Calcifediol Decreases Interleukin-6 Secretion by Cultured Human Trophoblasts From GDM Pregnancies

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Gestational diabetes mellitus (GDM) is often characterized by low maternal calcifediol (25OHD) and high inflammation levels. This study aimed to determine whether placental protein expressions of CYP27B1, vitamin D receptor (VDR), and CYP24A1 are impaired in GDM and to investigate the effect of a 25OHD treatment on IL-6 secretion by GDM trophoblasts compared with normoglycemic (NG) trophoblasts. Placental tissue samples were harvested to determine protein expression of CYP27B1, VDR, and CYP24A1 by immunoblots. Isolated trophoblasts were stimulated with 25OHD concentrations (25 to 2000 nM) once a day for 3 days and IL-6 secretion was quantified (ELISA). We recruited 17 NG women, 19 women with GDM treated with diet and exercise alone (GDM-d) and 9 women with GDM who necessitated insulin therapy (GDM-i). Protein expressions of CYP27B1 and VDR were significantly higher in placental tissue from GDM-d women compared with NG women (both $P = 0.02$), whereas no differences were detected between GDM-i and NG placental tissues. In cultured trophoblasts (two groups; n = 5 NG and n = 5 GDM-d), exposure to increasing 25OHD concentrations significantly decreased IL-6 secretion in the GDM-d group only ($P = 0.006$). After treatment with 25OHD (2000 nM), IL-6 secretion was lower in the GDM-d group compared with the NG group ($P = 0.03$). Our results suggest an upregulation of the VDR-1,25$(OH)_2$D complex bioavailability in GDM-d placentas, possibly reflecting a compensatory mechanism aiming to ensure that vitamin D can exert its genomic and nongenomic effects in the target cells of the placental-fetal unit. Our findings support an anti-inflammatory effect of vitamin D at the feto-maternal interface in GDM-d pregnancies.

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Freeform/Key Words: placenta, trophoblast, vitamin D, inflammation, IL-6, gestational diabetes

Gestational diabetes mellitus (GDM) is defined as glucose intolerance that begins or is first recognized during pregnancy [1]. GDM is associated with short- and long-term deleterious consequences for mothers and offspring [2, 3]. Currently, the two main strategies for the treatment of GDM include a diet and exercise intervention and insulin therapy if the glucose levels are not well controlled with diet and exercise alone. Although the exact mechanisms of

Abbreviations: 1,25$(OH)_2$D, calcitriol; 25OHD, calcifediol; BMI, body mass index; CHUS, Centre Hospitalier Universitaire de Sherbrooke; FBS, fetal bovine serum; GDM, gestational diabetes mellitus; GDM-d, women with gestational diabetes mellitus treated with diet and exercise; GDM-i, women with gestational diabetes mellitus receiving insulin therapy; IFN-γ, interferon-γ; LC-MS/MS, liquid chromatography/electrospray tandem mass spectrometry; MKP5, MAPK phosphatase 5; NG, normoglycemic; OGTT, oral glucose tolerance test; VDR, vitamin D receptor.

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GDM pathophysiology are poorly understood, a growing body of evidence suggests that inflammatory pathways might be implicated [4–9].

Vitamin D is a secosteroid hormone well known for its classic roles in phosphate and bone metabolisms. Emerging evidence has pointed out its nonclassic roles, including glucose homeostasis and immune regulation [10]. Several studies including one by our group have shown that calcifediol (25OHD) levels are lower in GDM women than in normoglycemic (NG) pregnant women [11–16]. Moreover, our group and others have demonstrated that lower levels of 25OHD during the first trimester were associated with higher subsequent risk of developing GDM [11–13, 16, 17]. Although we found a modest inverse association between 25OHD levels and insulin resistance in pregnancy [11], it appears that the association between 25OHD levels and GDM could also involve the anti-inflammatory effects of vitamin D.

Vitamin D is believed to play an important role in pregnancy, especially at the feto-maternal interface [18–23]. The dominant circulating form of vitamin D (25OHD) crosses the placental barrier more easily than its active form, calcitriol [$1,25(OH)_2D$]. In addition, the placental trophoblast cells express all the vitamin D metabolic components, so that 25OHD is metabolized by the CYP27B1 enzyme to $1,25(OH)_2D$. The active hormone $1,25(OH)_2D$ interacts with the vitamin D receptor (VDR) to exert genomic and nongenomic biological functions [24, 25]. Moreover, both 25OHD and $1,25(OH)_2D$ are catabolized by the CYP24A1 enzyme into inactive metabolites. Interestingly, a placenta-specific methylation has been reported in the *CYP24A1* promoter region, which was also observed in cultured trophoblast cells, leading to the downregulation of its transcription [26]. However, an increase in CYP24A1 mRNA and protein expressions has been shown in placentas from GDM pregnancies when compared with healthy pregnancies [27].

Trophoblasts express and secrete inflammatory cytokines [28, 29]. In trophoblasts from healthy pregnancies, it has been shown that $1,25(OH)_2D$ downregulates production of TNF-α–induced inflammatory cytokines such as IL-6 and IFN-γ [30]. However, insight on the regulation of proinflammatory cytokines in trophoblasts obtained from GDM pregnancies is still lacking.

We first assessed whether placentas from GDM pregnancies are characterized by impaired protein and mRNA expression of vitamin D metabolic components. Then, we investigated the effect of 25OHD exposure on IL-6 secretion by cultured trophoblasts from GDM pregnancies to compare with trophoblasts from NG pregnancies. Finally, we evaluated the effect of 25OHD exposure on protein expression of vitamin D metabolic components in trophoblasts.

