Loss of placental thrombomodulin in oocyte donation pregnancies

Manon Bos, B.Sc.,[a] Hans J. Baelde, Ph.D.,[a] Jan A. Bruijn, M.D., Ph.D.,[a] Kitty W. M. Bloemenkamp, M.D., Ph.D.,[b] Marie-Louise P. van der Hoorn, M.D., Ph.D.,[c] and Rosanne J. Turner, B.Sc.[a]

[a] Department of Pathology, Leiden University Medical Centre, Leiden; [b] Department of Obstetrics, University Medical Center Utrecht, Wilhelmina Children's Hospital, Utrecht; and [c] Department of Obstetrics, Leiden University Medical Centre, Leiden, the Netherlands

Objective: To investigate whether thrombomodulin dysregulation is involved in the development of preeclampsia after oocyte donation (OD). Women who become pregnant after OD are prone to develop preeclampsia, a syndrome characterized by an aberrant immunologic response, hypercoagulability, and endothelial dysfunction. A mediator of inflammation and coagulation is thrombomodulin, which has a possible role to play in this syndrome.
Design: Case-control study.
Setting: Not applicable.
Patient(s): Placentas from 82 women with an uncomplicated pregnancy (48 naturally conceived, 21 IVF, and 33 OD pregnancies) and 9 women with an OD pregnancy complicated by preeclampsia have been studied.
Intervention(s): None.
Main Outcome Measure(s): Abundances of thrombomodulin protein and vitamin D receptor (VDR) were determined using immunohistochemistry; mRNA expression was determined using quantitative polymerase chain reaction.
Result(s): Placental thrombomodulin protein abundance was lower in OD pregnancies (diffuse pattern in 45%) than in controls (diffuse pattern in 96%). Placental thrombomodulin mRNA expression was lower in OD pregnancies complicated by preeclampsia (0.72 ± 0.47) compared with in uncomplicated OD pregnancies (0.43 ± 0.18). Thrombomodulin expression correlated with inflammation and coagulation. VDR expression was decreased in OD pregnancies complicated by preeclampsia and was correlated with thrombomodulin mRNA.
Conclusion(s): Pregnancies conceived through OD lose placental thrombomodulin expression. This loss is associated with an increased coagulation and inflammation and indicates that endothelial protection is diminished in OD pregnancies, which might be an explanation for the increased risk for preeclampsia. Vitamin D metabolism is dysregulated in OD pregnancies and might be a target for therapy. (Fertil Steril® 2017;107:119–29. ©2016 by American Society for Reproductive Medicine.)
Key Words: Preeclampsia, oocyte donation, placenta, thrombomodulin, vitamin D

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Oocyte donation (OD) is a technique that enables women with diminished ovarian reserve to conceive. Pregnancy is a challenging state for the mother's immune system, where a controlled environment with a regulated immune response to the semiallogeneic fetus has to be established. After OD, the fetus is completely allogeneic; this even more challenging environment for the immune system is presumed to contribute to the increased number of obstetrical complications observed after OD (1, 2). These complications can be explained, in large part, by the increased prevalence of pregnancy-induced hypertension and preeclampsia (3–8).

Preeclampsia, a hypertensive disorder during pregnancy, is a leading cause of maternal and neonatal morbidity and mortality worldwide (9). The pathophysiology of preeclampsia is not fully understood, but the syndrome is characterized by impaired placental development and subsequent shedding of syncytial trophoblast. This results in the release of antiangiogenic factors such as soluble Flt-1, which binds to vascular endothelial growth factor in the circulation (10). These factors contribute to a maternal intravascular systemic inflammatory response, leading to generalized endothelial dysfunction, enhanced leukocyte and complement activation, and coagulation (10).

The role of the placenta in the pathogenesis of preeclampsia in OD pregnancies is presumed to be different from that in preeclampsia in naturally conceived pregnancies (11–13). Although these patients are subject to an increased risk of preeclampsia because of older age (9), it is also

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Reprint requests: Manon Bos, B.Sc., Leiden University Medical Centre, Department of Pathology, L1 Q, P.O. Box 9600, P0-107, 2300 RC Leiden, the Netherlands (E-mail: m.bos@lumc.nl).

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known that OD is an independent risk factor for hypertensive complications of pregnancy (14, 15). Furthermore, the clinical presentation of these patients is different from patients with preeclampsia after a naturally conceived pregnancy: growth restriction after OD pregnancies complicated by preeclampsia is less severe (6, 12, 16, 17). Moreover, the pathophysiology of preeclampsia after naturally conceived pregnancies seems to be of a more vascular origin (10), whereas preeclampsia after OD presumably has a more immunological origin (1, 11, 12, 18).

