

Getting too sweet: galectin-I dysregulation in gestational diabetes mellitus

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ABSTRACT: Galectin-I (gal-I) is a prototype carbohydrate-binding protein, whose dysregulation is associated with adverse pregnancy outcomes such as spontaneous abortion and pre-eclampsia. Furthermore, it is known that faulty gal-I protein production or gene regulation can be caused by single-nucleotide polymorphisms in the *LGALS1* gene. Gestational diabetes mellitus (GDM) is also an adverse pregnancy outcome and the most common metabolic disorder during gestation. However, gal-I expression patterns during GDM remain largely unknown. Our aims were to define local and peripheral gal-I expression patterns during pregnancy, and to investigate *LGALS1* gene polymorphisms in GDM patients. Circulating gal-I levels were determined by ELISA in GDM patients and normal pregnant controls, and *LGALS1* gene polymorphisms were assessed for association with GDM. Placental tissues were collected from control and GDM term pregnancies to evaluate local gal-I expression by immunofluorescence. Our results show that GDM is associated with a failure to increase circulating gal-I levels during the second and third trimester, as well as overexpression of gal-I in placental tissue. Additionally, the *LGALS1* polymorphism rs4820294 was associated with the development of GDM. In pregnancies complicated by GDM, we observed gal-I dysregulation both locally in the placenta and peripherally in the circulation. Furthermore, the association between the *LGALS1* polymorphism and GDM may indicate a genetic contribution to this adverse pregnancy outcome.

Key words: galectin-I / gestational diabetes / glucose

Introduction

Gestational diabetes mellitus (GDM) affects 1–14% of pregnant women and is defined by glucose intolerance that is first diagnosed during pregnancy (Hadar and Hod, 2010). Pregnancy is associated with a pronounced physiological decrease in peripheral insulin sensitivity due to the high levels of steroid hormones, and is hence considered a diabetogenic condition. However, women with GDM experience an impaired functional adaptation of beta-cells, resulting in deficient insulin secretion to maintain normal glycemia (Dahlgren, 2006). Since GDM is usually asymptomatic, mandatory screening is conducted for all pregnant women at 28 weeks of gestation by means of a 75-g oral glucose tolerance test (OGTT).

GDM is also an important factor responsible for increased fetal and maternal morbidity and mortality. For GDM mothers, the short-term impact of this condition includes an increased risk of hypertension, pre-eclampsia, urinary tract infections and Caesarean delivery; whereas longitudinal studies have shown that GDM increases the risk to develop type 2 diabetes later in life (Bellamy *et al.*, 2009; Getahun *et al.*, 2010). In addition, maternal hyperglycemia triggers an excessive fetal insulin production increasing the risk of embryopathy including macrosomia, birth trauma and perinatal death. Maternal hyperglycemia is not only associated with excessive fetal growth, but also with tissue damage (e.g. endothelial dysfunction). Therefore, current therapies aim to restore blood glucose levels close to normal range (Barrett *et al.*, 2013).

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Galectin-I (gal-I) is a carbohydrate-binding protein with an affinity for β -galactosides (Kasai and Hirabayashi, 1996) that has been recently implicated in regulating processes associated with adaptation to pregnancy (Blois *et al.*, 2007; Freitag *et al.*, 2013; Tirado-Gonzalez *et al.*, 2013). Regulated by steroid hormones, gal-I concentrations increase throughout gestation to promote angiogenesis and materno-fetal tolerance (Choe *et al.*, 1997; Blois *et al.*, 2007; Freitag *et al.*, 2013; Tirado-Gonzalez *et al.*, 2013). Furthermore, gal-I facilitates mechanisms involved in placentation such as trophoblast invasion, syncytium formation and expression of placental HLA-G proteins (Fischer *et al.*, 2010; Kolundzic *et al.*, 2011; Tirado-Gonzalez *et al.*, 2013). Dysregulation of gal-I expression has been identified in various adverse pregnancy outcomes such as spontaneous abortion and pre-eclampsia (Freitag *et al.*, 2013; Tirado-Gonzalez *et al.*, 2013), as well as in the plasma of type 2 diabetes patients (Liu *et al.*, 2009). However, our understanding of the role of gal-I in GDM is still incomplete. This study examines local and peripheral gal-I expression during the development of GDM and investigates the association between *LGALS1*, which encodes gal-I, single nucleotide polymorphisms (SNP) and GDM.