1. Methods

A. Subjects

Pregnant women were recruited at the end of the third trimester during their visit before elective cesarean delivery. Exclusion criteria were age <18 years old, pregestational diabetes (type 1 or 2), drug and/or alcohol abuse, multiple pregnancy, intrauterine growth restriction, and any major medical conditions that could influence inflammation and/or glucose regulation. Demographic information including maternal age, prepregnancy body mass index (BMI), and ethnicity were obtained from participants. The study was approved by the ethics committee of the Centre Hospitalier Universitaire de Sherbrooke (CHUS), Québec, Canada, and written informed consent was obtained from all women before their inclusion in the study, in accordance with the Declaration of Helsinki.

Among the 45 participants, 17 were NG and 28 had a diagnosis of GDM, of whom 19 women were treated with a diet and exercise intervention (GDM-d) and 9 women required insulin therapy (GDM-i) to achieve glycemic control. GDM diagnosis at the second trimester was made if: (i) glucose level was $\geq$10.3 mmol/L 1 hour after the 50-g oral glucose challenge test; (ii) during the 2-hour oral glucose tolerance test (OGTT) (75 g), one value of glucose was $\geq$5.1 mmol/L fasting, $\geq$10.0 mmol/L 1 hour after OGTT or $\geq$8.5 mmol/L 2 hours after OGTT; or (iii) capillary blood glucose levels taken four times a day during a week showed $\geq$50%

abnormal values (≥5.3 mmol/L fasting, ≥7.8 mmol/L 1 hour after a meal, or 6.7 mmol/L 2 hours after a meal) at one moment during the day [31].

B. Sample Collection

Immediately after delivery, placenta and venous cord blood samples were collected. Placenta tissue samples (2 cm^3) were harvested, dissected, and snap frozen in liquid nitrogen or stored in RNA*later* (Qiagen, Toronto, ON, Canada) at −80°C. The next morning after delivery, the mother's blood sample was collected. All blood samples were centrifuged (2500*g* at 4°C for 10 minutes), and the plasma was aliquoted and stored at −80°C for subsequent analyses.

C. Trophoblast Cell Isolation and Culture

Samples of the placenta (>35 g) of 5 NG and 5 GDM-d participants were harvested for immediate isolation of villous cytotrophoblasts. Maternal and fetal membranes were removed, and the remaining placental tissues were dissected. After several PBS washes, cytotrophoblasts were isolated by an enzyme digestion method followed by Percoll separation, as previously described [32, 33], with minor modifications for optimization of cell cultures [34].

Cells were seeded in triplicate at a density of 3.5×10^6 cells per 35-mm cell culture dish containing DMEM low-glucose medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Wisent Inc, St-Bruno, QC, Canada), 1% penicillin/streptomycin/neomycin (Thermo Fisher Scientific, Waltham, MA), 2 mM L-glutamine (Wisent), and 44 mM sodium bicarbonate (Wisent). Cells were incubated in humidified 5% CO_2–95% air at 37°C and allowed to attach overnight. While cytotrophoblasts were differentiating and fusing into syncytiotrophoblasts [32], they were stimulated every day with several 25OHD (Tocris Bioscience, Avonmouth, Bristol, UK) concentrations (25, 50, 100, 300, 500, 750, 1000, 2000 nM) or its vehicle (dimethyl sulfoxide; 0.1%) for 3 consecutive days. As previously described by Kliman *et al.* [32], a majority of syncytiotrophoblasts was observed after 3 days of culture. Medium was collected and proteins were isolated from syncytiotrophoblasts 24 hours after the last 25OHD stimulation.

D. Biochemical Assays

Plasma levels of $25OHD_2$ and $25OHD_3$ were determined by liquid-liquid extraction followed by liquid chromatography/electrospray tandem mass spectrometry (LC-MS/MS) (Quattro Micro Mass Spectrometer; Waters, Milford, MA) [35]. The limit of detection was 1.25 nmol/L for $25OHD_2$ and 6.25 nmol/L for $25OHD_3$. Intra-assay and interassay coefficients of variation were 7.4% and 4.1% for $25OHD_2$ and 4.7% and 2.9% for $25OHD_3$, respectively. Total 25OHD levels were calculated as the sum of $25OHD_2$ and $25OHD_3$ values.

After 25OHD exposure, the cell supernatant was collected and measurement of IL-6 secretion was performed with the Human IL-6 Precoated ELISA kit (Cedarlane, Burlington, Ontario, Canada) [36] according to the manufacturer's recommendations. Sensitivity was 2 pg/mL, and intra-assay and interassay coefficients of variation were <10%.