In women with preeclampsia in naturally conceived pregnancies, serum levels of the breakdown product of thrombomodulin are higher in comparison with uncomplicated naturally conceived pregnancies (19), and placental thrombomodulin protein and mRNA expression in naturally conceived pregnancies is decreased (20). Thrombomodulin is a protein essential for the maintenance of endothelium; it inhibits inflammatory pathways and apoptotic pathways in endothelial cells, and it inhibits coagulation (21). The pathways through which thrombomodulin is regulated in the placenta are currently not precisely known, but the angiogenic imbalance, as seen in preeclampsia, has been shown to decrease thrombomodulin expression (20). Another possible regulator of placental thrombomodulin is vitamin D; decreased vitamin D levels are associated with an increased incidence of preeclampsia (22), and vitamin D increases thrombomodulin expression in endothelial aorta cells (23).

Despite the fact that the placenta is presumed to have a different role in the pathophysiology of preeclampsia after OD pregnancies and naturally conceived pregnancies; the placental thrombomodulin expression might be alterd in OD pregnancies complicated by preeclampsia as well. In both naturally conceived and OD pregnancies, preeclampsia is characterized by endothelial dysfunction, inflammation, and hypercoagulability (12). Therefore, our objectives are to investigate placental thrombomodulin expression, downstream effects of thrombomodulin, and the regulation of thrombomodulin in women with preeclampsia after OD and in women with uncomplicated pregnancies that were either naturally conceived (20), induced by IVF, or induced by OD, as control subjects. We hypothesize that thrombomodulin expression is altered in OD pregnancies complicated by preeclampsia.

MATERIALS AND METHODS

Patients Who Underwent OD and Control Groups

A case-control study with 56 placentas from women pregnant after OD in the Leiden University Medical Centre (LUMC) and teaching hospitals in the region between 2004 and 2013 was performed; 40 placentas were from pregnancies without hypertensive complications, and 16 placentas were from women with preeclampsia according to International Society for the Study of Hypertension in Pregnancy (ISSHP) guidelines (24). Patients who had an OD with at least information on maternal age, gestational age, birth weight, highest diastolic blood pressure, and available paraffin-embedded placenta samples were included. Twenty-eight uncomplicated naturally conceived pregnancies and 21 IVF-induced pregnancies were selected from available patients as controls. Using IVF pregnancies and naturally conceived pregnancies as controls for OD pregnancies has been described before in comparable studies (12, 18). Controls were selected on the basis of mode of delivery, because mode of delivery has a broad impact on the placenta and influences gene expression (25). No other selection criteria were used.

Small for gestational age was defined as birth weight below the 10th percentile for gestational age according to the Dutch reference curves for birth weight by gestational age (26).

Patient characteristics were obtained from the medical records. From all placentas, paraffin-embedded samples were available for immunohistochemical staining. Frozen tissue, which was used for mRNA analysis, was available for 36 uncomplicated OD placentas, 16 placentas from an OD pregnancy complicated by preeclampsia, and 10 placentas from an uncomplicated naturally conceived pregnancy. As previously described, the placentas of twins and triplets were treated as individual samples, since placental pathology can be different in twins. Informed consent was obtained from all patients. This study was approved by the ethics committee of LUMC (P13.084).

Thrombomodulin and Maternal Age

Placentas from an additional group of older women ($n = 20$; maternal age >37) with an uncomplicated naturally conceived pregnancy were included to investigate the effect of maternal age on placental thrombomodulin protein expression.

Histochemical Staining

Histological phosphotungistic acid-hematoxylin (PTAH) staining was performed to investigate the presence of fibrin depositions. Sections were incubated in 0.25% potassium permanganate for 15 minutes followed by 5% oxalic acid for 5 minutes. Sections were then incubated in PTAH for 24 hours at room temperature.

Immunohistochemistry

Immunohistochemical staining was performed to investigate the placental protein abundance of thrombomodulin and the vitamin D receptor (VDR). We choose to determine placental VDR expression since decreased placental VDR expression has been described as a proper measure for disturbances in vitamin D signaling before (27, 28).

Sections were deparaffinized, and antigen retrieval was performed. Sections were incubated with anti-thrombomodulin mouse monoclonal antibody (1:200; Leica Biosystems) or an anti-VDR mouse monoclonal antibody (1:1,500) for 1 hour at room temperature. Binding of the primary antibody was visualized with a PO-labeled anti-mouse polymer (DAKO) and diaminobenzidine as a chromogen.

