

Hyperglycemia induces inflammatory mediators in the human chorionic villous

Simone Corrêa-Silva^{a,b,c,2}, Aline P. Alencar^{b,2}, Juscielle B. Moreli^{a,d,g}, Alexandre U. Borbely^{b,e}, Larissa de S. Lima^f, Cristóforo Scavone^f, Débora C. Damasceno^a, Marilza V.C. Rudge^a, Estela Bevilacqua^{b,*}, Iracema M.P. Calderon^{a,*}

^a Graduate Program in Gynecology, Obstetrics, and Mastology, Botucatu Medical School, São Paulo State University-UNESP, São Paulo, Brazil

^b Department of Cell and Developmental Biology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil

^c Paulista University, Institute of Health Sciences, São Paulo, Brazil¹

^d Post-Graduation in Structural and Functional Biology, Federal University of São Paulo, São Paulo, SP, Brazil¹

^e Institute of Health and Biological Sciences, Federal University of Alagoas, Alagoas, Brazil¹

^f Department of Pharmacology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil

^g Faceres School of Medicine, São José do Rio Preto, SP, Brazil

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ABSTRACT

This study was based on the hypothesis that IL-1 β and its central regulator, the inflammasome, may play a role in the inflammatory condition exhibited by placental tissues from mothers with different gestational hyperglycemia levels. Pregnant women were classified according to the glycemic reference as non-diabetic (n = 15), mild gestational hyperglycemia (n = 15), gestational diabetes mellitus (n = 15) and type 2 diabetes mellitus (n = 15). We investigated levels of pro-inflammatory factors in maternal plasma and placental tissues (by ELISA or immunohistochemistry) and, NF κ B activity (by electrophoretic mobility shift assay) and inflammasome protein expression (by Western blot) in chorionic villous. Maternal plasma and placental levels of inflammatory factors (IL-1 β , IL-6, and MCP-1) were increased during all hyperglycemic conditions. Villous stroma cells showed strong immunoreactivity to CD68. In addition, with syncytiotrophoblast, the villous stroma cells were also stained to detect iNOS, MCP-1, TLR2, and TLR4. Although the levels of protein had fluctuated in the groups, NLRP1, NLRP3, ASC, and Caspase 1 were up-regulated in all hyperglycemic groups suggesting the inflammasome may be assembled in these pregnant women. The NF κ B activity also exhibited higher levels in hyperglycemic groups, which might imply in pro-inflammatory cytokines production. In summary, increased maternal glucose levels during pregnancy changed systemic and placental inflammatory patterns, which occurred in parallel with the expression of inflammasome factors and processing and secretion of the pro-inflammatory cytokine IL-1 β . These results suggest an inflammatory condition in all gestational hyperglycemic conditions, even in hyperglycemia that is less severe than gestational or overt diabetes, likely associated with inflammasome activation and inflammatory cytokine secretion. Inflammasome activation as a possible source of inflammatory factors may be an important target to be considered while managing hyperglycemia and preventing adverse pregnancy outcomes.

Abbreviations: IL-1 β , interleukin-1 β ; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; TNF- α , tumor necrosis factor alpha; IL-10, interleukin 10; iNOS, inducible nitric oxide synthase; TLR, Toll-like receptors; TLR2, Toll-like receptor 2; TLR4, Toll-like receptor 4; AIM2, interferon-inducible protein absent in melanoma 2; NLRC4, NLR family CARD domain containing 4; NLRs, Nod-like receptors; NLRP1, NLR family pyrin domain containing 1; NLRP3, NLR family pyrin domain containing 3; ASC, apoptosis speck-like protein; NF κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; PAMPs, pathogen-associated molecular patterns; DAMPs, damage-associated molecular pattern molecules; EMSA, electrophoretic mobility shift assay; CARD, caspase activation and recruitment domain; DM1, type 1 diabetes mellitus; DM2, type 2 diabetes mellitus; GDM, Gestational diabetes mellitus; MGH, mild gestational hyperglycemia; GM, glycemic mean; GP, glucose profile; GTT, glucose tolerance test

* Corresponding authors at: Department of Cell and Developmental Biology, Institute of Biomedical Sciences, University of São Paulo, Av. Prof. Lineu Prestes, 1524, Butantan, São Paulo, SP 05508-000, Brazil. (E. Bevilacqua). Department of Obstetrics and Gynecology, Botucatu Medical School, UNESP—São Paulo State University, Distrito de Rubião Jr. s/n, CEP 18618-000, Botucatu, São Paulo, Brazil. (I.M.P. Calderon).

E-mail addresses: bevilacq@usp.br (E. Bevilacqua), calderon@fmb.unesp.br (I.M.P. Calderon).

¹ Present address.

² Equal contributions and credits.

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1. Introduction

Pregnancies complicated by hyperglycemia are associated with short-term and long-term risk of adverse outcomes in both mother and offspring, which includes disturbances in embryonic and fetal development, type 2 diabetes mellitus (DM2), and cardiovascular diseases [1–4]. Implications for adverse intra-uterine programming have also been largely discussed in the literature [5]. These complications are not restricted to pregnancies associated with pre-gestational (type 1 diabetes mellitus, DM1, or DM2) or gestational diabetes (GDM). It also occurs in pregnant women diagnosed with mild gestational hyperglycemia (MGH), a condition in which the pregnant woman has a normal glucose tolerance test, but altered glycemic profile [5,6]. Increased rates of perinatal mortality (tenfold compared with non-diabetic pregnant women) and augmented incidence of infants born large-for-gestational-age or with macrosomia (rates similar to those seen in diabetic pregnant women [2,7]) and, incidence of type 2 diabetes (DM2) after pregnancy equivalent to GDM have been reported in pregnant women with MGH [8].