E. Immunoblot Analysis

Placental tissues and trophoblast cells were lysed in radioimmunoprecipitation assay buffer [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1.0% Triton X-100] containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich, Oakville, ON, Canada). Placental tissues were homogenized with tissue homogenizer (Thermo Fisher Scientific). Protein concentrations were measured with detergent-compatible protein assay (Bio-Rad Laboratories, Mississauga, ON, Canada). Equal amounts of total proteins (40 μg for tissues and 10 μg for cells) were separated by SDS-PAGE (10% acrylamide/bisacrylamide), transferred to polyvinylidene fluoride membranes and blocked with 5% skim

milk. Membranes were incubated overnight at 4°C with primary antibodies against human CYP27B1 (H-90; 1:250 for tissues and 1:200 for cells) [37], VDR (D-6; 1:5000 for tissues and cells) [38], and CYP24A1 (H-87; 1:500 for tissues and 1:200 for cells) [39] (all from Santa Cruz Biotechnology Inc, Santa Cruz, CA). Then, membranes were washed three times with TBS-Tween 0.1%, incubated with horseradish peroxidase–conjugated secondary antibodies (1:10,000) ([40] for CYPs, [41] for VDR), and then visualized with Clarity Western ECL (Bio-Rad Laboratories). Immunoblots were detected with the Bio-Rad ChemiDoc MP System (Bio-Rad Laboratories) and quantified with Bio-Rad Image Laboratory software (Bio-Rad Laboratories). Results were normalized against total protein (TGX Stain-Free technology; Bio-Rad Laboratories) [42] because it is more stable than actin or glyceraldehyde 3-phosphate dehydrogenase for intragroup and intergroup variations when studying placentas from normal and GDM pregnancies [43].

F. RNA Isolation and Real-Time PCR Analysis

Real-time PCR analysis was performed to quantify mRNA expressions of *CYP27B1, VDR, CYP24A1, IL-6, IL-1β, IFN-γ,* and *TNF-α* in placental tissues as previously described [44]. *YWHAZ* RNA expression was used for normalization, because it is one of the most stable endogenous genes in the placenta [45, 46]. Total RNA was extracted from placental tissue with TRI Reagent, as described by the manufacturer (Molecular Research Center, Inc, Cincinnati, OH). RNA integrity was monitored with an agarose gel [47] for samples of four randomly chosen participants. The RNA was treated with deoxyribonuclease I (DNase I; Invitrogen, Carlsbad, CA) to remove any genomic DNA contamination. Approximately 1 μg RNA was used to generate cDNA via Moloney murine leukemia virus reverse transcriptase (Invitrogen) and random primers (Invitrogen). Sequences of primers used for quantitative PCR analyses are listed in an online repository [48]. Melting curves were obtained for each reaction to confirm the specificity and the accuracy of primers.

G. Statistical Methods

Characteristics of participants are presented as median and interquartile ranges or percentages. Participant characteristics were compared between the NG, GDM-d, and GDM-i groups via Kruskal-Wallis test. A Mann-Whitney test was used to compare tissue mRNA and protein levels between two groups and IL-6 levels in culture media at baseline and after exposure to 2000 nM 25OHD between NG and GDM-d groups. A trend test via one-way repeated-measures ANOVA was performed for the NG and GDM-d groups separately to assess linearity after 25OHD concentrations (0 to 2000 nM) were added in the culture media for (i) IL-6 concentrations in culture media and, (ii) protein levels in trophoblast cells. A Wilcoxon matched-pairs signed rank test was used to compare (i) IL-6 concentrations in culture media and, (ii) protein levels in trophoblast cells within a group (NG or GDM-d), after the different 25OHD exposures (0 to 2000 nM). Differences were considered statistically significant at $P < 0.05$.

2. Results

A. Characteristics of Study Participants

Demographic and biochemical data for NG, GDM-d, and GDM-i participants are summarized in Table 1. Participants were mostly white, and GDM-i participants had a higher median prepregnancy BMI compared with GDM-d and NG participants ($P = 0.004$ and $P = 0.0002$, respectively). The plasma levels of 25OHD in cord blood and maternal blood were lower in the GDM-i group than in the GDM-d and NG groups, but differences did not reach statistical significance ($P = 0.59$ and $P = 0.07$, respectively). Baseline characteristics of participants (n = 5 NG and n = 5 GDM-d) whose placentas were used for trophoblast cell culture experiments (data not shown) were representative of the study cohort, except for the ethnicity of

Table 1. Clinical and Biochemical Characteristics of Participants

	NG (n = 17)	GDM-d (n = 19)	GDM-i (n = 9)	*P*
Age, y	31.0 [28.0–33.0]	32.0 [29.0–36.0]	34.0 [30.0–35.5]	0.11[a]
Prepregnancy BMI, kg/m^2	22.7 [21.1–25.7]	25.7 [20.0–30.0]	42.3 [30.7–55.0]	0.001[a]
Ethnicity, % white	88.2	84.2	100.0	n/a
Vitamin D supplementation, %	62.5	73.7	100.0	n/a
Maternal 25OHD levels, nmol/L	80.2 [60.1–106.2]	94.2 [86.6–125.3]	59.6 [29.5–87.9]	0.07[a]
Cord blood 25OHD levels, nmol/L	54.8 [37.6–69.9]	48.7 [38.5–69.1]	46.0 [24.3–76.3]	0.59[a]
Gestational age at delivery, wk	38.7 [38.5–39.1]	38.6 [38.1–39.0]	38.3 [37.9–38.6]	0.08[a]
Birth weight, kg	3.42 [3.15–3.68]	3.48 [3.24–3.71]	3.31 [3.08–3.57]	0.49[a]

Data are presented as median [interquartile range] or percentage. For cord blood 25OHD levels, n = 16 NG, n = 17 GDM-d. For maternal 25OHD levels, n = 10 NG, n = 11 GDM-d, n = 6 GDM-i. For birth weight, n= 16 NG, n = 17 GDM-d, n = 8 GDM-i.
[a]Kruskal-Wallis test.

the NG group, in which three out of five participants were white. In addition, twice more participants took vitamin D supplementation in the GDM-d group (four out of five) than in the NG group (two out of five).