Scoring of Staining Patterns

Slides were scored by two observers blinded with respect to cases and control groups. Twenty percent of cases were scored differently between observers, and for those, consensus was

obtained during a consensus meeting. Thrombomodulin protein abundance at the surface of viable syncytiotrophoblast was scored semiquantitatively as absent (present on <10% of viable syncytiotrophoblast), focal (present on 10%–50% of viable syncytiotrophoblast), or overall (present on >50% of viable syncytiotrophoblast) as described elsewhere (20). Viability of syncytiotrophoblast was confirmed using hematoxylin-eosin staining. The presence of fibrin depositions on the villi was scored similarly.

VDR abundance at the surface of syncytiotrophoblast was scored semiquantitatively as overall (>90% of syncytiotrophoblast positive for VDR), decreased (90%–50% of syncytiotrophoblast positive VDR), and absent (<50% of syncytiotrophoblast positive for VDR).

Quantitative Polymerase Chain Reaction (PCR)

Quantitative PCR was performed to quantify placental mRNA expression of thrombomodulin, VDR, intercellular adhesion molecule-1, tumor necrosis factor-alpha, factor VIII, tissue factor, vascular endothelial growth factor, and soluble FLT-1. Primer sequences can be found in Supplemental Table 1. RNA isolation was performed with TRIzol (Lifetechnologies). Synthesis of cDNA was performed with AMV reverse transcriptase (Roche), and SYBR green quantitative PCR was performed according to the manufacturer's protocol (Bio-Rad Laboratories). Expression was measured by the comparative threshold cycle method and normalized to expression of housekeeping genes hypoxanthine phosphoribosyltransferase and GAPDH. A melting curve analysis was performed to verify the specificity of amplification.

Cell Culture Experiments

Cell culture experiments were performed to investigate placental thrombomodulin regulation through vitamin D signaling. The human choriocarcinoma cell line BeWo (CCL-98, ATCC) was cultured in RPMI medium supplemented with 10% fetal calf serum. Short tandem repeat analysis with GenePrint (Promega) confirmed the identity of the cells. Cells were used within 25 passages.

Forskolin (50 μM, dissolved in dimethyl sulfoxide 0.1%; Sigma-Aldrich) was used to stimulate VDR expression (29). Vitamin D (dissolved in ethanol 1%, Sigma-Aldrich) was added to the medium with a final concentration of 100 nM (29).

Cells were cultured for 24 hours, and then forskolin alone or forskolin with 1,25-di-hydroxyl-vitamin D was added to refreshed medium (29). Cells were cultured for 48 hours before mRNA was isolated (29). Normalized mRNA expression of thrombomodulin and VDR was measured. Experiments were repeated 4 times.

Statistical Analysis

For statistical analyses, placentas from OD pregnancies complicated by preeclampsia were compared with placentas from uncomplicated OD pregnancies. Placentas from uncomplicated OD pregnancies were compared with placentas from uncomplicated naturally conceived and uncomplicated IVF-induced pregnancies.

Factors considered potential confounders were maternal age at pregnancy, body mass index (BMI), smoking, gravidity, parity, and twin or triplet pregnancy. Continuous data were compared between groups using the independent *t*-test for normally distributed data or a Mann-Whitney *U*-test for skewed distributions. Discontinuous data were analyzed with the χ^2-test of Fisher's exact test.

Correlations between thrombomodulin mRNA and other mRNA levels or clinical parameters were determined with the Spearman's rho test or Pearson test. $P<.05$ was considered statistically significant. Analyses were performed with the IBM SPSS statistics software package (ver. 21; IBM).

RESULTS

Patient Characteristics

Patient characteristics can be found in Table 1. The mean age of women who had a naturally conceived pregnancy (33.7 years) or a pregnancy after IVF (35.0 years) was significantly lower than the mean age of women with an uncomplicated OD pregnancy (38.5 years; $P<.05$ for both). The average age of women with OD pregnancies complicated by preeclampsia was 42.9 years; this was significantly higher compared with women with uncomplicated OD pregnancies ($P<.05$). The mean BMI of women with a naturally conceived pregnancy was significantly higher than the mean BMI of women with an uncomplicated OD pregnancy (respectively, 25.81 and 21.37; $P<.05$ for both). The mean BMI of women with preeclampsia during OD pregnancies was higher than the mean BMI of women with uncomplicated OD pregnancies ($P=.044$). Medical histories from previous pregnancies were available for all control cases and for 46 out of 56 OD pregnancies. In none of our cases was a history of hypertension or preeclampsia reported in the medical records. Furthermore, none of the patients with an OD-induced pregnancy had diabetes mellitus, gestational diabetes, or disturbances in coagulation according to the medical records. Three women with an uncomplicated OD pregnancy had proteinuria, and five had a diastolic blood pressure > 85 mmHg. Nevertheless, none of these women fulfilled the ISSHP criteria (24) for the diagnosis of preeclampsia nor were they treated for these symptoms.