Changes in the maternal environment have received particular attention in the last decades as they can program permanent effects on the fetal physiology and metabolism. This fetal programming may result from different stimuli throughout pregnancy or at specific periods and increases the prevalence of coronary heart disease and diabetes, among many other conditions during adulthood [9]. Metabolic adaptations to fail or exceed the fetal nutritional demand (as for glucose in diabetic pregnancies), stress, chronic and acute inflammation are some of the aspects that are being studied [10]. The unbalanced expression of inflammatory mediators alter the maternal environment and metabolism during gestation and increase the potential for gestational systemic insulin resistance, participating in the development of DM2 and GDM [11–12]. Inflammation at the placental territory also contributes to the fetal developmental environment and therefore has been the subject of numerous studies in hyperglycemic gestations [9].

During diabetes, high levels of serum glucose are also associated with increased levels of lipid serum [11] and both induce the expression of Toll-like receptors (TLR) 2 and TLR4 in macrophages and placenta [13]. These receptors recognize conserved pathogens-associated molecular patterns (PAMPs), inducing innate immune responses which are essential for host defenses [14]. They can also be activated by endogenous molecules (damage-associated molecular patterns, DAMPs) released under conditions of cellular stress or tissue injury [15] as free fatty acids and glycation end products [16]. TLRs and nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs) trigger the formation of an inflammasome in the cytoplasm, a multi-protein complex, when active, recruits inactive procaspase-1 molecules to form activated caspase-1 [17].

The assembly of the inflammasome complex requires a particular NLR family protein (or AIM2), the Apoptosis-associated Speck-like protein containing a CARD (ASC) adaptor protein and pro-caspases-1, 5 and 8 [17]. Among the known inflammasome complexes (NLRP1, NLRP3, NLRC4, and AIM2) the best-characterized inflammasome is NLRP3, whose activation is thought to be dependent on NF κ B signaling [18].

Caspase-1-mediated cleavage activates the pro-inflammatory cytokines interleukin (IL)-1 β and IL-18 into their secreted active forms [13,17]. IL-1 β acts as a signal cytokine of the local injury and is involved in secondary cascades for the production and release of additional pro-inflammatory cytokine and therefore influencing local and systemic immune responses to resolve the injury-triggering factor. The mechanisms associated with the inflammasome in diabetes have been widely discussed in the literature [19], but little is known about gestational hyperglycemia that is not characterized as DM2 or GDM.

During gestation, the maternal blood flows into the placental intervillous space, allowing the exchange of oxygen and nutrients between fetal and maternal organisms and exposing the maternal blood

microenvironment to the fetal component of the placenta, the chorionic villi [20]. Changes in this environment, such as increased glucose levels, whether enough to induce inflammasome activity and consequently inflammatory cytokines production may, therefore, affect gestation and impact the development and adult life of the neonate.

In this context, we herein compare the levels of pro-inflammatory factors in serum and fetal placental compartment (chorionic villous) in different hyperglycemic gestational conditions (mild gestational hyperglycemia, gestational diabetes mellitus, and type 2 diabetes). In addition, we analyze the expression of inflammasome proteins in order to understand whether it may have a potential role in the subclinical inflammatory condition that is also seen in the mild gestational hyperglycemia.

2. Methods

2.1. Study design and subjects

The Research Ethics Committee on human beings from Botucatu Medical School, UNESP, Brazil have approved all the procedures used in this cross-sectional study. Written informed consent was obtained from all subjects according to the principles of the Declaration of Helsinki.

Sixty pregnant women, allocated in four study groups, participated in the study. Pregnant women with DM2 (n = 15) were referred to the Diabetes and Pregnancy Service of Botucatu Medical School with a confirmed diagnosis. The diagnosis of GDM or MGH was established between 24 and 28th gestational weeks, by the 75-g glucose tolerance test (75-g-GTT) according to ADA's criteria [26] and/or the glucose profile (GP - fasting glucose \geq 90 mg/dL or postprandial glucose \geq 130 mg/dL) test according to Rudge [6]. From the data, the women were classified into the following study groups: non-diabetic (ND; normal 75-g GTT and GP; n = 15), MGH (n = 15; normal 75-g GTT and abnormal GP) GDM (abnormal 75-g GTT first reported during the pregnancy; n = 15).

According to our treatment protocol, the glycemic control was achieved with fasting glucose < 95 mg/dL, 1 h post-prandial < 140 mg/dL, and 2 h post-prandial < 120 mg/dL. At this time, MGH and GDM pregnant women were initially treated with diet and exercise; insulin was administered only if necessary. All DM2 pregnant women were treated with diet, exercise and insulin at the first attendance in our service, and were readjusted weekly [6,7,12].

2.2. Exclusion criteria and subject characterization

The exclusion criterion for this study was as follows: women with DM1, multiple pregnancies, fetal malformations, deliveries before the 37th week, self-reported as current daily smokers, active or past infection with hepatitis C, hepatitis B, HIV or tuberculosis, family history of cancer, diagnosed coronary artery disease or females who are under treatment for a medical condition requiring chronic use of medications that were unrelated to the DM2 condition [6,7].