Materials and Methods

Determination of circulating gal-I levels

Human gal-I levels were measured with a specific sandwich ELISA protocol, as reported previously (Tirado-Gonzalez *et al.*, 2013). For analyses of gal-I levels during normal and GDM pregnancies, blood samples were collected from healthy ($n = 155$) and GDM ($n = 94$) pregnant women in the first, second and third trimester of pregnancy at their planned visits to the Department of Obstetrics, Sao Paulo Federal University (UNIFESP), Brazil. All patients involved in this work were properly informed about the purpose of our research and gave their written consent before the sampling. The study was approved by the ethics committee of Sao Paulo Federal University (UNIFESP). Characteristics of the recruited participants are summarized in Table I. Diagnosis of GDM was based on the criteria proposed by the

World Health Organization: fasting glucose ≥ 126 mg/dl and/or ≥ 140 mg/dl 2 h after the ingestion of 75 g of glucose (OGTT). Control population consisted of 155 healthy pregnant women without any maternal or fetal disorders. Groups were matched by ethnicity (self-referred). Inclusion criteria for both groups were: singleton pregnancy with living fetus; gestational age between 6 and 36 weeks. Exclusion criteria for both groups were: autoimmune diseases, pre-existing diabetes, uterine malformation, pregnancy resulting from *in vitro* fertilization, placental abruption, infection, cancer or any other systemic disease, including pre-existing hypertension. We also excluded women with solid organ transplantation and in the use of steroids, antibiotics, immunosuppressants, antihistamines or anti-inflammatory medication.

Characterization of gal-I expressing peripheral lymphocytes by flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated from GDM patients ($n = 10$) and healthy controls ($n = 35$) during the third trimester of gestation. Isolated PBMC were stored at -80°C until flow cytometric analysis was performed analyzing gal-I expression in leukocytes following the standard method described previously (Molvarec *et al.*, 2011). Briefly, isolated mononuclear cells were washed twice with PBS and viability was assessed by trypan blue exclusion ($>90\%$). Non-specific binding sites were blocked by incubation with 10% mouse serum for 10 min at room temperature (RT). Four color flow cytometry was performed using the following antibodies: anti-CD3 FITC, anti-CD56 PC5, anti-CD8 FITC and anti-CD4 PC5 mouse monoclonal antibodies (BD Biosciences, USA) for 15 min at RT. After washing, red blood cells were lysed with Lysing Solution (BD Biosciences, USA) for 10 min at RT. Next, cells were treated with fluorescent antibody cell sorting (FACS) Permeabilizing Solution (BD Biosciences, USA) and subsequently incubated with anti-gal-I biotinylated antibody followed by a streptavidin PE conjugate (Vector Laboratories, Burlingame, CA, USA) for 30 min at RT. As controls, cells were stained with the corresponding isotype-matched Ab. Flow cytometric analysis was performed on a FACSCalibur flow cytometer and data were processed using the CellQuest Pro software (BD Biosciences). A real-time gate was set around the viable lymphocytes based on their forward scatter/side scatter profile.

Table I Characteristics of the human subjects recruited for determination of gal-I peripheral levels.

Parameter	Controls			GDM		
Gestational age (weeks)	6–13	14–28	29–36	6–13	14–28	29–36
Maternal age (years)	29.9 \pm 7.4	27.7 \pm 6.7	26.8 \pm 6.6	32.1 \pm 7.8	32.9 \pm 6.9	31.4 \pm 6.2
N	31	61	63	9	27	58
BMI (pre-pregnancy)	25.7 \pm 3.9	26.3 \pm 5.1	26.7 \pm 5.0	30.7 \pm 4.6	29.3 \pm 3.6	29.9 \pm 6.6*
Glucose (mg/dl)	82 \pm 5	81 \pm 5	82 \pm 6	96 \pm 16	101 \pm 17***	117 \pm 19***
Progesterone (mg/ml)	228 \pm 22	559 \pm 41	1041 \pm 53	178 \pm 19	961 \pm 37*	1415 \pm 46*
β -hCG (mIU/ml)	17 590 \pm 3793	2964 \pm 156	2119 \pm 249	16 850 \pm 4187	5223 \pm 529	12 281 \pm 768
Ethnicity (%)						
Caucasian	39.3			31.9		
Mulatto	43.8			50		
Black	15.4			17		
Native-Brazilian	0.6			–		
Asian	–			1		

Data are presented as mean values \pm SD. * $P < 0.05$ and *** $P < 0.001$ using one-way ANOVA, Tukey's test. BMI, body mass index; GA, gestational age; GDM, gestational diabetes mellitus.

