetus. Fetal hyperglycemia occurs and stimulates insulin secretion. This leads to hyperinsulinemia and the associated growth of insulin-sensitive organs such as the liver, adipose tissue, and heart. (32) Immediately after the delivery, insulin is still in high concentrations in the baby´s blood, but the glucose level decreases, leading to neonatal hypoglycemia. (33)

Excessive fetal growth can be described as either macrosomia or large for gestational age (LGA), both associated with GDM pregnancies. Macrosomia is typically defined as an absolute birth weight of over 4000 to 4500 grams, while LGA refers to a birth weight greater than the 90th percentile for gestational age. These conditions increase the risk of asphyxia, perinatal death, shoulder dystocia, hypoglycemia, and respiratory distress. (34)

According to a landmark study by Barker et al., 1990, 1993, there is a significant connection between in-utero development and the proper functioning of organ systems later in life. (19) Therefore, an abnormal in-utero stimulus can disrupt normal fetal programming and cause changes in structure and metabolic activities, predisposing to chronic diseases later in life. As such, GDM is associated with a predisposition for cardiovascular disease, hypertension, and T2DM. (35)

1.6   Inflammatory changes in GDM

Hyperglycemia in GDM causes inflammation in the placenta through several mechanisms. The hyperglycemic environment induces: (i) the production of proinflammatory cytokines; (ii) increased placental infiltration of macrophages and T cells; and (iii) synthesis of reactive oxygen species (ROS) precursors, such as NADPH oxidase 4 (NOX 4), resulting in

increased oxidative stress. (36) During GDM pregnancy, the body enters a state of low-grade systemic inflammation, which is exacerbated by the placenta's secretion of inflammatory cytokines, such as NF-κB, IL-6, IL-8, IL-1β, and TNF-α. These cytokines impair maternal insulin resistance, disrupt pancreatic beta-cell function, and contribute to chronic inflammation, further exacerbating the condition. (37)

Moreover, the lack of ability of GDM women to compensate for oxidative stress is associated with increased insulin resistance and reduced insulin secretion.(36) ROS triggers the production of inflammatory cells and mediators, and this inflammation, in turn, amplifies ROS release, exacerbating oxidative stress and causing a vicious circle to ensue. (38) Furthermore, inflammation in the GDM placenta alters the synthesis of calcitriol, the active form of vitamin D, which compromises beta-cell function and affects immune responses. This highlights the intricate interplay between inflammation, insulin resistance, and beta-cell dysfunction in GDM. (36)

1.6.1 Mechanisms implicated in GDM-induced inflammation

IL-1β, a proinflammatory cytokine, can induce apoptosis in pancreatic β cells and stimulate through the NK-κB pathway the release of IL-6, IL-8, and other inflammatory factors. (37) Studies prove increased IL-1β expression in GDM in pancreas and blood. (39) It is intriguing that IL-1β antagonism could potentially become a target of therapeutic strategy for the treatment of GDM, as it leads to enhanced glycemia in mouse models with GDM. (40)

Moreover, IL-6 and IL-8 have the capacity to stimulate various inflammatory cells, thereby exacerbating the inflammatory response.(37) IL-6 in serum is increased in women with GDM compared to women without GDM (41). However, the data regarding IL-8 levels in GDM presents a more varied picture. While certain studies indicate elevated IL-8 levels in GDM patients compared to those with healthy pregnancies, others observe no noteworthy differences. The inconsistencies may be due to variations in the methodologies and assays used in different studies. (41)

IL-18 seems to be increased in women with GDM compared to women with normal glucose tolerance. Furthermore, correlations between IL-18 and markers of insulin resistance have been reported, which indicates association between IL-18 and this pathophysiological

condition. (42) In addition, a genetic study identified specific polymorphisms within the IL-18 gene, indicating a potential genetic predisposition for altered IL-18 production. This genetic susceptibility may also play a role in the development of GDM. (43) Lastly, TNF-α, a pro-inflammatory cytokine implicated in GDM and contributing to the inflammatory state, shows no difference in serum levels between women with and without GDM. Nonetheless, the placenta exhibits elevated TNF-α levels, influencing local placental processes without affecting the body systemically. (44)

Apart from proinflammatory cytokines, Ines Mrizak et al. have reported an elevation in immune cells like cluster of differentiation (CD) 68+ or CD14+ cells in GDM placentas, along with increased expression of proinflammatory factors such as TLR, and TGF-β in GDM placenta. (37) Collectively, this underscores the significant involvement of immune cells and the cytokines they release in the development of GDM. Nevertheless, the precise role of placental inflammation in GDM remains unclear. (37)

1.6.2 Sterile inflammatory responses mediated by the inflammasome; the NLRP3 inflammasome

Sterile inflammation occurs without the presence of microorganisms - nonetheless, it can also be triggered by external substances like silica and asbestos. This phenomenon is commonly linked to the detection of intracellular components DAMPs (damage-associated molecular patterns) released from damaged or necrotic cells by receptors involved in inflammatory signaling. (6) Microbes identifying host receptors, particularly pattern recognition receptors (PRRs), such as the Toll-like receptors (TLRs) and NOD-like receptors (NLRs) are activated not only by infectious agents but also by internal and non-infectious triggers, leading to sterile inflammatory responses. Interestingly, host receptors not primarily linked to recognizing pathogens, such as the receptor for advanced glycation end products (RAGE), can also perceive sterile stimuli. (6)

PRRs recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). PAMPs are structural elements found in microorganisms, such as lipopolysaccharide (LPS), while DAMPs are released under cellular

stress and damage. Subsequently, PPRs activate a signaling cascade that ultimately activates the inflammasome. (7)

The NLRP3 inflammasome is an intracellular multimeric protein complex of the innate immune system, whose expression and activity have also been reported also in the human placenta. (45, 46) Its activation leads to the secretion of proinflammatory cytokines IL-1β, IL-18, and caspase-1 in response to cellular damage and microbial infection. (46) The NLRP3 inflammasome is so far the most studied of all inflammasomes due to its wide range of activators and its implication in many inflammatory diseases. (47)

The NLRP3 inflammasome is comprised of the NLRP3 protein, apoptosis-associated speck-like protein (ASC) and pro-caspase-1. (7)The NLRP3 protein contains a pyrin domain PYD, while the ASC contains PYD and CARD (caspase recruitment domain). When activated, the NLRP3 protein via PYD interacts with PYD domain of ASC, and the CARD domain of ASC binds to the CARD domain of pro-caspase-1, resulting in the assembly of the NLRP3-ASC-pro-caspase-1 complex called NLRP3 inflammasome. (47)

The assembly of the NLRP3 inflammasome is shown in Figure 2. The first signal primes the NLRP3 inflammasome, and the second activates it. (46) The priming stage includes the transcriptional upregulation of NLRP3 and proinflammatory cytokines – pro-IL1β and pro-IL-18. The central mediator of the priming signal is the nuclear-factor kappa-light-chain-enhancer of activated B cells (NF-kB), whose target is the NLRP3 gene in nucleus. The priming phase is crucial because, in most cell types, the levels of NLRP3 are inadequate for activating the inflammasome. (48) During this phase, ASC is ubiquitinated and phosphorylated. (45)