Pregnant women in this study were characterized by age, weight gain during pregnancy, glycated hemoglobin levels (HbA1c) during the third trimester (high-performance liquid chromatography—D10™ Hemoglobin Testing System, Bio-Rad Laboratories, Hercules, USA) and glycemic mean (GM). For the pregnant women classified as DM2, GDM, and MGH, the GM was calculated by the arithmetic mean of all plasma glycemic levels evaluated in all GP performed during pregnancy. For the ND pregnant women, the GM was calculated by the arithmetic mean of all plasma glycemic levels assessed in the diagnostic GP. All plasma glucose levels were evaluated by the glucose oxidase method (Glucose Analyzer II Beckman®, Fullerton, CA, USA) [6,7].

2.3. Samples collection

Maternal blood samples were collected at 37–40 gestational weeks, before the beginning of labor, in Vacutainer tubes (Becton Dickinson, USA) containing separator gel or EDTA. For cytokine analyses, the plasma was separated by centrifugation at 4 °C for 30 min at 1.000g, and stored at –80 °C until analyzed. Aliquots of EDTA tubes were separated for immediate analyses of the glucose and HbA1c levels.

Term placental tissues were obtained after elective cesarean sections. Small fragments of chorionic villi (fetal placental area) were isolated under a stereomicroscope and exhaustively washed in PBS to remove blood cells. Samples were fixed for immunohistochemistry or frozen in liquid nitrogen for protein evaluation by Western blot or NFκB activity by electrophoretic mobility shift assay. All reagents were purchased from Sigma Chemical Co. (St Louis, MO) unless otherwise specified.

2.4. Placenta homogenate and protein assay

Frozen placental tissues were homogenized in RIPA buffer (1% NP-40, 0.25% Na-deoxycholate, 1 mM NaF, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na₃VO₄, 50 mM Tris-HCl, pH 7.4) with protease inhibitors before being centrifuged at 6.000g at 4 °C. The supernatants were collected and frozen at –80 °C. Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific Inc. Waltham, MA, USA) measured total protein concentration.

2.5. Plasma and placental IL-1β, IL-6 and MCP-1 analysis

Plasma and placental IL-1β and IL-6 concentrations were determined by immune enzyme assays using commercial kits (Human IL-1β and IL-6 DuoSet ELISA kits, BD Biosciences, USA). MCP-1 was measured using the dual antibody sandwich technique, as described by the manufacturer (R&D, USA). Tetramethylbenzidine and hydrogen peroxide were used as substrates in the peroxidase reaction. Plates were read at 450 nm in a SpectraMax Plus384 Absorbance Microplate reader, using Soft Max Pro Data Acquisition & Analysis Software. Concentrations of the cytokines were calculated in picograms/mL using a standard curve.

2.6. Placental immunolocalization of MCP-1, iNOS, TLR2, TLR4 and CD68

Placental fragments were fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) overnight and routinely embedded in paraffin. Five-μm sections were dewaxed, rehydrated, and immunostained through the following sequential incubations:

- (i) 3% hydrogen peroxide in methanol for 5 min at room temperature to deactivate endogenous peroxidase activity.
- (ii) 3% BSA-PBS for 1 h at 37 °C to block non-specific reactions.
- (iii) Primary mouse anti-human monoclonal antibodies anti-MCP-1 (1:100), iNOS (1:100), TLR2 (1:67) and TLR4 (1:67) are from R&D System, USA; anti-CD68 (1:100) from Invitrogen, USA for 18 h at 4 °C.
- (iv) Secondary biotinylated antibodies and anti-mouse (KPL, CA, USA) for 1 h at 25 °C.
- (v) Avidin-biotin-peroxidase complex (Vectastain Elite ABC Kit, Vector, Burlingame, CA, USA) for 45 min at 25 °C.

Peroxidase activity was detected by color development with diaminobenzidine-hydrogen peroxide as the chromogen (Sigma Chemical Co., St. Louis, MO, USA). The sections were lightly counterstained with Mayer's hematoxylin. Control reactions omitted the primary antibody. At least 15 placental fragments from each experimental group with 3 different randomly histological sections per fragment were subjected to immunohistochemistry reactions, converted to digital images (captured

at a 100× magnification) and quantified using Image J (v. 1.43, NIH, Bethesda, MD, USA) software.

2.7. Proteins of the inflammasome pathway

Inflammasome proteins in placental tissues were investigated by Western blot. Villous placental tissue samples were previously washed and lysed with a Precellys homogenizer (Bioamerica Inc., Corporate Headquarters, USA) in RIPA buffer supplemented with protease inhibitors (Sigma). Samples were centrifuged (13.000 rpm, 4 °C for 10 min) and the soluble fractions used to detect NLRP1, NLRP3, ASC, and Caspase-1. Samples were resolved by SDS-PAGE (12.5% acrylamide, Bio-Rad Inc., USA) and proteins were transferred to nitrocellulose membranes (0.45 m, Hybond ECL, GE Healthcare, USA). The membranes were blocked for 1 h in TBS-T-milk 3% (140 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.1% Tween-20, 3% powdered milk) and incubated overnight, at 4 °C, with the primary antibodies (anti-NLRP1 and NLRP3, both from Cell Signaling Technology Inc., USA, anti-ASC, Merck Millipore Inc., USA) diluted 1:1000 in the same buffer. Anti-Caspase-1 were purchased from Biologend Inc., USA and diluted 1:250. Membranes were then incubated for 1 h with the HRP-conjugated secondary antibody (diluted 1:1000, KPL, Synapse Biotechnology, Brazil). Bound antibodies were visualized (peroxidase activity) using the ECL method. Western blots were scanned, and densitometric analysis of the bands were carried out using Image J program (NIH, Bethesda, USA). The density of each band was normalized with beta-actin (as housekeeping protein) as arbitrary units (AU) values.