B. Placental Protein Expression of CYP27B1, VDR, *and* CYP24A1

To further understand the mechanism that altered the vitamin D levels in women with GDM, placental protein expression of the vitamin D metabolic components was evaluated. Protein levels of CYP27B1 and VDR in placental tissues of GDM-d participants were significantly higher than in placental tissues of NG participants ($P = 0.02$ for both; Fig. 1A and 1B). In addition, VDR placental protein expression was higher for GDM-d participants than for GDM-i participants (Fig. 1B). No differences were observed between the three groups in CYP24A1 placental protein expression (Fig. 1C) nor between GDM-i and NG groups for the protein expression of the three vitamin D metabolic components.

C. Placental mRNA Expression of CYP27B1, VDR, *and* CYP24A1

To corroborate our protein level findings, the mRNA expression of *CYP27B1, VDR,* and *CYP24A1* was examined in placental tissues. Levels of *CYP27B1* mRNA in placental tissues of GDM-d participants were similar to those in placental tissues of NG participants [49]. Placental *VDR* mRNA expression was higher in the GDM-d group than in the NG and GDM-i groups, but differences did not reach statistical significance [49]. Moreover, *CYP24A1* mRNA levels were higher in the GDM-d group than in the NG and GDM-i groups, but we did not detect significant differences between groups [49].

D. Placental mRNA Expression of IL-6, IL-1β, IFN-γ, *and* TNF-α

To verify the basal placental inflammatory phenotype for each group, the mRNA expression of *IL-6, IL-1β, IFN-γ* and *TNF-α* was assessed in placental tissues. Levels of *IL-6* mRNA were significantly higher in placental tissues of GDM-d participants than in placental tissues of NG participants ($P = 0.048$; Fig. 2A). Placental *IL-6* mRNA expression was higher in the GDM-i group than in the NG group, but the difference did not reach statistical significance (Fig. 2A). No differences were observed between the three groups in the placental mRNA expression of *IL-1β* (Fig. 2B), *IFN-γ* (Fig. 2C), and *TNF-α* (Fig. 2D).

E. IL-6 Secretion by Trophoblast Cells After 25OHD Stimulation

To assess the anti-inflammatory properties of 25OHD, isolated trophoblast cells (from 5 NG and 5 GDM-d participants) were exposed for 3 days to various concentrations of

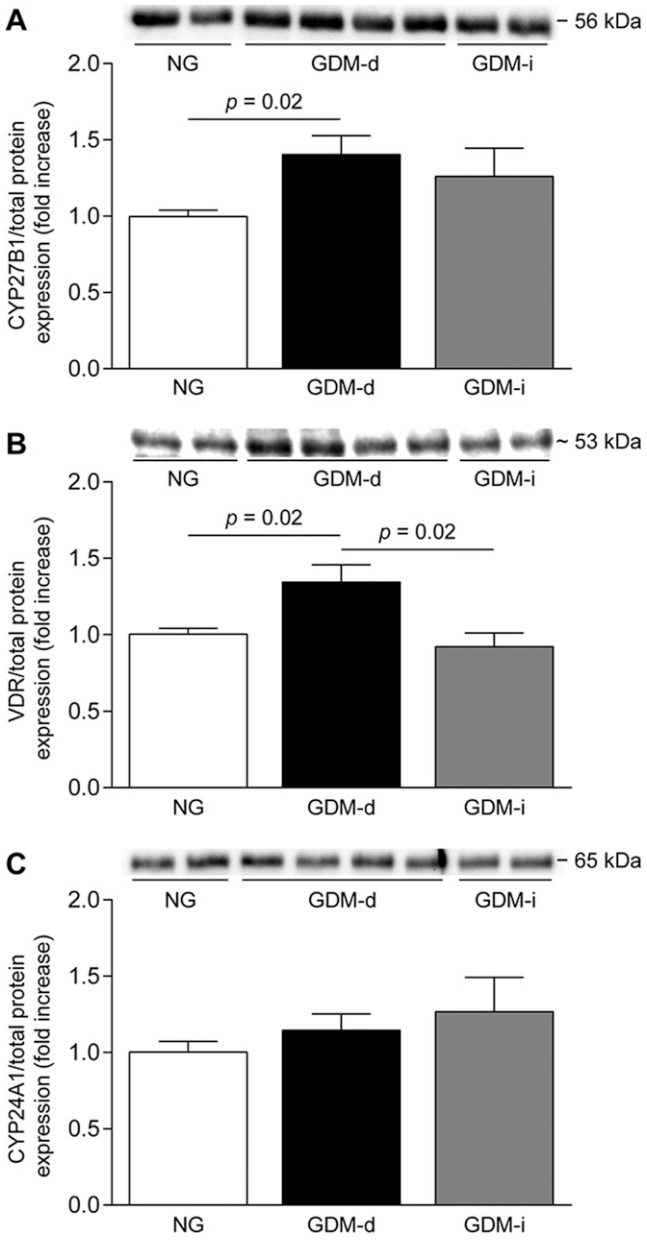

Figure 1. Quantification of placental (A) CYP27B1, (B) VDR, and (C) CYP24A1 protein levels for NG participants (n = 17), GDM-d participants with (n = 19), and GDM-i participants (n = 9). Results were normalized against total protein (TGX Stain-Free technology).