In the second stage, the NLRP3 inflammasome is assembled through activation of several stimuli, such as adenosine triphosphate (ATP), pathogens, uric acid crystals, asbestos, silica, ionic flux, mitochondrial dysfunction, reactive oxygen species (ROS) and lysosomal damage. (7) DAMPs or PAMPs most commonly trigger this step, leading to inflammasome formation and cleavage of pro-caspase 1. Subsequently, caspase-1 is activated, which processes pro-IL-18 and pro-IL-1β into their active forms. Moreover, active caspase-1 cleaves gasdermin D (GSDMD), creating membrane pores that facilitate the release of inflammatory cytokines and eventually induce pyroptosis. (49)
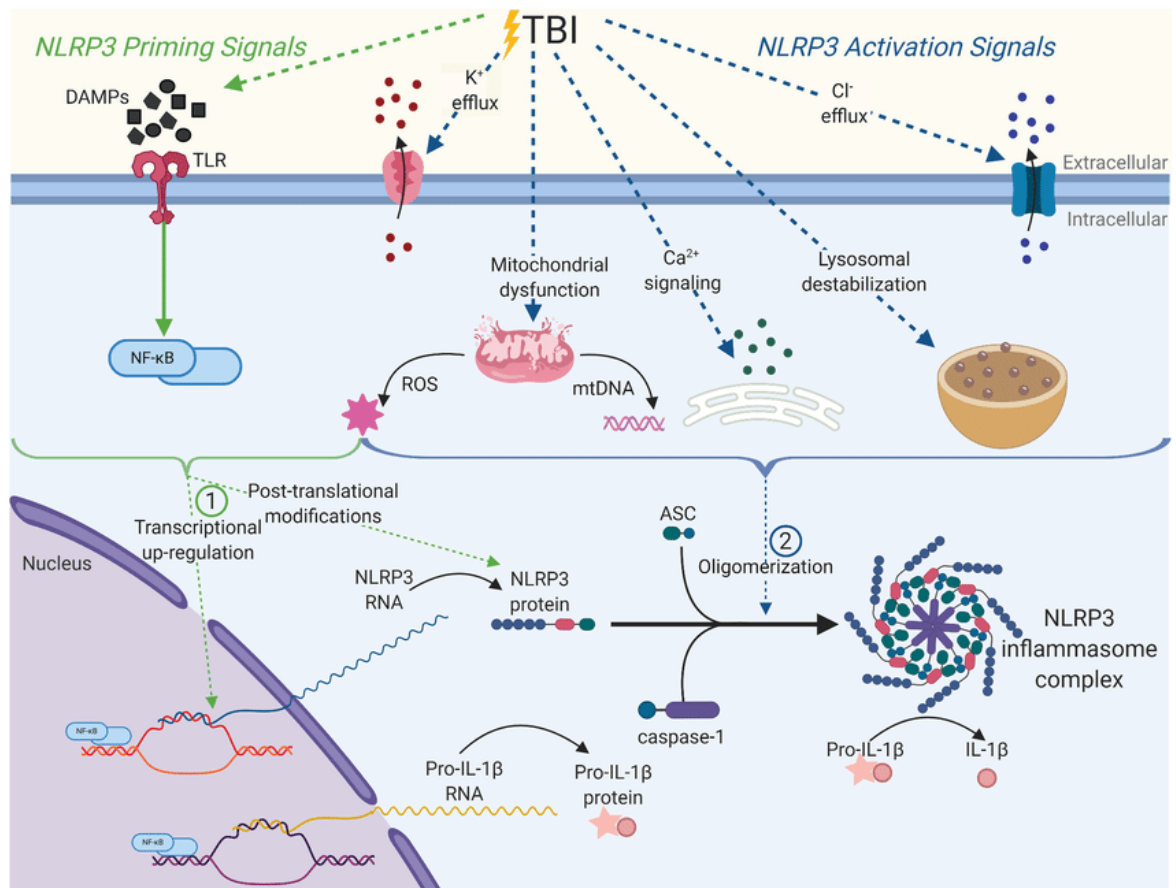
Figure 2 NLRP3 inflammasome priming and activation.

Over the past five years, there has been growing interest in studying the role of the inflammasome in placental disorders; however, there has been limited investigation into the connection between GDM, hyperglycemia, and the NLRP3 inflammasome. So far, evidence only exists from studies on adipose tissue, which revealed heightened expression of active caspase-1 and increased production of IL-1β in pregnancies with GDM (51). Notably, experiments involving pregnant adipose tissue treated with IL-1β showed a significant reduction in insulin signaling, suggesting a potential role of caspase-1/IL-1β signaling in the development of GDM (51). Studies have also highlighted elevated levels of DAMPs like palmitate, advanced glycation end-products, and uric acid in GDM pregnancies (52). Moreover, recent findings indicate that maternal hyperglycemia during pregnancy elevates placental levels of IL-1β, IL-6, and inflammasome proteins (53). This suggests a possibility that the increased presence of DAMPs and the inflammatory milieu in these pregnancies might activate the placental NLRP3 inflammasome, contributing to sustained low-grade inflammation associated with GDM and hyperglycemia.

1.7 Current treatments for GDM

The basis of treatment of gestational diabetes mellitus is a change in diet. The most important is to monitor carbohydrates because they have the most significant effect on glycemia – women should exclude those, that are quickly absorbed. At the same time, the glycemic index of diet is another aspect to consider, and foods with a lower glycemic index should be chosen. When the target glycemia values are not reached, pharmacological treatment is used – primarily insulin or metformin. (13)

The advantage of insulin is that it does not cross the placental barrier, so it does not affect the fetus. Unfortunately, insulin treatment is associated with significant disadvantages, such as the risk of hypoglycemia, injection application, and the need for regular monitoring. (13) On the other hand, metformin (N,N – dimethyl biguanide) belongs to the biguanide class of antidiabetic drugs and is currently used mainly as a first-line therapy for T2DM. (54) Probably the most important clinical advantage of metformin is that it does not induce hypoglycemia and weight gain, in addition to its cardiovascular safety. (54) Metformin is efficacious both as monotherapy and in combination with insulin and other oral antidiabetics. (54)

1.7.1 Metformin use in pregnancy

The global usage of metformin during pregnancy is increasing due to randomized controlled trial data that support its safety and effectiveness. Metformin treatment results were comparable to insulin treatment, with lower maternal weight gain and higher patient acceptability. Metformin can also have a positive effect on obese, non-diabetic pregnant women. (55)

The recommendations for treatment in pregnancy differ worldwide. For example, the National Institute for Health and Care Excellence (NICE) in the United Kingdom suggests the treatment with metformin for women with GDM if glucose levels are not achieved through dietary interventions within 1-2 weeks. On the other hand, the American Diabetes Association (ADA) recommends insulin as a first-line therapy for GDM due to a lack of long-term safety data for metformin. (55) Based on new safety profile studies (56), the Summary of Product

(SmPC) was changed in February 2022, and metformin can now be used during pregnancy. (57)

Like all medicines, metformin can cause side effects, including nausea, vomiting, and diarrhea, but generally, metformin is considered a safe drug with good patient tolerance. (55) One possible risk of metformin is lactic acidosis – fortunately, the incidence in therapeutic doses is rare (less than three cases per 100.000 patients in a year). (54)