2.8. Placental NFκB activity

NFκB activity in placental samples was evaluated by electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared from each tissue homogenate. A double-stranded cDNA encoding 5'-AGT TGA GGG GAC TTT CCC AGG C-3' NFκB was generated and end-labeled with γ-³²P-ATP and T4 polynucleotide kinase. Binding reactions were prepared in a final volume of 20 mL with 50 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, 5 mM MgCl₂, 2.5 mM DTT, 300 mM NaCl, 0.25 μg/μL poly (dIdC) and 20% glycerol. The nuclear extracts (10 μg) and radiolabeled NFκB probe (2.5 × 10⁴ cpm/μL) were incubated at room temperature, for 30 min. Bound complexes were separated by electrophoresis on 5.5% non-denaturing polyacrylamide gel in 90 mM Tris, 1 mM EDTA, and 90 mM boric acid. The membranes were visualized by autoradiography. Pierce BCA Protein Assay (Thermo Fisher Scientific Inc., Waltham, MA, USA) used an equivalent amount of each sample to quantify the protein concentration.

2.9. Statistical analysis

Analysis of variance and the Tukey's multiple comparison test were used for quantitative variables with normal distributions. For those with an abnormal distribution, the generalized linear model with gamma distribution and the log-link function, and the LSMeans test were used for multiple comparisons. Data were presented as means ± SD/SEM, with significance set at *p* < 0.05.

3. Results

3.1. Maternal characteristics

Maternal characteristics are summarized in Table 1. Non-diabetic mothers were significantly younger than the hyperglycemic mothers (*p* < 0.05). Weight gain was similar among studied groups. As expected, plasmatic glucose levels were significantly increased among all hyperglycemic mothers (ND vs. MGH, GDM and, DM2; *p* < 0.001), as well glycated hemoglobin (HbA1c) levels in the diabetic groups (ND vs. GDM and DM2; *p* < 0.001). Analysis of this data in light of the insulin

Table 1
Maternal data.

	ND (n=15)	MGH (n=15)	GDM (n=15)	DM2 (n=15)
Age (years)	23.54 ± 6.74	32.05 ± 5.77*	32.27 ± 5.81*	33.11 ± 5.60*
Weight gain (Kg)	13.18 ± 3.20	11.47 ± 6.06	10.31 ± 7.98	8.95 ± 4.15
HbA1c,% (mmol/mol)	5.3 ± 0.5 (34 ± 3)	5.7 ± 0.8 (39 ± 4)	6.1 ± 0.7 (43 ± 4)**	6.5 ± 0.8 (47 ± 5)**
GM (mg/dL)	81.08 ± 8.70	96.72 ± 7.30**	107.20 ± 13.26**	113.72 ± 19.61**

Weight gain during pregnancy; GM: glycemic mean, HbA1c: glycated hemoglobin. Data are presented as mean ± standard deviation. * $p < 0.05$ when compared to ND group; ** $p < 0.001$ when compared to ND group.

therapy, showed differences only in GM and HbA1c, being higher in mothers who received insulin (data not shown).

3.2. Maternal and placental inflammatory profile changes in hyperglycemic conditions

In the fetal compartment of the placenta, depending on the antigen investigated, immunoreactivity was found in the syncytiotrophoblast, stromal cells, and endothelial cells.

CD68-stained cells in the chorionic villous showed an irregular shape, rounded nuclei, and granulated cytoplasm. A substantial increase in CD-68 reactivity was seen in classical diabetic groups (GDM and DM2, Fig. 1A, c-d) in comparison with the normoglycemic group (Fig. 1B). This finding suggests that hyperglycemia may influence the density of these fetal macrophages.

A stronger immunoreactivity to iNOS and MCP-1 were seen in the chorionic villi of hyperglycemic groups (Fig. 1A, e-l). The iNOS reactivity was stronger in hyperglycemic placentas in comparison with the reaction in placentas from ND group. iNOS was immunolocalized in stromal cells and cytoplasm of the syncytiotrophoblast (Fig. 1A, e-h). MCP-1 immunostaining was intense in the vascular endothelium and the cytoplasm of the syncytiotrophoblast and stromal cells (Fig. 1A, i-l). Image analysis showed the prevalence of the inflammatory markers iNOS and MCP-1 in placentas of mothers with hyperglycemic disturbances (Fig. 1C-D, respectively). A significant increase of IL-1 β , IL-6 and MCP-1 levels ($p < 0.05$) in placental tissues (Fig. 1E-G) and maternal plasma (Fig. 1H-J) suggest the development of a local and systemic inflammatory profile in all hyperglycemic groups.