25OHD (ranging from 25 to 2000 nM) or its vehicle, and IL-6 secretion was measured in the culture media. We observed that baseline levels of IL-6 were not different between trophoblasts isolated from the placentas of NG and GDM-d participants (P = 0.31; Fig. 3A). In addition, the exposure of trophoblasts from NG pregnancies to increasing doses of 25OHD did not influence the secreted IL-6 levels (P for trend test = 0.26; Fig. 3B). However, measurement of IL-6 secretion by trophoblasts from GDM-d pregnancies revealed a significant decreasing linear trend after treatment from 0 to 2000 nM of 25OHD (P = 0.006; Fig. 3C). After exposure to 2000 nM of 25OHD, IL-6 levels were lower in trophoblasts from GDM-d pregnancies than in trophoblasts from NG pregnancies (P = 0.03; Fig. 3D).

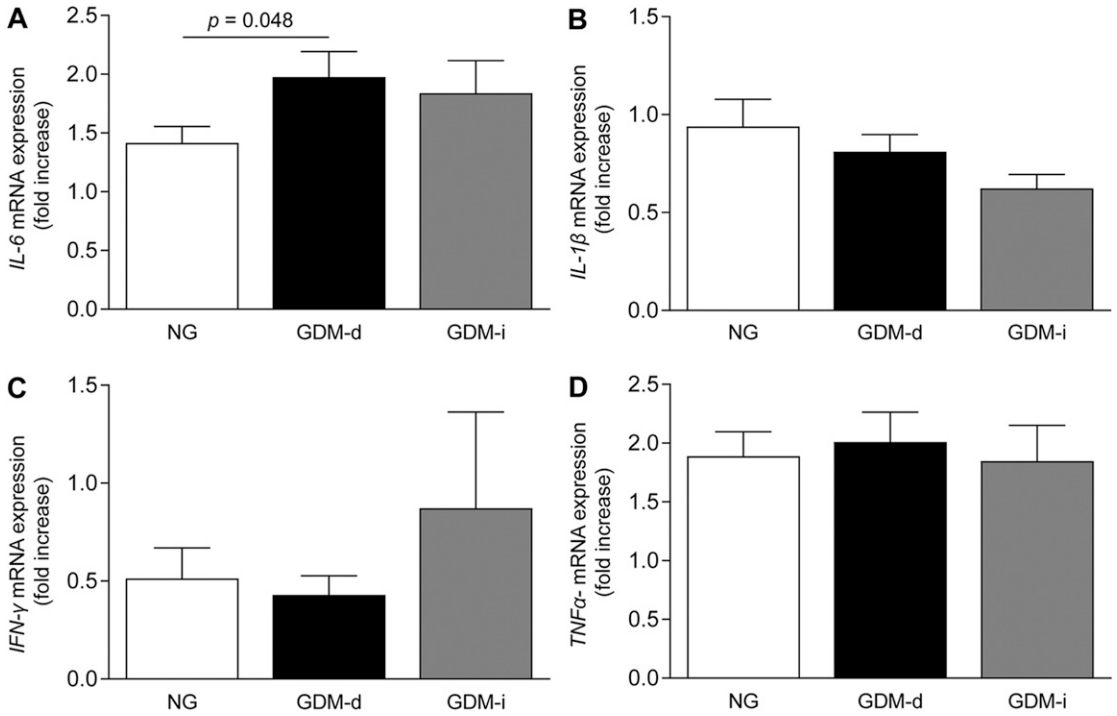

Figure 2. Quantification of placental (A) IL-6, (B) IL-1β, (C) IFN-γ, and (D) TNF-α mRNA levels for NG participants (n = 17), GDM-d participants (n = 19), and GDM-i participants (n = 8). Results were normalized against *YWHAZ* gene expression.

F. Protein Expression of CYP27B1, VDR, and CYP24A1 in Trophoblast Cells After 25OHD Stimulation

Because treatment with 25OHD decreased IL-6 secretion by trophoblasts from GDM-d pregnancies, we used the GDM-d and NG trophoblasts exposed to increasing concentrations of 25OHD to determine whether a linear trend or a specific pattern in protein expression of vitamin D metabolic components could be detected within each group. For the NG group, variations observed in CYP27B1 and VDR protein expression after 25OHD exposures were not statistically significant, and no linear trend was observed (Fig. 4A and 4C). In addition, CYP24A1 protein expression remained stable on average after 25OHD exposures in trophoblasts from NG pregnancies (Fig. 4E). For the GDM-d group, variations in protein expression of CYP27B1 and CYP24A1 were observed. However, no linear trend was detected, and no comparison between baseline and 25OHD exposures reached statistical significance (Fig. 4B and 4F). VDR protein expression in trophoblasts from GDM-d pregnancies was decreased after the addition of 25OHD (Fig. 4D). The comparison between baseline and treatment with 2000 nM of 25OHD almost reached statistical significance (P = 0.06), whereas the linear trend test was not significant (P = 0.18).