LGALS1 SNP genotyping assay

Four SNPs in the *LGALS1* gene were analyzed in a subset of subjects of GDM ($n = 43$) and healthy controls ($n = 90$). We included the two SNPs that have been previously analyzed in human disease (rs4820293, rs4820294). Other SNPs were selected based on their gene location (rs4887 and rs3208174). For SNP measurement, 4 ml of peripheral blood was collected from each participant in tubes containing EDTA, and genomic DNA was extracted by the DTAB/CTAB method. Genotypes were determined using TaqMan[®] SNP Genotyping Assays for *LGALS1* rs4820293 (5' regulatory), rs4820294 (5' regulatory), rs4887 (*LGALS1*-Trp69*) and rs3208174 (*LGALS1*-Asn57Lys) according to manufacturer recommendations (Assay IDs: C_2495792_10, C_2495793_10, C_11475053_10 and C_27479779_20, respectively; Applied Biosystems, Foster City, CA, USA).

Dual immunofluorescence staining

Placental tissues were obtained from GDM diagnosed ($n = 40$) and control ($n = 40$) women giving birth at the Department of Obstetrics and Gynaecology of the Ludwig Maximilian University (LMU) of Munich. The study was approved by the ethics committee of the LMU Munich, Germany, and informed consent was obtained from all patients. Demographic and clinical data of the study population are summarized in Table II. One aliquot of placental tissue was frozen immediately after delivery and stored at -80°C . A second placental tissue aliquot was fixed immediately after delivery in 4% buffered formalin for 24 h and subsequently embedded in paraffin. Cytokeratin-7 (CK7) and gal-I dual immunofluorescent staining was performed as previously described (Jeschke et al., 2007). Briefly, gal-I-expressing cells were characterized in decidual and placental tissue by examining cryosections. Slides were fixed with acetone, blocked by Ultra V Block (Lab Vision, Fremont, USA) and incubated with primary antibody overnight at 4°C in the following antibody combinations: gal-I (R&D, AFI 152, 1:50) and cytokeratin 7 (CK7, Novocastrol, OV-TL12/30, 1:30) as a marker for extravillous trophoblast cells. After washing, slides were secondarily labeled with Cy2-labeled goat anti-mouse IgG and Cy3-labeled goat anti-rabbit IgG (both Dianova), diluted 1:200. Finally the slides were embedded in mounting buffer containing 4',6-diamino-2-phenylindole (DAPI) and examined with a Zeiss Axiophot photomicroscope (Jena, Germany).

Ex vivo placenta perfusion with D-glucose

A single sided placenta perfusion system was developed, which allows simultaneous separate and independent perfusion of two cotyledons of the same placenta. In all experiments one cotyledon was perfused with low glucose (5.5 mM) or high glucose (25 mM) medium. Briefly, two cotyledons from each placenta were cut out surrounded by sufficient tissue for fixation in the chambers. On the upside, the maternal tissue was penetrated by four blunt metal cannulae through the decidual plate into the intervillous space.

The fetal tissue remained untouched. The perfusion medium consisted of NCTC-135 tissue culture medium (Cambrex, Verviers, Belgium) diluted 2:1 with Earl's-Buffer (Biochrom, Berlin, Germany), and supplemented with bovine serum albumin (40 g/l; MP Biomedicals, Illkirch, France), amoxicillin (250 mg/l; Sigma-Aldrich), heparin (500 $\mu\text{l/l}$, equivalent to 25 000 N, Ratiopharm, Ulm, Germany) and dextran FP40 (10 g/l, Serva, Heidelberg, Germany), plus D-glucose 5.5 mM (low glucose: 0.33 g/l) or 25 mM (high glucose: 4.17 g/l; Merck, Darmstadt, Germany), adjusted to a pH of 7.4 by NaOH (Roth, Karlsruhe, Germany). For perfusion, the medium was warmed up to 37°C . The flow rate was 12 ml/min during the entire perfusion period. The experimental cotyledon was perfused with physiologic solution (0.9% NaCl) for 1 h followed by perfusion medium containing low or high glucose (5.5 and 25 mM, respectively) for further 6 h. Perfusion solution was collected at 30-min intervals for further analysis. Supernatants were stored in aliquots at -80°C until analysis.