1.7.2 Placental transfer and metformin pharmacokinetics in pregnancy

The oral bioavailability of metformin is 50-60% and with typical doses of 1g to 2g a day, the plasma concentrations range from ~10 µM to ~40 µM. (58) Metformin crosses the placenta, and its transplacental transfer has been studied using *ex-vivo* placental perfusion models. According to a study by Kovo et al., the maternal-fetal transport rate of metformin (1 µg/ml) in the human placenta is 10.61± 2.85%. (59) Metformin is a highly polar, positively charged drug, so its transplacental transfer is facilitated by a carrier belonging to the organic cation transporters (OCT) family. (59)

1.7.3 Mechanisms of action of metformin

The mechanisms of action of metformin are not fully understood, but the general consensus is that metformin inhibits gluconeogenesis in the liver, increases insulin-mediated glucose utilization in muscle and adipose tissue, and increases glycogen synthesis in muscle. (58, 60)

The mechanism of gluconeogenesis inhibition is widely studied, where metformin inhibits mitochondrial complex I, which leads to activation of AMPK (adenosine-monophosphate protein kinase). This enzyme is responsible for cells' sensitivity to insulin and is a key substance that regulates the metabolism of glucose and lipids in cells. AMPK thereby reduces insulin resistance. However, this mechanism has only been observed in high concentrations of metformin (>1 mM), which are not used in clinical practice. The gluconeogenesis inhibition mechanism that occurs in clinical concentrations (50- 100 µM) is redox-dependent, whereby metformin inhibits glycerol-3-phosphate dehydrogenase and causes

an increased cytosolic redox state. (58) Moreover, recently metformin has also been discussed in connection with its antitumorigenic effects due to its AMPK-driven inhibition of the mammalian target of rapamycin (mTOR). (61)

1.7.4 The effect of metformin on the NLRP3 inflammasome

Metformin has demonstrated diverse effects on the NLRP3 inflammasome, among others, due to its ability to stimulate AMPK and inhibit mTOR, essential signaling molecules in NLRP3 activation. One study on diabetic cardiomyopathy proved elevation in phosphorylated (activated) AMPK levels and reduced mTOR and NLRP3 levels. (62) In addition, metformin was recently reported to positively regulate the expression of sirtuins (SIRTs), a family of signaling proteins involved in cell differentiation, cellular metabolism, and recognized for their anti-inflammatory properties. (63) SIRT1 also shows an effect on NLRP3 inflammasome – specifically, this histone deacetylase suppresses the activation of NLRP3 inflammasome and subsequent inflammatory responses. Conversely, SIRT1 knockdown leads to the NLRP3 formation. (64) Apart from its action in AMPK and SIRT1 signaling, metformin was shown to block the TLR4/NF-kB signaling pathway, resulting in the inhibition of NLRP3 inflammasome. (65)

The Trx1/TXNIP pathway plays a significant role in NLRP3 inflammasome activation. Thioredoxin-1 (Trx1) interacts with thioredoxin interacting protein (TXNIP) to modulate the NLRP3 inflammasome. Under stressful conditions like redox imbalance, TXNIP separates from Trx1 and binds to NLRP3, initiating the formation of the NLRP3 inflammasome. Research indicates that metformin effectively inhibits the expression of TXNIP and the interaction between TXNIP and NLRP3. (49)

Collectively, metformin has attracted interest in its potential to modulate inflammasome activation and reduce the levels of proinflammatory cytokines (66). As the NLRP3 inflammasome pathway represents a promising target for various placental pathologies, deciphering the therapeutic potential of metformin beyond its glucose-lowering properties is of significant importance for future clinical practice.

2 AIMS OF STUDY

Based on our comprehensive review of existing literature, we have developed several hypotheses regarding the interplay between hyperglycemia during pregnancy, placental inflammation, and the potential impact of metformin intervention. Firstly, we propose that hyperglycemia in pregnancy correlates with heightened levels of DAMPs, activation of the placental NLRP3 inflammasome, and an increased secretion of proinflammatory cytokines. Additionally, we hypothesize that LPS induces pathogen-associated activation of the NLRP3 inflammasome in human placental tissue. Furthermore, we suggest that metformin may mitigate the activation of the placental NLRP3 inflammasome.

To address these hypotheses, the aims of this thesis are twofold. Firstly, we aim to characterize the expression and activation of the inflammasome in placental tissue exposed to high glucose and LPS. Secondly, we intend to evaluate the impact of metformin on the activation of the placental NLRP3 inflammasome. To achieve these aims, placental explant cultures have been employed as an optimal model system, facilitating the investigation of various tissue functions, including cellular uptake, production, and release of secretory components, as well as the inflammatory response to diverse stimuli.

3 METHODOLOGY

3.1 Reagents

D-(+)-Glucose solution and LPS (Escherichia coli O111:B4) were obtained from Sigma Aldrich, USA. D-Mannitol was obtained from Penta Chemicals, Czech Republic. Metformin hydrochloride was purchased from MedChemExpress (New Jersey, USA). All other chemicals were of analytical grade and were obtained from Penta Chemicals (Prague, CZ).

3.2 Human placenta collection

For this thesis, placental tissue samples were obtained from healthy-term pregnancies immediately after delivery at the Department of Obstetrics and Gynecology, University Hospital in Hradec Kralove, Czech Republic. All pregnant women provided written informed consent and the Ethics Committee of the University Hospital Hradec Kralove approved this research (approval no. 202205 P09).

3.3 *Ex-vivo* human placental explants

Human placental explants were isolated as previously reported by our team. (67) Briefly, fragments of cotyledons were carefully dissected from various placental areas, with the chorionic plate and decidua removed. Villous tissue was then dissected into approximately 30 mg explants. Samples of villous tissue, roughly 0.5cm × 0.5cm in size, were cleaned, rinsed with sterile saline, and placed in 12-well plates (approximately 100 mg/well) containing DMEM Low glucose - 1g/l (Capricorn Scientific, Germany) supplemented with fetal bovine serum, penicillin 100 U/ml, streptomycin 0.1 mg/ml, and amphotericin B 2.5 μg/ml. The explants were incubated under specific conditions (8% O_2, 5% CO_2, 87 % N_2) at 37°C for 4 hours. After media change, the explants were allowed to recover for 18-24 hours before experiments were conducted.