3. Discussion

The main findings of the current study include an upregulation of CYP27B1 and VDR protein expression in GDM-d placental tissues and a decrease in IL-6 secretion after 25OHD stimulation in the GDM-d cultured trophoblasts compared with the NG group. Our results demonstrated that placental protein expressions of CYP27B1 and VDR were higher in GDM-d pregnancies, whereas CYP24A1 expression was similar when compared with NG pregnancies. Taking together, these results suggest a rise in the ability to convert 25OHD into its active form, $1,25(OH)_2D$, without any change in its catabolism. In addition, elevated

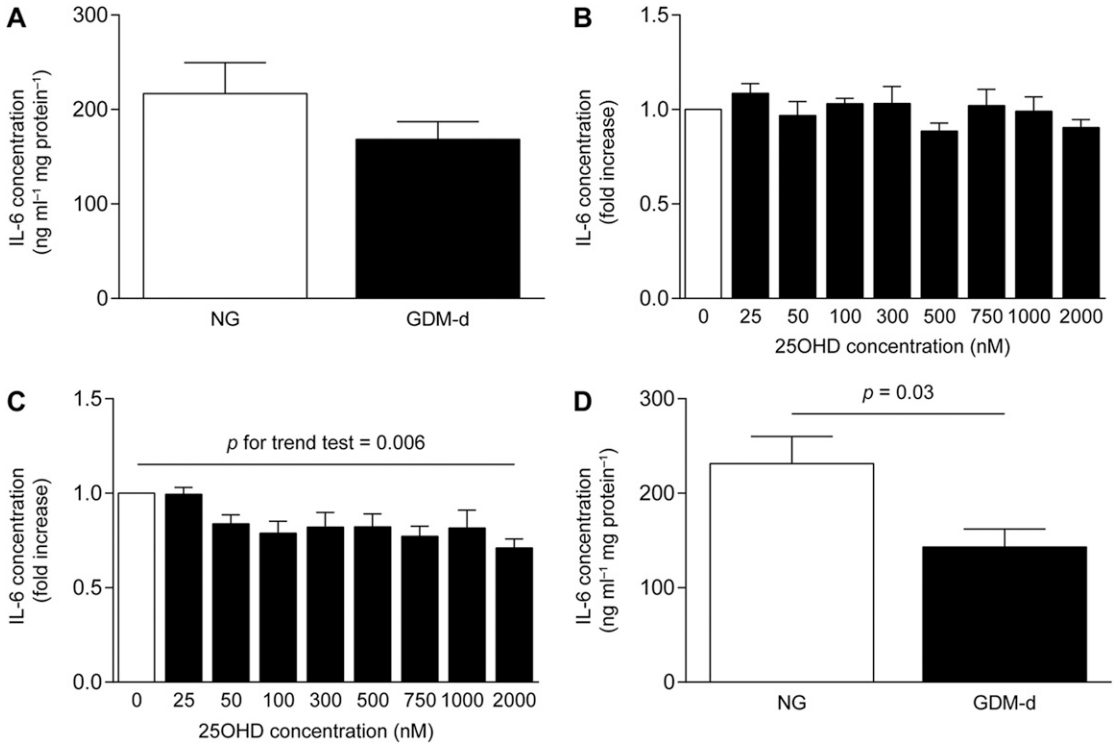

Figure 3. Regulation of IL-6 secretion by primary trophoblast cells isolated from placentas of NG participants (n = 5) and GDM-d participants (n = 5). (A) Baseline IL-6 levels in both groups. Levels of IL-6 after 25OHD exposures in (B) the NG group and (C) the GDM-d group. (D) Levels of IL-6 after exposure to 2000 nM 25OHD in both groups. (B, C) Results are expressed as fold change of control (vehicle-treated) cells, arbitrarily assigned a value of 1.0.

expression of VDR may favor the receptor bioavailability in GDM-d placentas. This upregulation of the receptor-ligand complex VDR-1,25$(OH)_2$D bioavailability in GDM-d placentas might reflect the need to enhance the effect of active vitamin D within the placental tissue in women with GDM.

In our study, the GDM group was divided into two subgroups to further investigate the effect of insulin therapy (GDM-i) compared with a diet and exercise intervention (GDM-d). In contrast to GDM-d, we found no differences in protein expression for any of the vitamin D metabolic components between GDM-i and NG placental tissues. The lack of difference between GDM-i and NG groups could be explained by the anti-inflammatory properties of insulin [50, 51]. It is possible that the inflammatory state is decreased at the feto-maternal interface in GDM-i women compared with GDM-d women. Therefore, the anti-inflammatory properties of insulin might override potential anti-inflammatory effect of vitamin D that we could observe. For this reason, we did not include a GDM-i group for experiments related to trophoblast cell cultures, because we wanted to assess the 25OHD anti-inflammatory properties without any bias from insulin's anti-inflammatory properties. Considering GDM treatment seems to be of great importance when studying vitamin D metabolic components in GDM pathophysiology.

Only two previous studies assessed placental protein expression of vitamin D metabolic components between normal and GDM pregnancies. Cho *et al.* [27] demonstrated an increase in CYP24A1 protein expression in GDM compared with normal placental tissues, whereas no differences were shown in CYP27B1 and VDR protein expression. A potential explanation for the disparity of the protein expression profile between our data and those of Cho *et al.* could be the dissimilar treatment received in the GDM groups in our study and the unknown GDM treatment in the Cho *et al.* study. In another study, Knabl *et al.* [52] assessed placental VDR protein expression by immunohistochemistry in insulin-treated women with GDM and NG