Statistical analysis

All data are shown as mean and SEM except where indicated. Data were analyzed with the GraphPad Prism 5.0 software using the one-way analysis of variance (ANOVA) with Tukey's post test, except where indicated. Values were considered significantly different when $P < 0.05$. Hardy-Weinberg equilibrium (HWE) tests were performed by calculating the expected frequencies of each genotype and comparing them with the observed values. Single genotype frequencies (obtained by direct count) were analyzed by Fisher exact test or χ^2 tests, with significance set at $P < 0.05$. Correlations between variables were calculated using the Spearman rank correlation coefficient (r_s).

Results

Changes in gal-I levels during the development of GDM

A total of 249 pregnant women were included in the current study, of which 94 were diagnosed with GDM, clinical data shown in Table I. As expected, GDM patients had a significantly higher BMI, as well as increased progesterone and β -hCG circulating levels compared with control pregnant women (Table I). In healthy pregnant women, gal-I peripheral levels increased during gestation, reaching a maximum level at the second trimester. In contrast, GDM patients did not show any significant changes in gal-I levels during gestation (Fig. 1A). Interestingly, the frequency of peripheral blood gal-I expressing T cells (i.e. CD3^+ , CD4^+ and CD8^+ cells) and NK cells (CD56^+ cells) showed no significant differences between healthy and GDM pregnant women during the third trimester (Fig. 1B).

Table II Characteristics of the samples used for local analysis of gal-I expression.

Parameter	Controls ($n = 40$)		GDM ($n = 40$)		Statistic
Maternal age (years)	30.3 \pm 6.1	32.0 \pm 6.1	31.5 \pm 4.1	33.2 \pm 5.3	$P = 0.177$
GA (weeks)	39.80 \pm 1.54	39.75 \pm 1.16	39.67 \pm 1.30	39.83 \pm 1.40	$P = 0.943$
BMI (pre-pregnancy)	21.92 \pm 3.97	25.04 \pm 7.90	29.38 \pm 8.03	26.96 \pm 4.73	*** $P < 0.001$
Birthweight (g)	3339.8 \pm 568	3294 \pm 440	3662.1 \pm 562	3635.9 \pm 661	** $P < 0.01$
Umbilical artery pH	7.28 \pm 0.10	7.30 \pm 0.08	7.30 \pm 0.07	7.30 \pm 0.10	$P = 0.826$
Child gender	Male	Female	Male	Female	

Data are presented as mean values \pm SD. ** $P < 0.01$ and *** $P < 0.001$, as analyzed by Mann-Whitney rank sum test. BMI, body mass index; GA, gestational age; GDM, gestational diabetes mellitus.