3.3. The effects of hyperglycemia on TLR2 and 4 expressions and NFKB activity in chorionic villi

Toll-like receptors (TLRs) play a significant role in the progression of diabetes, recognizing PAMPs and DAMPs, leading to the production of cytokines such as IL-1 β [19]. The relevance of these receptors in other hyperglycemic conditions was investigated through the immunolocalization of TLR2 and 4 in placental tissues. Both surface receptors were detected in chorionic villi of healthy pregnant women, particularly in the syncytiotrophoblast (Fig. 2A). In placentas of hyperglycemic mothers (MGH, GDM, and DM2), the reactivity was also seen in the villous stromal cells (Fig. 2A, b-f, g-k). Quantification showed that hyperglycemia is associated with increased TLR2 (Fig. 2B) and TLR4 (Fig. 2C) reactivity in the placenta.

Several studies have highlighted the activation of NFKB in diabetes and its association with the increase of transcription and production of pro-IL-1 β [31–33]. Herein, the activation of NFKB was evaluated by EMSA. As seen in Fig. 2D, all different degrees of the hyperglycemic condition resulted in NFKB activation in chorionic villi.

3.4. Inflammasome components increase in placental tissues from hyperglycemic women

There was a significant increase in protein expression of NLRP1 (Fig. 3A), NLRP3 (Fig. 3B) and Caspase-1 (Fig. 3D) in the fetal portion of placentas from hyperglycemic groups (MGH, GDM, and DM2, $p < 0.05$) when compared with ND group. Protein expression of ASC increased only in placentas from diabetic pregnancies (Fig. 3C).

4. Discussion

Chronic proinflammatory cytokines are considered to be pathologic mediators of diabetogenic metabolic disorders, associated with insulin resistance and pancreatic islet cell death [8–10,21,22]. Recent studies have shown the participation of inflammasome complexes triggered by mechanisms able to sense specific metabolic-changed danger signals and control the secretion of proinflammatory cytokines such as IL-1 β and IL-18 in this aberrant proinflammatory response [23]. Previous studies also shown in DM2 patients with increased levels of the basal NLRP3 inflammasome, elevated caspase-1 activation and higher levels of IL-1 β and IL-18 in monocytes derived from peripheral blood and myeloid cells, respectively [14]. However, the question remained whether inflammasome complexes might also be associated with mild hyperglycemia during gestation, somehow mimicking the well-characterized DM2 or GDM [6–7].

Collectively, our studies demonstrate IL-1 β , IL-6, iNOS, and MCP-1 as key proinflammatory mediators in pregnancies complicated by hyperglycemia. In contrast, Hara et al. [24] also found increased levels of inflammatory cytokines, but not IL-1 β in mild gestational hyperglycemia. Since our measurements resulted from a direct analysis of placental tissues, one possible explanation for this difference is the procedures used to isolate placental cells in Hara et al.'s studies, which may interfere with the integrity of molecules necessary for the inflammasomes to fully function and therefore, interfere with the IL-1 β levels.

A great focus was placed on the production of IL-1 β by placental cells in this study. It is primarily involved in the signaling cascade that triggers translocation of the transcription factor nuclear factor-kappa B (NFKB) into the nucleus where it induces the transcription of pro- and anti-inflammatory genes including IL-6, iNOS, and COX-2 [25]. Changes in IL-1 β levels associated with the inflammatory biomarkers iNOS and MCP-1, as we found, suggest that lower mean blood glucose activates the expression of the inflammatory cascade at both plasma and placenta levels.

TLRs and inflammasome complexes have recently been proposed to have a pivotal role in several inflammatory diseases, likely through NFKB activation [26]. Expression of Toll-like receptors 2 and 4 in term placenta has been previously demonstrated [13] and was herein immunolocalized in chorionic villi, and particularly augmented in all hyperglycemic conditions. Binding of TLR-2 or TLR-4 to cognate ligands (chaperones (HSP60/70), extracellular matrix components and glycation end products (among many others) stimulates the production of