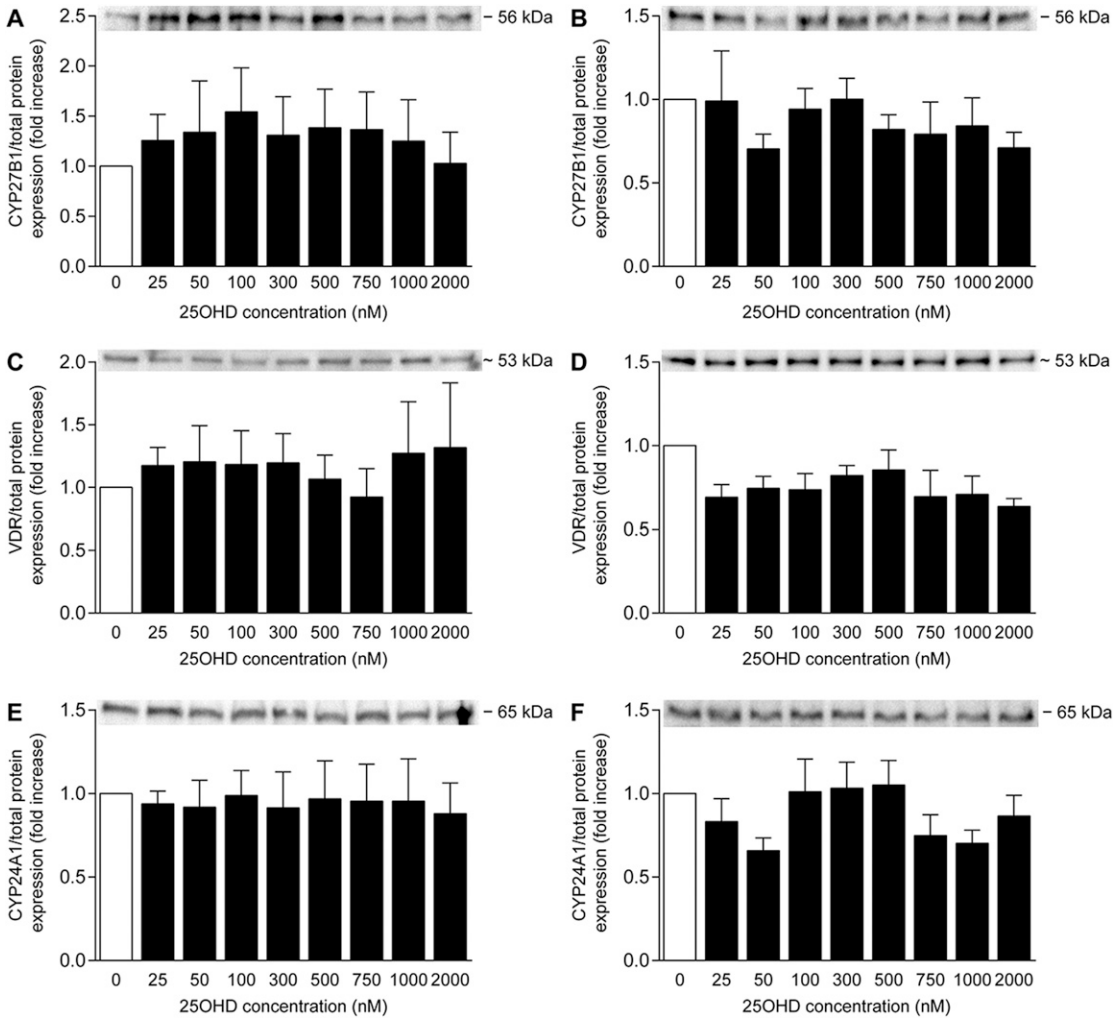

Figure 4. Quantification of (A, B) CYP27B1, (C, D) VDR, and (E, F) CYP24A1 protein levels in trophoblast cells isolated from placentas of (A, C, and E) NG participants (n = 5) and (B, D, and F) GDM-d participants (n = 5), respectively. Results were normalized against total protein (TGX Stain-Free technology).

women. In contrast to our results, they reported an upregulation of VDR expression in the villous syncytiotrophoblasts and in the villous endothelial capillaries in insulin-treated GDM compared with normal placental tissues. This disparity might be explained by differences in participants' characteristics, such as prepregnancy BMI and mode of delivery. In our study, most GDM-i participants were morbidly obese before pregnancy (median prepregnancy BMI of 42.3 kg/m^2), whereas insulin-treated participants with GDM in the Knabl *et al.* study were overweight (mean prepregnancy BMI of 28.1 kg/m^2). In addition, Knabl *et al.* included a majority of women who went into labor and delivered vaginally, whereas only women who planned to give birth by elective cesarean delivery were recruited in our study. This approach was chosen to eliminate the possible bias of increased inflammation at delivery, which can modify the placental expression of vitamin D metabolic components [53]. Another potential confounder that may explain the differences between our results and those of Cho *et al.* and Knabl *et al.* is vitamin D supplementation. Indeed, vitamin D could induce genomic changes during pregnancy that could modify gene/protein expression [54, 55]. We reported here the number of women taking vitamin D supplements in each group, whereas this information was not provided in the Cho *et al.* and Knabl *et al.* studies.

Our data on mRNA placental expression of the vitamin D metabolic components were different than those on protein expression. *CYP27B1* and *VDR* mRNA results were not affected in GDM-d placental tissues compared with NG placental tissues. Thus, the elevated protein expression of CYP27B1 and VDR in GDM-d placentas does not seem to be a consequence of an increased gene transcription. This observation led us to speculate that protein turnover (degradation) of CYP27B1 and VDR could be affected in GDM-d placentas compared with NG and GDM-i placentas. However, this hypothesis will need to be tested in further studies.