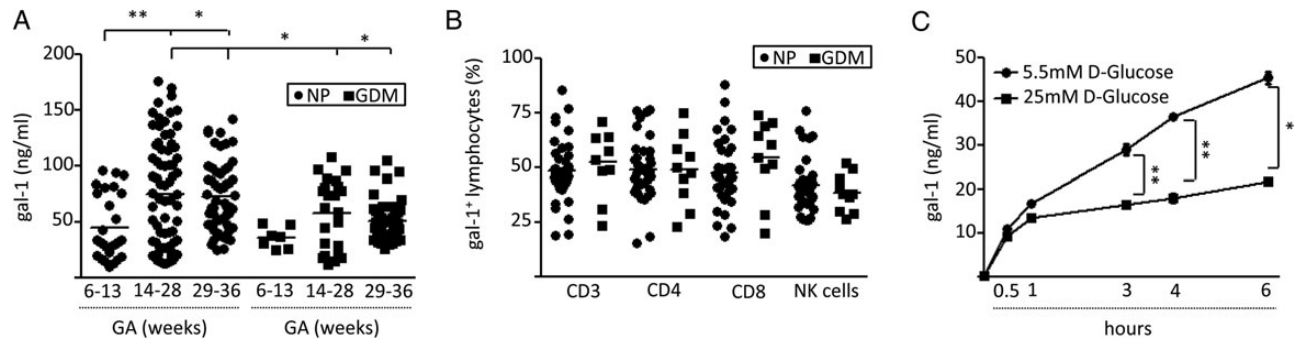


Figure 1 Galectin-I peripheral levels failed to rise during the course of GDM. **(A)** Circulating gal-I levels evaluated by ELISA in a cohort of patients ($n = 249$) during the first, second and third trimester, where some women subsequently developed GDM during the second trimester ($n = 94$). $*P < 0.05$ and $**P < 0.01$ using one-way ANOVA, Tukey's test. **(B)** Characterization of gal-I expression of peripheral blood CD3⁺, CD4⁺, CD8⁺ and CD56⁺ (NK cells) lymphocytes in healthy pregnant women ($n = 35$) and GDM patients ($n = 10$) during the third trimester. **(C)** gal-I levels in placental fluid upon 5.5 mM (low) or 25 mM (high) D-glucose medium perfusion during 0.5, 1, 3, 4 or 6 h, as measured by ELISA. $*P < 0.05$ and $**P < 0.01$ using two-tailed t-test. Data are expressed as mean \pm SEM from six to eight placentas per each D-glucose medium analyzed in duplicate. NP, normal pregnancy; GDM, gestational diabetes mellitus; GA, gestation age.

Table III Correlation analysis of gal-I peripheral levels and BMI, glucose, progesterone and β -hCG levels.

Variables tested	Spearman correlation coefficient	P-value
NP gal-I – BMI	0.3657	0.0462 (NS)
NP gal-I – Glucose	0.2293	0.2598 (NS)
NP gal-I – Progesterone	0.2156	0.3480 (NS)
NP gal-I – β -hCG	0.1727	0.4540 (NS)
GDM gal-I – BMI	-0.01951	0.0406*
GDM gal-I – Glucose	-0.03012	0.0312*
GDM gal-I – Progesterone	0.1158	0.5498 (NS)
GDM gal-I – β -hCG	0.0296	0.8790 (NS)

NP, normal pregnancy; BMI, body mass index; GDM, gestational diabetes mellitus; NS, not significant ($P > 0.05$) and $*P < 0.05$ as analyzed by Spearman test.

To further characterize gal-I peripheral levels as a marker for GDM outcome estimation, our next aim was to analyze its correlation with BMI, glucose, progesterone and β -hCG levels. Table III shows that no significant correlation was found between gal-I serum levels and either BMI, glucose, progesterone or β -hCG in normal pregnancy patients. Interestingly, gal-I peripheral levels were found to correlate inversely with BMI and glucose values (BMI $r_s = -0.01951$, $P < 0.05$; glucose $r_s = -0.03012$, $P < 0.05$, Table III) only in GDM patients.

Hyperglycemia regulates placental gal-I secretion

Since maternal glucose levels correlated inversely with peripheral gal-I exclusively in GDM patients, an *ex vivo* placenta perfusion with low or high concentration glucose medium was performed to elucidate whether hyperglycemia is able to modify the pattern of gal-I secretion. As shown in Fig. 1C, we found that placental gal-I secretion is induced

during perfusion with both low and high concentration glucose solutions. However, placental gal-I secretion was less sensitive to high glucose concentration (25 mM), showing a slower kinetics of response when compared with perfusion with low concentration glucose (5.5 mM) solution (Fig. 1C). Additionally, immunofluorescence analysis in term choriodecidual tissue showed that GDM was associated with decreased gal-I expression in extravillous trophoblast cells (EVT) within the decidua (CK7⁺ gal-I⁺ cells, Fig. 2A and B). In contrast, placental expression of gal-I (as noted by CK7⁺ gal-I⁺ cells, Fig. 2A) was significantly increased in GDM patients respect to controls (Fig. 2B).

Association between *LGALS1* rs4820294 SNP and GDM

SNP analysis was performed to investigate the association of gal-I (*LGALS1*) gene polymorphisms in GDM patients and ethnically matched healthy pregnant controls. All allelic variants in control group were in HWE. A significant difference was found in the genotype frequencies of the 5' regulatory *LGALS1* rs4820294 polymorphism between patients with GDM and controls. The percentage of TT genotype was significantly higher in GDM patients than in control pregnant women (OR = 4.5; CI 1.406–14.406, $P < 0.01$; Table IV). The 5' regulatory *LGALS1* rs4820293 polymorphism showed no association with GDM (Table IV). The *LGALS1*-Trp69* and *LGALS1*-Asn57Lys SNPs were homogenous throughout the population (100% CC and 100% GG, respectively).