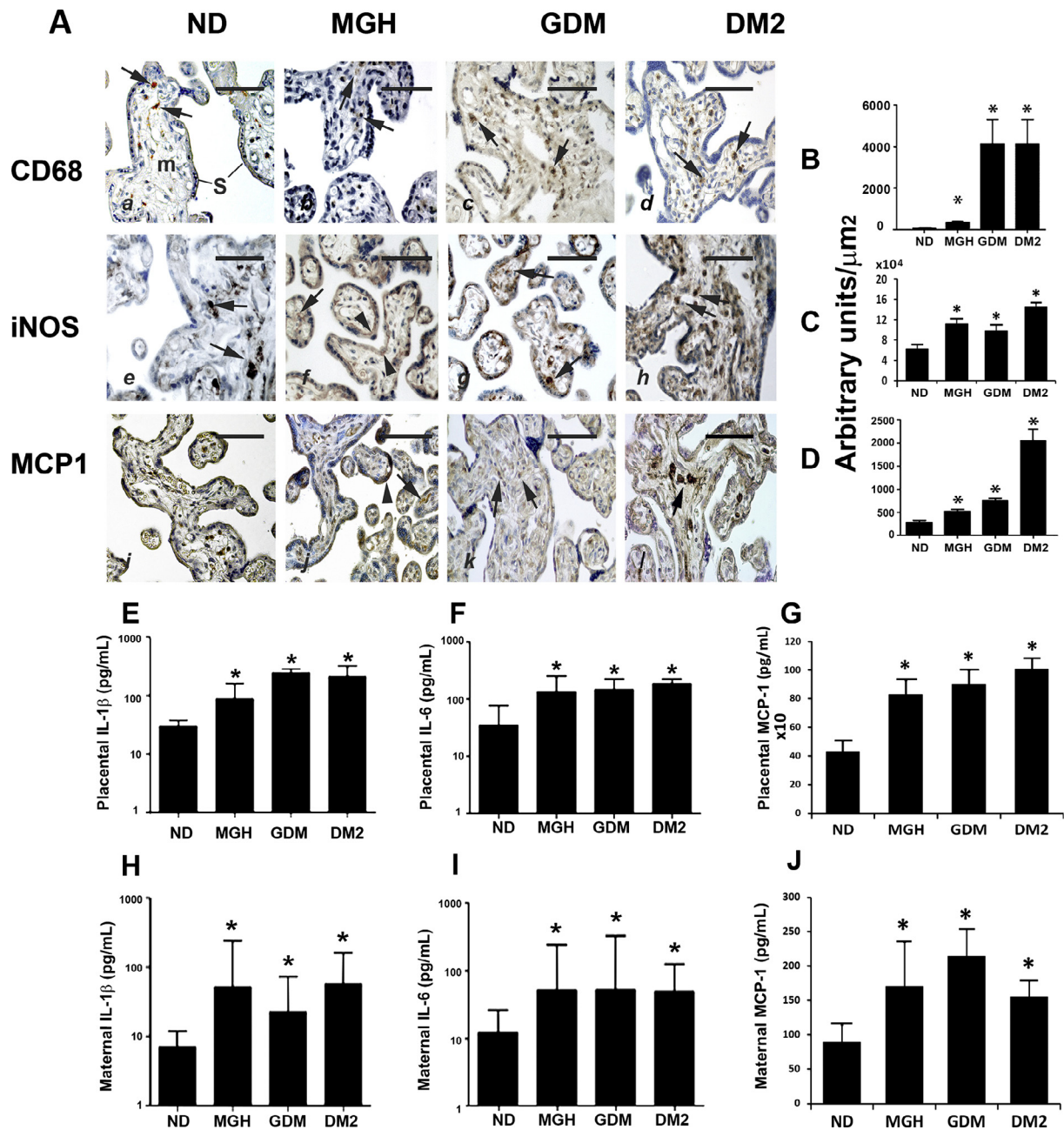


Fig. 1. Hyperglycemia induces the maternal and placental inflammatory profile. Immunolocalization (A) and quantification (B–D) of CD68 cells (Aa–d; B), iNOS (Ae–h; C) and MCP-1 (Ai–l; D) in chorionic villi. Immunoperoxidase counterstained with Mayer's hematoxylin. Bar = 150 μm. Reactive stromal cells (arrows) and syncytiotrophoblast (arrowhead) are depicted in the photomicrographs. Quantification of the immunohistochemical staining was performed by intensity of the brown immunostaining generated by Image J software. The values given are in arbitrary units/μm² (mean ± SD). **p* < 0.05 compared to ND group. Levels (pg/mL) of IL-1β (E), IL-6 (F) and MCP-1 (G) were evaluated by ELISA, in homogenate of placentas. Levels (pg/mL) of IL-1β (H), IL-6 (I) and MCP-1 (J) were evaluated by ELISA in maternal plasma. Data are expressed as mean ± SD. **p* = < 0.005 compared to ND group. (ND) non-diabetic, (MGH) mild gestational hyperglycemia, (GDM) gestational diabetes mellitus and, (DM2) type 2 diabetes mellitus.

cytokines leading to a typical pro-inflammatory profile as seen in diabetes [26–29], which also may explain our results. The relationship between TLRs and this profile was reinforced in TLR4KO mice, in which NFKB activity and release of proinflammatory cytokines are both abrogated in diabetic condition [30].

The transcription factor NFKB is a multifunctional regulator and a critical component of innate and adaptive immune inflammatory responses [31] and, it is of relevance to the pathobiology of diabetes [32,33]. The potential impact of hyperglycemia on NFKB activation was also seen in an ex vivo isolated peripheral blood mononuclear cells [31]. NFKB activates the expression of pro-inflammatory cytokines and

chemokines and participates in inflammasome regulation [32]. In this regard, we found an increase in nuclear translocation of NFKB in placentas in all hyperglycemic groups, suggesting a role not only in well-characterized diabetes but also in vivo in all glucose-changed metabolic disorder.

To expand on our initial observations, we also evaluated the levels of NLRP1, NLRP3, and ASC and found higher expressions in all hyperglycemic groups. A relevant increase in mRNA and protein expression of NLRP3, ASC and proinflammatory cytokines (IL-1β maturation and IL-18 secretion) was shown in subjects diagnosed as DM2 in monocyte-derived macrophages [14]. As in other tissues, the overall