3.4 Treatment plan

In the first set of experiments (Figure 3), explants were exposed to either a high concentration of glucose (HG) or lipopolysaccharide (LPS) for varying durations: 1, 3, 6, 12, 24, and 48 hours. Additionally, concentration-dependency studies were conducted for glucose at concentrations of 10, 15, 20, 25, 30, and 35 mM, and for LPS at concentrations of 0.1, 1, and 10 µg/ml. To ensure changes in osmolarity were not affecting the HG treatment, separate experiments were conducted using mannitol at the same concentrations as glucose, which is inert and not utilized by mammalian cells.
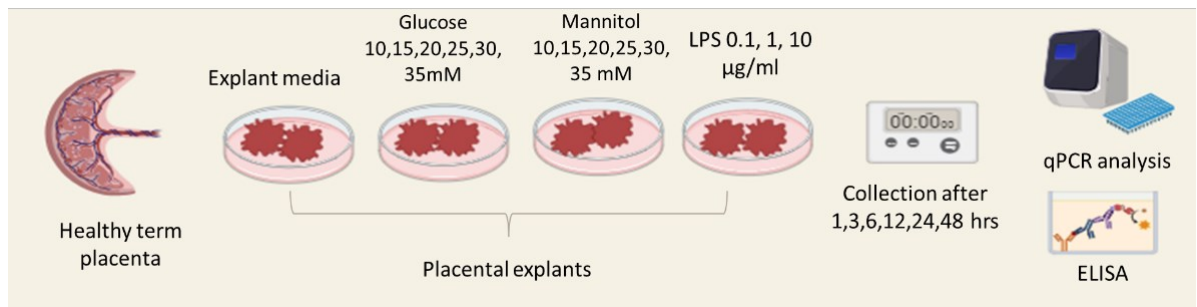

Figure 3 Time- and concentration-dependent treatment plan for glucose and LPS. The figure is reproduced with permission from Fiona Kumnova, the author of the figure.

In the second set of experiments (Figure 4), explants were treated with metformin at concentrations of 1, 10, 100 µM and 1 mM. After a 24-hour incubation period, the same doses of metformin were added to the explants along with 1 µg/ml of LPS. Our aim was to investigate whether metformin could mitigate the effects of LPS on NLRP3 inflammasome activation, hypothesizing that metformin might interfere with the priming and subsequent activation stages of the inflammasome pathway. This approach allowed us to assess the potential modulatory effects of metformin across a range of concentrations, providing insight into its dose-dependent impact on placental inflammatory responses.

In all experiments, the control sample represents the untreated explants which contained only 5 mM glucose concentration present in the media.
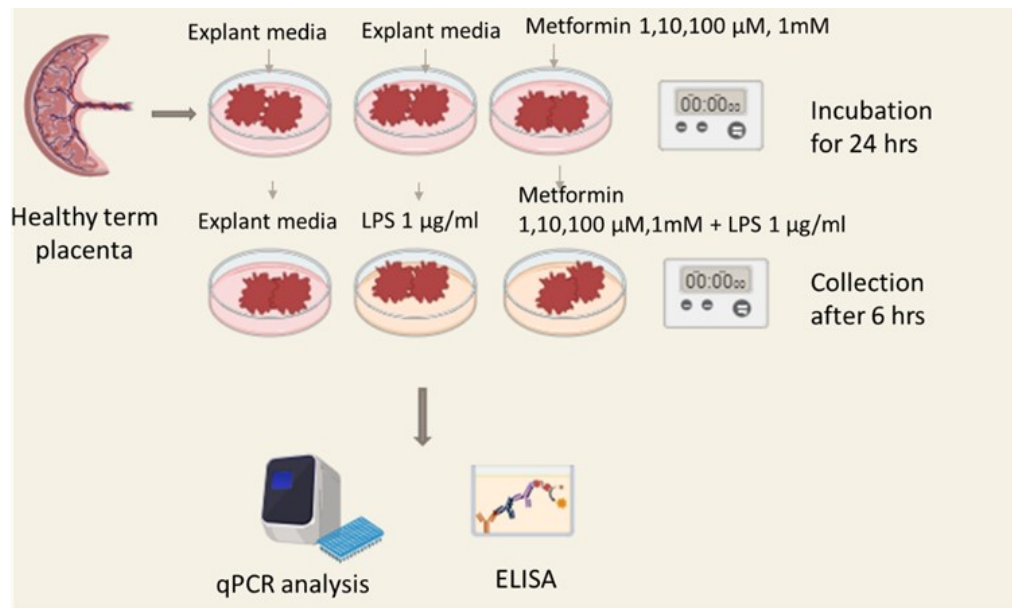
Figure 4 Metformin treatment plan. The figure is reproduced with permission from Fiona Kumnova, the author of the figure.

After the specified incubation periods were finished, the tissue was harvested, washed with cold saline solution, and stored at -80 °C. The supernatant was collected and centrifuged at 10,000 xg for 15 minutes at 4 ºC. The resulting cell-free supernatant was then stored at -80°C until further analysis.

3.5 RNA isolation

RNA was isolated as previously reported in our team's studies (68-70). To the 100 mg of placental tissue was added 1 ml of TRI Reagent and the samples were homogenized in homogenizer for 20 seconds, twice. Next step was to add 200 µl of chloroform, shake in hand for 15 seconds and centrifuge at 12,000 xg for 15 minutes at 4°C, which separated the mixture into 3 layers, shown also in Figure 5:

i)   Aqueous phase with RNA on the top.
ii)   Interphase with DNA.
iii) Organic phase with lipids and proteins in the bottom.
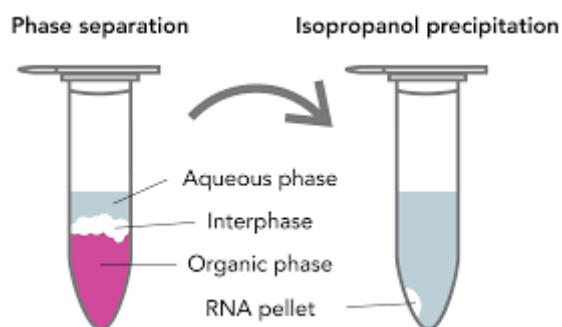
Figure 5 Phase separation in RNA isolation.

The top layer was collected into a new vial and for RNA precipitation was 500 µl of 2-propanol and 2 µl of glycogen added. Next step was to let them stand in room temperature for 10 minutes and centrifuge at 12,000 g for 10 minutes at 4°C. The result of this step was RNA precipitated as a white pellet on the wall of the vial. Subsequently, the pellet was washed with 1 ml of 75% ethanol and centrifuged at 12,000 g for 5 minutes at 4°C. The washing step was repeated once more for better purity. At this point, the pellet was dissolved in 20 µl of API (aqua pro injection).

To evaluate RNA concentration and purity, absorbance (A) was measured using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA). The concentrations were measured at absorbance of 260 nm and purity was evaluated by determining A260/280 ratio (for contamination of protein and DNA) and 260/230 ratio (for contamination of organic solvents). Samples were used in the study only if their 260/280 ratio was between 1.8-2.2 and 260/230 ratio > 1.7. At the end of this procedure, the samples were diluted to concentration of 500 ng/µl and stored at -80°C.

3.6 Reverse transcription

For this procedure, iScript Advanced cDNA Synthesis Kit (Bio-Rad, USA) was used and the procedure was conducted according to the manufacturer´s instructions. 750 ng of RNA in reaction mixtures with total volume of 20 µl was transcribed on a Bio-Rad T100™ thermal cycler. The conditions of this procedure were: 5 min at 25°C, 20 min at 46°C and 1 min at 95°C used for transcriptase inactivation. The obtained cDNA (187.5 ng/µl) was diluted to 12.5 ng/µl and stored at -20°C until use.