Discussion

The incidence of GDM has increased significantly worldwide and it is associated with a risk of subsequent overt metabolic syndrome and diabetes in mothers, as well as with adverse maternal, fetal and neonatal pregnancy-related outcomes (Hadar and Hod, 2010). Here we report that gal-I, a prototype carbohydrate-binding protein with multiple functions during human pregnancy, exhibits differential kinetics during the course of GDM. For instance, serum gal-I levels were reduced only in

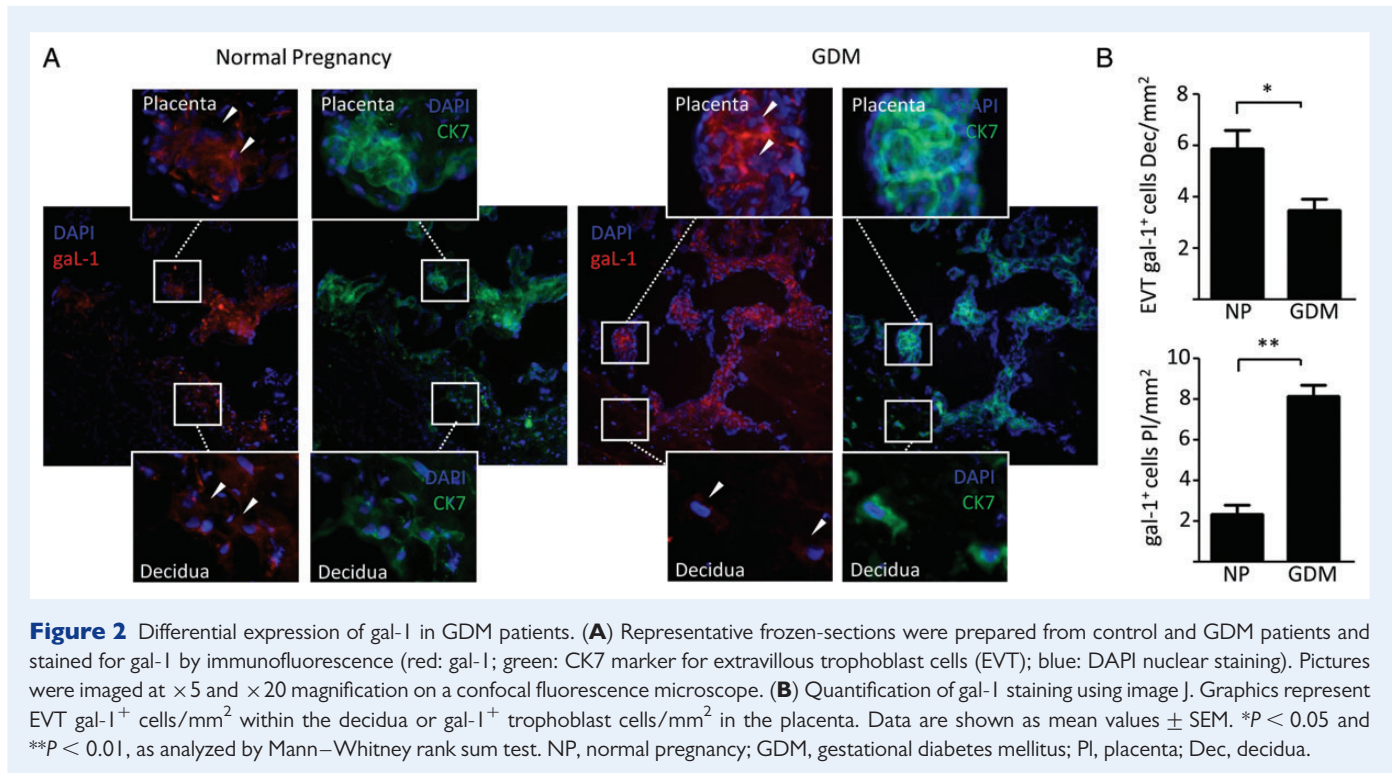


Table IV Gal-1 (LGALS1) single nucleotide polymorphisms (SNP) in healthy control and gestational diabetes mellitus (GDM) patients.

rs4820294	Genotypes	Controls n = 90	GDM n = 43	P-value
	CC+CT	85 (94.4)	34 (79.0)	0.0129 ^a
	TT	5 (5.6)	9 (21.0)	OR = 4.500
	HWE	2.02	5.10	CI 1.406–14.406