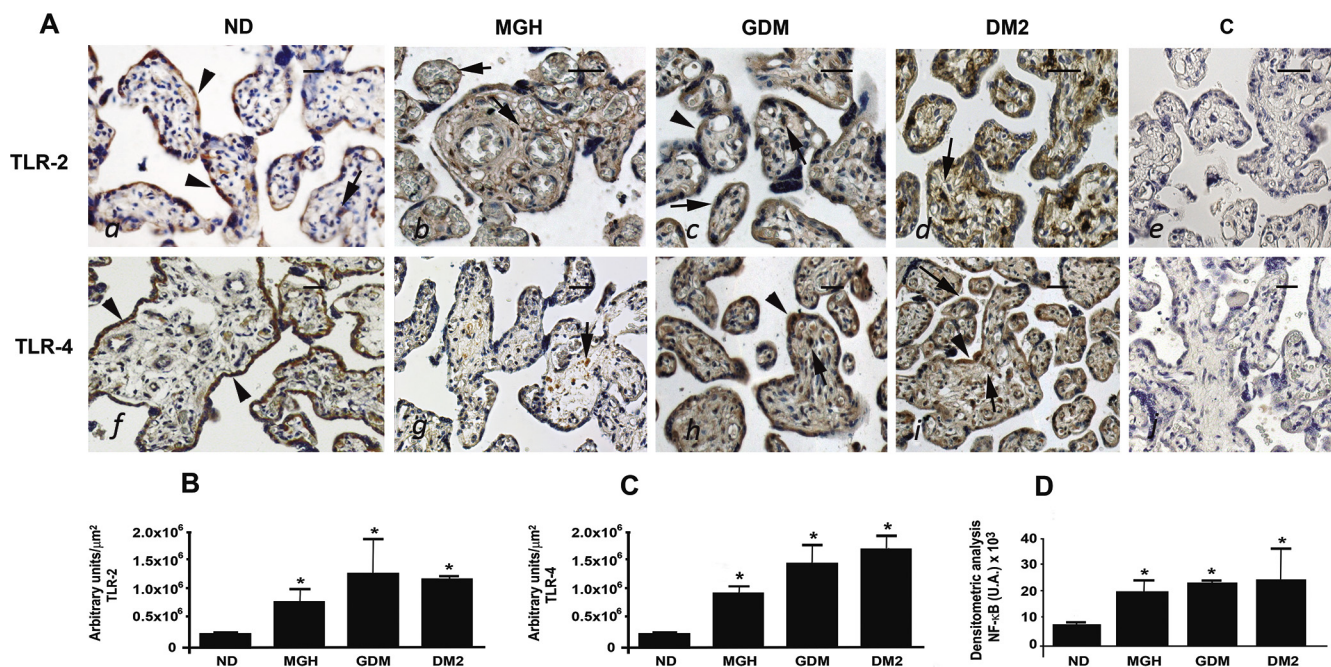


Fig. 2. Hyperglycemia increased TLR2 and TLR4 expression (A–C) and activation of NFKB in placental tissues (D). Placental immunolocalization (A) and quantification (B–C) of TLR2 (A, a–e; B) and TLR4 (A, f–j; C). Immunoperoxidase counterstained with Mayer's hematoxylin. Bar = 150 μm. Conventional negative controls in which primary antibodies have been omitted of the reaction (e, j). Quantification of the immunohistochemical staining (B–C) was performed by intensity of brownish-color immunostaining while using Image J software. The values given are in arbitrary units/μm² (mean ± SD). **p* < 0.05 compared to ND group. (D) Placental activation of NFKB evaluated by EMSA. Data are expressed as mean of arbitrary units × 10³ ± SD. **p* < 0.05 compared to ND group. (ND) non-diabetic; (MGH) mild gestational hyperglycemic; (GDM) gestational diabetes mellitus and, (DM2) diabetes mellitus type 2.

meaning of these findings in the placenta may be an ability to regulate cellular immune responses and protect the maternal-fetal interface at a molecular level through a delicate balance between the activation and inhibition of inflammation in order to allow the removal of dangerous elements without harming the functionality of the tissue.

In a more detailed analysis, our goal was to identify the levels of cleaved caspase-1. Our data also suggest high levels of cleaved caspase-1 in its two active subunits in hyperglycemic pregnant women (MGH, GDM, and DM2), corroborating the evidence on inflammasome by-products. The activated intracellular cysteine protease, caspase-1 participated in the inflammatory response by cleaving the precursors of IL-1 beta, IL-18, and IL-33 to a mature and active form, able to be secreted from the cell [34]. This activation is tightly controlled and is a central mechanism to restraining inflammation [35].

Finally, it is worth mentioning the potential role of fetal macrophages (Hofbauer cells, CD68 reactive cells). In all hyperglycemic conditions, the CD68-reactive cell (Hofbauer cell) population increases, characterizing a change in this functional compartment. In fact, previous studies have demonstrated the expression of a pro-inflammatory profile in this fetal macrophage population in diabetic gestations, leading to adverse consequences for the fetal-maternal axis [36]. Although we do not isolate this cell population from the mild hyperglycemic pregnancies to evaluate the cytokine profile, the augment in this population may reflect intrinsic disturbances as seen in classical diabetes. Studies on the elucidation of the role of these fetal macrophages in MGH have to be further considered for an appropriate comparison.

Our results, therefore, suggest that chorionic villous cells from placentas exposed to different hyperglycemic levels produce IL-1β and other pro-inflammatory factors, increase the expression of TLR2/4, activate the nuclear translocation of NFKB and exhibit a relative increase in the inflammasome components (NLRP1, NLRP3, ASC) and Caspase-1 activity.