3.7 qPCR analysis

Quantitative polymerase chain reaction (qPCR) was performed to evaluate gene expression in target genes in the human placenta explants, as previously described (68-70). cDNA was amplified in a 384-well plate with total reaction volumes of 5 μl per well using the TaqMan Universal Master Mix II no UNG (Thermo Fisher Scientific, USA) and predesigned TaqMan Assays (Thermo Fisher Scientific, USA) – see table 2 for full list of assays. Each sample was amplified in triplicate in QuantStudio 6 Flex (Thermo Fisher Scientific, USA). Firstly, polymerase activation at 95°C for 10 minutes was performed, followed by denaturation and annealing step - 40 cycles composed of 15 seconds at 95°C and 1 minute at 60°C. Four reference genes were used for normalizations of the target genes (Table 2).

The Ct values obtained were used to calculate the gene expression using the ΔΔCt method. The following formulas were used:

$$\text{Relative Quantity}_{\text{reference or target gene}} = 2^{(\text{Ct control} - \text{Ct treatment})}$$

$$\text{Normalized Expression}_{\text{target gene}} = \frac{\text{Relative Quantity}_{\text{target gene}}}{\text{Geometric mean of Relative Quantity}_{\text{reference gene}}}$$

Table 2 List of TaqMan gene assays (Thermo Fisher Scientific, USA).

Gene name	Gene symbol	Assay ID
Reference genes		
Eukaryotic initiation factor-4A	*EIF4A2*	Hs00756996_g1
Ubiquitin	*UBC*	Hs05002522_g1
DNA topoisomerase 1	*TOP1*	Hs00243257_m1
TATA-binding protein	*TBP*	Hs00427620_m1
Target genes		
Interleukin 6	*IL-6*	Hs00174131_m1
Interleukin 1-β	*IL-1β*	Hs0155410_m1
Interleukin 18	*IL-18*	Hs01038788_m1
NLR Family Pyrin Domain Containing 3	*NLRP3*	Hs00918082_m1
Tumor necrosis factor α	*TNF-α*	Hs00174128_m1
Caspase 1	*CASP1*	Hs00354836_m1
Thioredoxin-binding protein	*TXNIP*	Hs01006897_g1
Sirtuin 1	*SIRT1*	Hs01009006_m1

3.8 ELISA assay

The process of ELISA assay procedure is shown in Figure 6. The levels of specific proinflammatory cytokines were assessed through an Enzyme-linked Immunosorbent Assay (ELISA) Kits (Thermo Fisher Scientific, USA) - IL-6 (cat. no. KHC0061C), TNF-α (cat. no. KHC3011C), IL-1β (cat. no. BMS224-2). For this study, a solid-phase sandwich type of ELISA was used. The procedure involved introducing samples and standards into wells coated with a capture antibody and the antigen from the samples adhered to the antibody. Subsequently, the cytokine- biotin conjugate was introduced, attaching to the antigen. Finally, streptavidin-horseradish-peroxidase (HRP) was added, binding to the biotin conjugate. Following each incubation, the unbound fraction was removed by aspirating the liquid, and the wells were washed to prevent nonspecific binding. Upon adding the substrate (Stabilized Chromogen, Tetramethylbenzidine – TMB), absorbances were measured at 450 nm, and the color intensity

reflected the concentration of cytokines in the samples. The concentration was determined using a standard curve.
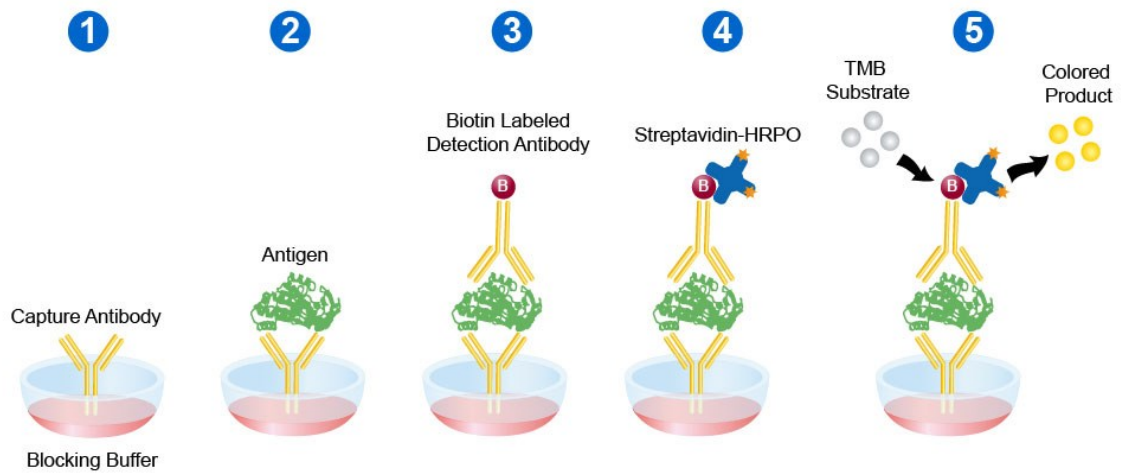
Figure 6 Schematic depiction of sandwich ELISA.

3.9 Statistical analysis

The thesis data presented encompass preliminary investigations conducted with biological replicates numbering ≤ 5. Due to the fact that human placenta tissue was used, notable interindividual variability was observed, particularly evident in gene expression analyses. Therefore, we recognize the necessity for additional biological replicates to accurately assess statistical significance, preferably employing a non-parametric approach. Nevertheless, to evaluate the thesis results, qPCR experiments were analyzed using One-Way ANOVA or multiple t-tests. No statistical analysis was performed in ELISA experiments due to high sample variability and low biological replicate number (n = 4). All analyses were conducted using GraphPad Prism 8.3.1 software (GraphPad Software, Inc., San Diego, USA). Significance levels in the figures are denoted by asterisks: * ($p \leq 0.05$), ** ($p \leq 0.01$), * ($p \leq 0.001$).

4 RESULTS

4.1 Effect of glucose and LPS on the expression of NLRP3-related genes: concentration-dependent studies

In the initial phase of the study, placental explants were exposed to varying concentrations of glucose (10 mM, 15 mM, 20 mM, 25 mM, 30 mM, 35 mM) and LPS (0.1 µg/ml, 1 µg/ml, 10 µg/ml), along with corresponding levels of mannitol as an osmotic control, for a duration of 12 hours. Subsequently, we evaluated the gene expression levels of proinflammatory cytokines (*IL-1β, IL-6, TNFα, IL-18*), NLRP3 components (*NLRP3, CASP1*), *TXNIP*, and *SIRT*.