Inflammation is one of the proposed physiological mechanisms leading to GDM pathophysiology [4–9]. Thus, we measured mRNA expression of proinflammatory cytokines in placental tissues. Interestingly, only the IL-6 mRNA expression in GDM-d placental tissues was higher than in NG placental tissues, whereas the expression of the other cytokines did not differ between groups. These findings might be partly explained by the study design, considering that we measured mRNA expression in placental tissues, whereas the majority of studies investigating the association between inflammation and GDM published to date measured the circulating (systemic) levels of inflammatory markers. Indeed, only a few studies have measured inflammatory cytokine expression in the placenta. For instance, Yu *et al.* [56] showed upregulation of *TNF-α* and *IL-6* mRNA expression in GDM compared with normal placental tissues. Our *IL-6* mRNA expression results corroborate those observed by Yu *et al.*

IL-6 has been shown to be elevated in GDM pathophysiology [4–7]. Therefore, we measured IL-6 levels as a marker of inflammation in trophoblasts, because these cells are capable of secreting this proinflammatory cytokine [30]. Accordingly, we expected an increased level of IL-6 in GDM-d trophoblast culture media, but we observed no difference in baseline IL-6 levels between GDM-d and NG trophoblasts. This result might be partly explained by the number of participants taking vitamin D supplementation. Indeed, twice more GDM-d participants whose placentas were used for trophoblast cultures were taking vitamin D supplementation compared with NG participants. As a result, IL-6 levels may have been lowered in GDM-d women. Moreover, primary isolated trophoblasts were differentiated in culture for 4 days with 10% FBS into the media to ensure optimal cell shape and avoid cell death. Therefore, we cannot exclude the possibility that IL-6 secretion might have been different between groups at an earlier time point and that adding 10% FBS into the culture media might have decreased the inflammatory cytokine production by trophoblasts because of the presence of survival factors such as insulin.

This study investigated the effect of exposure to the vitamin D dominant circulating form (25OHD) on IL-6 secretion by primary cultured human trophoblasts. A previous study by Díaz *et al.* [30] assessed the effect of a treatment with the active form of vitamin D [1,25$(OH)_2$D] on inflammatory cytokine expression and secretion of trophoblasts from normal pregnancies in response to TNF-α. The study showed that adding 1,25$(OH)_2$D to the culture media decreased mRNA expression and secretion of IL-6 induced by TNF-α. Our study provides additional insight by comparing the effect of 25OHD between trophoblasts from GDM-d and NG pregnancies. An important finding of the current study is that IL-6 concentrations in culture media after treatment with 2000 nM 25OHD were lower in GDM-d trophoblasts than in NG trophoblasts. Even more interestingly, data indicated a decreasing linear trend in secreted IL-6 levels after treatment from 0 to 2000 nM of 25OHD, observed only for GDM-d trophoblasts. Our results suggest that vitamin D–stimulated mechanisms leading to inhibition of IL-6 secretion may be more sensitive in GDM-d trophoblasts, possibly reflecting an increased necessity for the anti-inflammatory effect of vitamin D.

In line with these results, Diaz *et al.* [30] demonstrated that the decrease in IL-6 mRNA expression after treatment of NG trophoblasts with 1,25$(OH)_2$D involved a VDR-dependent mechanism, because the presence of a specific VDR antagonist prevented this effect. In addition, previous studies on prostate epithelial cells and adipocytes suggested that regulation of IL-6 production was dependent on the action of VDR [57, 58]. The authors proposed that the VDR-1,25$(OH)_2$D complex interacts with the vitamin D response element present in

the promoter region of the MAPK phosphatase 5 (MKP5) gene, resulting in enhanced gene transcription. MKP5 is known to specifically dephosphorylate and inactivate p38 MAPK, leading to decreased production of IL-6 and other inflammatory cytokines. Thus, according to the literature, the VDR-1,25$(OH)_2$D complex and its genomic actions seem to be involved in IL-6 regulation by vitamin D. Additional studies will be needed to determine the mechanistic pathway linking vitamin D and IL-6 secretion by trophoblasts.

In our study, as we observed differences in placental protein expression of vitamin D metabolic components between GDM-d and NG groups and a decreasing linear trend of the secreted IL-6 levels by GDM-d trophoblasts after adding increasing 25OHD concentrations, we sought to verify the protein expression of VDR, CYP27B1, and CYP24A1 in GDM-d and NG trophoblasts after the addition of the same increasing 25OHD concentrations. Surprisingly, no linear trend or constant pattern was detected for the expression of any of the three proteins in GDM-d and NG trophoblasts. Judging from the literature, we expected an increase in CYP24A1 protein expression after 25OHD treatments [59]. In both groups, trophoblasts were treated with 25OHD concentrations every day for 3 days, and then IL-6 secretion and protein expression of the vitamin D metabolic components were evaluated. We cannot exclude the possibility that modulation of protein expression of CYP27B1, VDR, and CYP24A1 may have occurred earlier.

In summary, our results showed upregulation of CYP27B1 and VDR protein expression in GDM-d placental tissues, which could lead to an increase in the VDR-1,25$(OH)_2$D complex bioavailability, underlying the importance of vitamin D in pregnancy and GDM pathophysiology. Our findings also suggest an anti-inflammatory effect of vitamin D at the feto-maternal interface in GDM-d trophoblasts. Because inflammation is likely to be a significant risk factor for GDM, it is of great interest to further investigate the potential therapeutic anti-inflammatory effect of vitamin D in GDM. Future studies are needed to investigate regulation of other inflammatory cytokines by vitamin D to corroborate our findings.

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