5. Conclusion

This study adds novel findings on inflammatory balance at the chorionic villi caused by various systemic maternal changes in blood glucose, which may be relevant for placental machinery involved in cell growth, apoptosis, invasion, and other activities fundamental to successful placentation and embryogenesis. Indeed, adverse changes reported here in the chorionic villi may be an additional factor affecting fetal development, with the ability to impact significantly the developmental programming of health outcomes and susceptibility to disease also seen in MGH pregnant women. Moreover, the knowledge of mechanisms that lead to inflammation by regulating inflammasome assembly and subsequent activation of IL-1β, even if subclinical, are relevant to the search for targets that can be used to improve the conditions of the pregnancies associated with all degrees of hyperglycemia.

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Authorship contributions

SCS, JBM, EB, and IC designed the study and wrote the paper; APA performed immunohistochemistry and Western blot analysis; IC, MVCR, DCD and JBM provided clinical information; IC and MVCR diagnosed and cared for the patients; AUB help in the immunohistochemistry protocols; LS and CS performed the

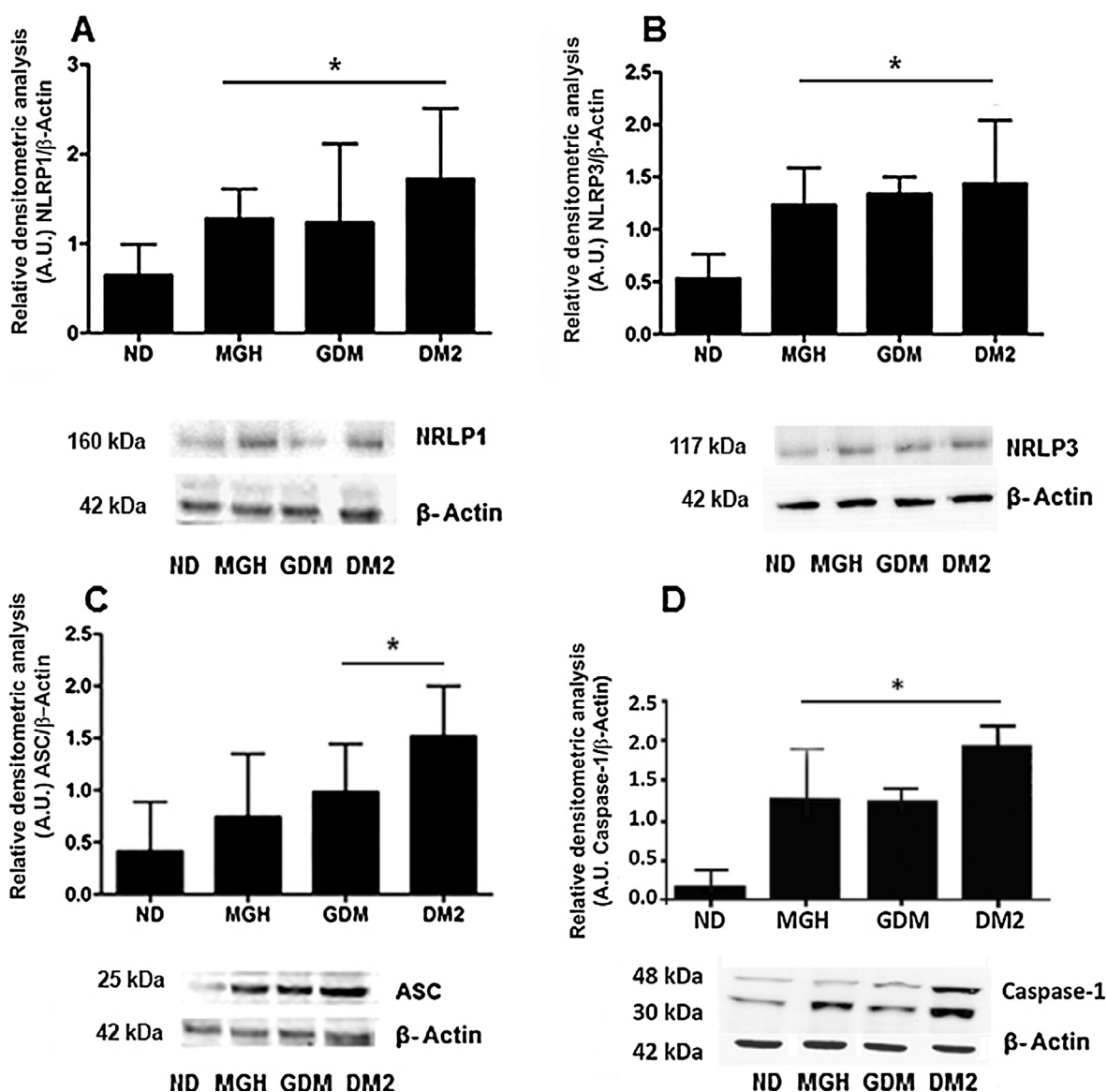


Fig. 3. Hyperglycemia increases NLRP1, NLRP3, ASC and Caspase-1 protein expression in placental tissues. Western blot analysis of NLRP1 (A), NLRP3 (B), ASC (C) and Caspase-1 (D) protein expression in placental homogenates of non-diabetic (ND), mild gestational hyperglycemic (MGH), gestational diabetic (GDM) and type 2 diabetic (DM2) pregnant women. Chorionic villi total lysate was subjected to immunoblot analysis using specific antibodies. Relative protein expression was quantified by densitometric scanning and normalized to the β -Actin. * $p < 0.05$ in comparison with ND.

electrophoretic mobility shift assays, SCS performed cytokine assays and analysis.

Conflict of interest

The authors have no conflicts of interest to disclose.

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