The test results indicate that LPS induces a classical pathogen-activated inflammatory response, leading to a statistically significant upregulation predominantly in the proinflammatory cytokine genes: *IL-1β* (Figure 7A)*, IL-6* (Figure 7C)*,* and *TNFα* (Figure 7D)*.* Conversely, glucose elicits a less pronounced response indicative of low-grade inflammation (Figure 7). Moreover, these results suggest that there is no strong correlation between glucose concentration and inflammatory effect.
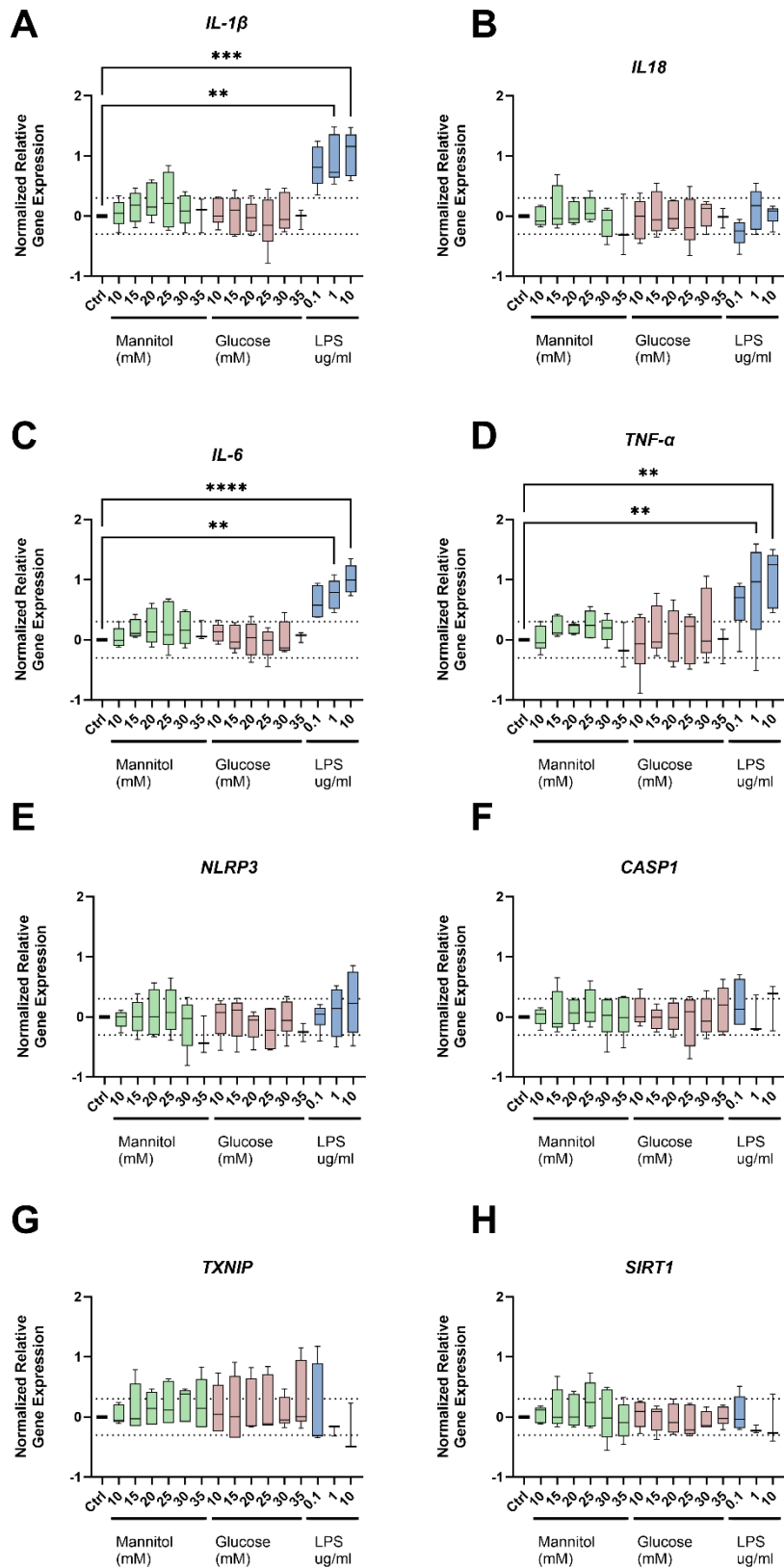
Figure 7 Expression of NLRP3-related genes in human placental explants exposed to mannitol, glucose, and LPS at different concentrations. The analysis of gene expression was conducted using qPCR analysis, and relative expression was normalized to the geometric

mean of *TOP1*, *TBP*, *UBC*, and *EIF4A*. Statistical analysis was performed using One-Way ANOVA. Data are presented as log2-transformed Tukey boxplots; n=5. ** ($p \leq 0.01$), * ($p \leq 0.001$).

4.2 Effect of glucose and LPS on the expression of NLRP3 inflammasome-related genes: time-dependent studies

Following concentration-dependent investigations, we were interested in examining whether the duration of exposure to glucose or LPS influences the gene expression of NLRP3 inflammasome-related genes. Therefore, we conducted experiments at various time points. Human placental explants were subjected to the highest concentrations of glucose (35 mM), LPS (10 µg/mL), and mannitol (osmotic control - 35 mM) for intervals of 1, 3, 6, 12, 24, and 48 hours. Subsequently, the gene expression levels of NLRP3-related genes were assessed.

We observed distinct time-dependent effects, particularly noticeable in explants treated with LPS, where peak regulation occurred at early time points (1 to 12 hours). However, due to considerable interindividual variability, the results did not reach statistical significance. Conversely, in the case of glucose treatment, no visible time-dependent effects were detected (Figure 8).
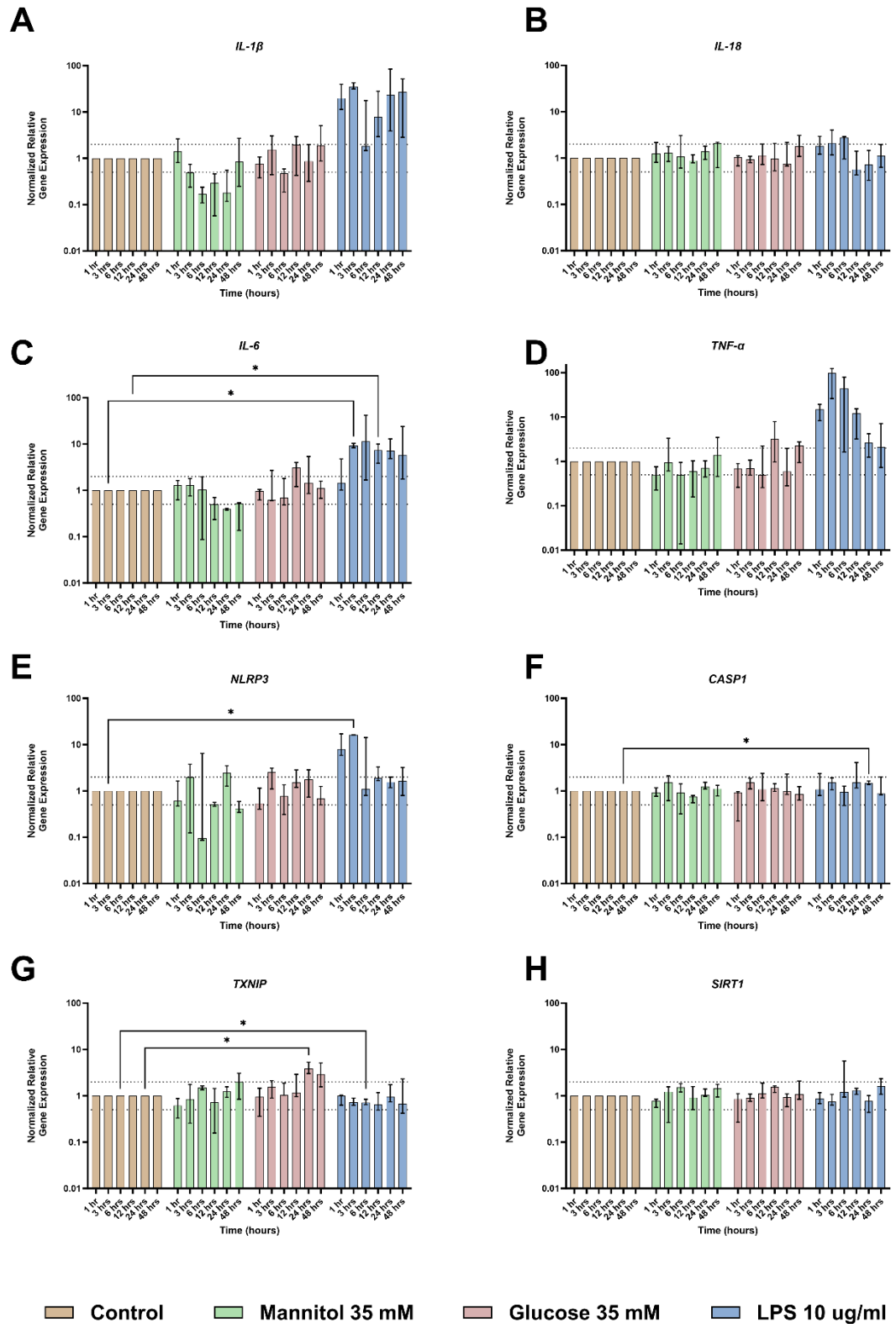
Figure 8 Expression of NLRP3-related genes in human placental explants exposed to mannitol, glucose, and LPS at different time points. The analysis of gene expression was conducted using qPCR analysis, and relative expression was normalized to the geometric mean