HWE, Hardy–Weinberg equilibrium; OR, odds ratio; CI, confidential interval.
^aFischer Exact test.

patients that developed GDM. This is in agreement with our previous work showing that normal pregnancy progression is associated with an increase of serum gal-1 levels over gestation (Tirado-Gonzalez et al., 2013). The differential kinetics observed in GDM pregnant women was only evidenced during the second and third trimesters, suggesting that gal-1 expression is sensitive to the hormonal and metabolic changes that characterize GDM. In this context, steroid hormone (estrogen and progesterone) regulation of gal-1 is particularly important during the phase of uterine receptivity (Choe et al., 1997). However, the present findings suggest that the supra-physiological progesterone and β -hCG levels observed in GDM patients play a role in down-regulation of the circulating gal-1 levels. Supporting this, we have previously demonstrated *in vitro* that gal-1 administration decreased β -hCG and progesterone production by BeWo cells, a trophoblast-derived choriocarcinoma cell line (Jeschke et al., 2004).

Accumulating evidence suggests that high glucose concentrations during GDM are associated with abnormal fetal development (Bellamy

et al., 2009). As noted above, the normal profile in successful pregnancy is increasing gal-1 peripheral levels throughout gestation, whereas an unchanged gal-1 secretion pattern seems to be associated with GDM. In this study, we demonstrated by *ex vivo* studies that alterations in glucose concentrations at the fetomaternal interface decrease gal-1 secretion by the placenta, results that agree with the inverse correlation between glucose and gal-1 found in GDM patients. In turn, due to the differential susceptibility of Th1 and Th2 cells to pro-apoptotic effects of the lectin (Motran et al., 2008), it is conceivable that reduced peripheral levels of gal-1 would affect the cytokine imbalance ensuring the Th2 predominance necessary to maintain pregnancy in GDM mothers. In support of this notion, recent studies have shown that while GDM is associated with increased peripheral levels of TNF- α , IL-6 and pro-inflammatory adipocytokines, a significant down-regulation of the Th1 to Th2 cytokine ratio is also a feature of these patients (Ategbro et al., 2006). On the other hand, over-expression of placental gal-1 in pregnancies complicated with GDM possibly counteracts the exacerbation of placental inflammatory-related genes during this pregnancy complication (Hauguel-de Mouzon and Guerre-Millo, 2006), due to its homeostatic function during immune responses (Camby et al., 2006). This is supported by evidence showing up-regulation of placental gal-1 in patients with late onset pre-eclampsia, which is also characterized by a maternal excessive inflammatory response (Jeschke et al., 2007; Freitag et al., 2013).

An additional objective of the present study was to determine the association between polymorphisms in the gal-1 LGALS1 gene and GDM. We report, for the first time, an association between an LGALS1 SNP and GDM complicated pregnancy. In our case–control cohort, we found a significant association between the 5' regulatory LGALS1 SNP rs4820294 (C/T) and GDM. This SNP is found in a regulatory region of the LGALS1 gene (Pal et al., 2010), and the TT genotype seems to

represent a risk factor for the development of GDM. Additional studies are needed to confirm the association between *LGALS1* polymorphism rs4820294 and GDM in a similar population (validation) and, due to the low frequency of the T allele and the large confidence interval reported in our study, sample sizes must be increased for future studies. The confirmation of this association in different ethnic groups will support the hypothesis that an *LGALS1* gene regulatory SNP is involved in GDM predisposition.

In summary, this study reveals that metabolic and hormone alterations during GDM influence gal-I expression in both peripheral and local environments and subsequently contribute to the pathogenesis of this pregnancy complication. We have also identified an association between a regulatory SNP in the *LGALS1* gene and GDM; though this connection should be confirmed in a larger study including different ethnic groups.

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Authors' roles

S.M.B. and S.D.: conceived and designed research; S.M.B., I.T.-G., N.F., G.B., B.Y.G.-S., M.R.T., R.M., M.L.C., L.U., J.K., G.T., A.M. and U.J.: performed research; M.R. and U.R.M.: contributed to essential reagents; I.T.-G., L.U., J.K., G.T., A.M., M.L.C. and S.M.B.: analyzed the data; M.L.C., S.D., U.J. and G.B.: gave input on writing the manuscript; S.M.B.: wrote the paper.

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