of *TOP1*, *TBP*, *UBC*, and *EIF4A.* Statistical analysis was performed using multiple t-tests. Data are presented as log2-transformed median with interquartile range; n=5. * ($p \leq 0.05$).

4.3   Effect of metformin on the expression of NLRP3 inflammasome-related genes

In the second phase of the study, we evaluated the gene expression of proinflammatory cytokines (*IL-1β, IL-6, TNFα, IL-18*), NLRP3 components (*NLRP3, CASP1*), *TXNIP*, and *SIRT* in placental explants treated varying concentrations of metformin for 24 hours, followed by the addition of LPS 1 µg/ml for an additional 6 hours. We observed a significant upregulation of several NLRP3-related genes upon exposure to LPS. However, to our surprise, metformin neither reduced nor prevented the inflammatory response induced by LPS, but instead increased it with increasing dose (Figure 9). In the case of *TNFα* (Figure 9D) and *CASP1* (Figure 9F), there was a significant increase in gene expression after treatment with metformin and LPS compared to LPS alone. A similar tendency was observed for *IL-6* (Figure 9C).
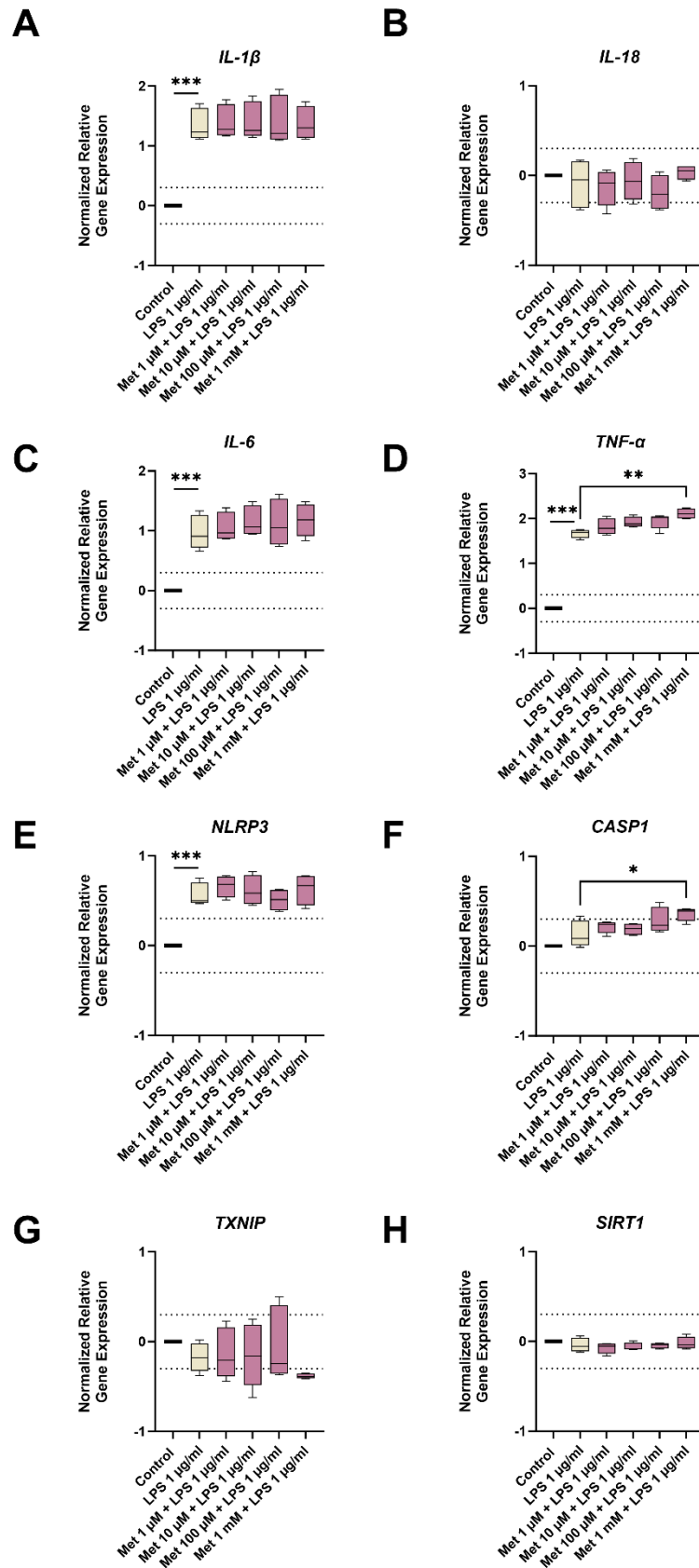
Figure 9 Expression of NLRP3-related genes in human placental explants exposed to metformin. Explants were treated with different doses of metformin (1 µM, 10 µM, 100 µM

and 1 mM) for 24 hours, followed by the addition of LPS 1 µg/ml for an additional 6 hours. The analysis of gene expression was conducted using qPCR analysis, and relative expression was normalized to the geometric mean of *TOP1*, *TBP*, *UBC*, and *EIF4A*. Statistical analysis was performed using One-Way ANOVA. Data are presented as log2-transformed Tukey boxplots; n=4. * ($p \leq 0.05$), ** ($p \leq 0.01$), * ($p \leq 0.001$).

4.4 Effect of metformin on the release of pro-inflammatory cytokines by human placental explants

In addition to gene expression results, we assessed the protein release of selected proinflammatory cytokines in the culture media of explants exposed to glucose and LPS. As illustrated in Figure 10, LPS treatment led to a high release of IL-1β, TNFα, and IL-6 into the culture media. Furthermore, we observed tendency for an increased release of proinflammatory markers in the media after treatment with metformin and LPS, which was also concentration dependent.
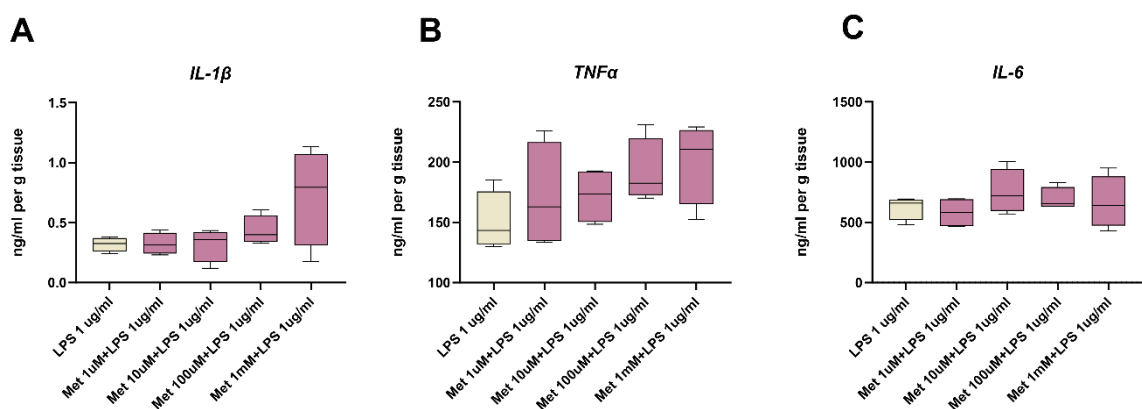

Figure 10 Protein levels of selected pro-inflammatory cytokines in human term placenta explants exposed to metformin and LPS. IL-1β (A), TNFα (B), and IL-6 (C) production by villous explants were analyzed by ELISA. Data are presented as Tukey boxplots; n = 4.

5 DISCUSSION

Our study aimed to investigate the inflammatory response of human placental tissue to high glucose levels and LPS exposure, as well as explore the potential anti-inflammatory effects of metformin. Through gene expression analysis using qPCR and protein expression analysis via ELISA, we obtained insights into the modulation of proinflammatory cytokines (*IL-6, TNF-α, IL-1β, IL-18*), NLRP3 inflammasome-related genes (*NLRP3, CASP1*), and selected genes involved in tissue stress response (*TXNIP, SIRT1*) in placental explants under various experimental conditions.

The results of our gene expression analysis indicated that LPS exerted effects on the inflammatory state of the placental tissue. Specifically, we observed a strong effect of LPS on the upregulation of pro-inflammatory cytokines *IL-6, TNFα,* and *IL-1β*, which was also concentration- and time-dependent. These results correspond to previous investigations employing LPS as a stimulus in various placental models and reporting elevated levels of proinflammatory cytokines, including TNF-α, IL-6, and IL-1β (73). LPS, derived from the outer membrane of Gram-negative bacteria, serves as a commonly employed tool in laboratory settings to induce inflammation (74). By engaging TLR4, LPS initiates a signaling cascade culminating in NF-κB activation and subsequent production of proinflammatory cytokines like IL-6, TNF-α, and IL-1β (75).

Previous investigations have utilized placental models to mimic hyperglycemic conditions. Given that the threshold for GDM lies between 5.1 and 6.9 mM in repeated fasting plasma glucose levels, we chose 5 mM glucose concentration to represent the control in this study. Conversely, concentrations ranging from 20 to 50 mM have commonly been used to simulate a GDM-like environment (76); hence, we selected 35 mM as the highest concentration used in our study. To distinguish whether the responses observed in our placental explant model are attributed to the direct effects of high glucose content rather than elevated osmolar pressure, additional controls for osmolarity were conducted using mannitol as a non-permeating sugar alcohol (77). However, our findings revealed no evident differences between control and mannitol-treated explants, indicating no effect of osmolarity.

We illustrate that glucose exposure elicits only a modest increase in the expression of numerous NLRP3-related genes in human placental explants and there was no clear linear relationship between glucose concentration and inflammatory effect. This finding aligns with

reports of sterile inflammation in GDM, often referred to as low-grade inflammation (2). This inflammatory response appears to be partly mediated by the NLRP3 inflammasome, suggesting a potential role in the pathophysiology of GDM. Furthermore, our results highlight the complexity of the inflammatory processes involved in placental pathology associated with hyperglycemic conditions during pregnancy.

Building upon these findings, our next aim was to evaluate the effects of metformin, a drug occasionally used for the management of GDM in pregnancy, on placental inflammation. To achieve this, we designed a follow-up series of experiments in which placental explants were exposed to increasing concentrations of metformin for a duration of 24 hours. Subsequently, the same doses of metformin were administered to the explants alongside with 1 µg/ml of LPS. This experimental strategy was established on the notion that metformin might mitigate the inflammatory effects induced by LPS on NLRP3 inflammasome activation, potentially by disrupting the priming and subsequent activation stages of the inflammasome pathway (78).

Contrary to our initial expectations, our findings revealed that metformin neither alleviated nor prevented the inflammatory response elicited by LPS; instead, it exhibited a dose-dependent exacerbation of the inflammatory response. Particularly noteworthy was the observation that TNFα, both in terms of gene expression and cytokine release, was significantly upregulated in the metformin together with LPS treatment group compared to LPS alone. This unexpected outcome suggests a complex interplay between metformin and LPS-induced inflammation, possibly implicating metformin in the activation phase (phase two) of NLRP3 inflammasome activation rather than the priming phase (phase one).

Interestingly, a recent study by Ryssdal et al. examined the impact of metformin on maternal immunological status in pregnant women with polycystic ovary syndrome. Results showed that metformin treatment led to higher serum levels of several multifunctional cytokines throughout pregnancy, suggesting that metformin has immunomodulating rather than anti-inflammatory properties during pregnancy (79). This finding, in line with our own results, challenges the conventional perception of the role of metformin in inflammation management.

Collectively, these finding requires further investigation to elucidate the precise mechanisms underlying the interactions between metformin and inflammation during pregnancy and in the placenta. Future experiments will be essential to distinguish whether the

involvement of metformin in placental inflammation extends beyond its interaction with LPS. We are currently performing another set of experiments whereby placental explants are exposed to metformin alone. Such investigations aim to conclusively determine whether metformin is also implicated in the priming stage of NLRP3 inflammasome activation or if its effects are confined solely to the activation phase.

6 CONCLUSION

In summary, our study investigated the inflammatory response of human placental tissue to high glucose levels and LPS exposure, alongside exploring the potential anti-inflammatory effects of metformin. Notably, LPS induced a robust upregulation of proinflammatory cytokines in placental tissue, consistent with existing literature on LPS-mediated inflammation. Additionally, our examination of glucose exposure revealed a modest increase in NLRP3-related gene expression, corresponding to the low-grade inflammatory response in GDM. Unexpectedly, metformin exacerbated rather than alleviated the inflammatory response induced by LPS, suggesting a complex interaction between metformin and the NLRP3 inflammasome in the placenta. This finding highlights the need for further investigation into the precise mechanisms underlying these interactions. Future studies in our team are aimed at elucidating the role of metformin in placental inflammation and its potential implications for pregnancy-associated disorders.

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