Pregnancy Characteristics

Mean gestational age was significantly lower in children born after OD pregnancies complicated by preeclampsia ($P<.001$). In OD pregnancies, more twins and triplets were seen compared with in naturally conceived pregnancies ($P<.01$), and women pregnant with twins or triplets more often had preeclampsia ($P<.01$).

Fetal Characteristics

The mean birth weight of children was lower in IVF pregnancies compared with in naturally conceived pregnancies and compared with in OD pregnancies complicated by preeclampsia ($P<.001$). When corrected for gestational age, these differences were no longer significant.

TABLE 1

Patient characteristics.

	NC (n = 28)	IVF (n = 21)	OD (n = 33)	OD PE (n = 9)
Maternal characteristics				
Maternal age (y), mean (SD)	33.7 (4.0)	35.0 (3.9)	38.5 (5.8)[a,b]	42.9 (4.7)[c]
Maternal BMI (kg/m^2), mean (SD)	25.81 (4.9)	24.65 (5.2)	21.37 (1.9)[a]	24.21 (0.9)[c]
Smoking (%)				
Yes	1 (3.6)	2 (10)	1 (6.7)	1 (16.7)
No	27 (96.4)	18 (90)	14 (93.3)	5 (83.3)
Gravidity, mean (range)	2.9 (1–7)	2.2 (1–5)[a]	2.1 (1–7)[a]	2.1 (1–5)
Parity, mean (range)	1.4 (0–5)	0.5 (0–2)[a]	0.4 (0–2)[a]	0.2 (0–1)
Highest diastole, mmHg (SD)	75 (70–80)	80 (65–95)[a]	80 (60–90)[a]	99 (85–115)[c]
Proteinuria, n (%)				
Yes	0 (0)	1 (5.6)	3 (23.1)	9 (100)
No	28 (100)	17 (94.4)	10 (76.9)[a]	0 (0)[c]
Prior history of hypertension or preeclampsia, n (%)				
Yes	0 (0)	0 (0)	0 (0)	0 (0)
No	28 (100)	21 (100)	25 (100)	7 (100)
Pregnancy characteristics				
Gestational age at delivery, wk + d (SD [d])	39 + 4 (9)	38 + 5 (17)	39 + 2 (15)	34 + 2 (21)[c]
Term (%)				
A term	28 (100)	15 (78.5)	30 (93.8)	2 (22.2)
Preterm	0 (0)	4 (21.5)[a]	2 (6.2)	7 (77.8)[c]
Mode of delivery, n (%)				
Caesarean section	16 (57.1)	9 (45)	17 (54.8)	6 (66.7)
Vaginal delivery	12 (42.9)	11 (55)	14 (45.2)	3 (33.3)
Twin or triplet, n (%)				
Yes	0 (0)	1 (5)	7 (21.2)	6 (66.7)
No	28 (100)	19 (95)	26 (78.8)[a]	3 (33.3)[c]
Fetal characteristics	n = 28	n = 21	n = 40	n = 16
Sex of child, n (%)				
Male	12 (42.9)	10 (47.6)	20 (54.1)	9 (56.3)
Female	16 (57.1)	11 (52.4)	17 (45.9)	7 (43.9)
Birth weight, g (SD)	3,597 (435)	3,113 (701)[a]	3,297 (717)	1,952 (660)[c]
Small for gestational age, n (%)				
Yes	2 (7.1)	3 (14.3)	3 (8.1)	0 (0)
No	26 (92.9)	18 (85.7)	34 (91.9)	14 (100)
Placenta weight, g (SD)	638 (140)	611 (149)	698 (204)	863 (238)

Note: NC = naturally conceived; OD PE = OD pregnancies complicated by preeclampsia.
[a] $P<.05$ compared with uncomplicated NC pregnancies.
[b] $P<.05$ compared with uncomplicated IVF pregnancies.
[c] $P<.05$ compared with uncomplicated OD pregnancies.

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Thrombomodulin Protein Expression

Placental thrombomodulin staining was observed in three distinct patterns; representative examples of these patterns are depicted in Figure 1A–1C. Syncytiotrophoblast thrombomodulin protein abundance was decreased more often after uncomplicated OD pregnancies and after OD pregnancies complicated by preeclampsia compared with pregnancies conceived through IVF and naturally conceived pregnancies ($P<.001$; Fig. 1D).

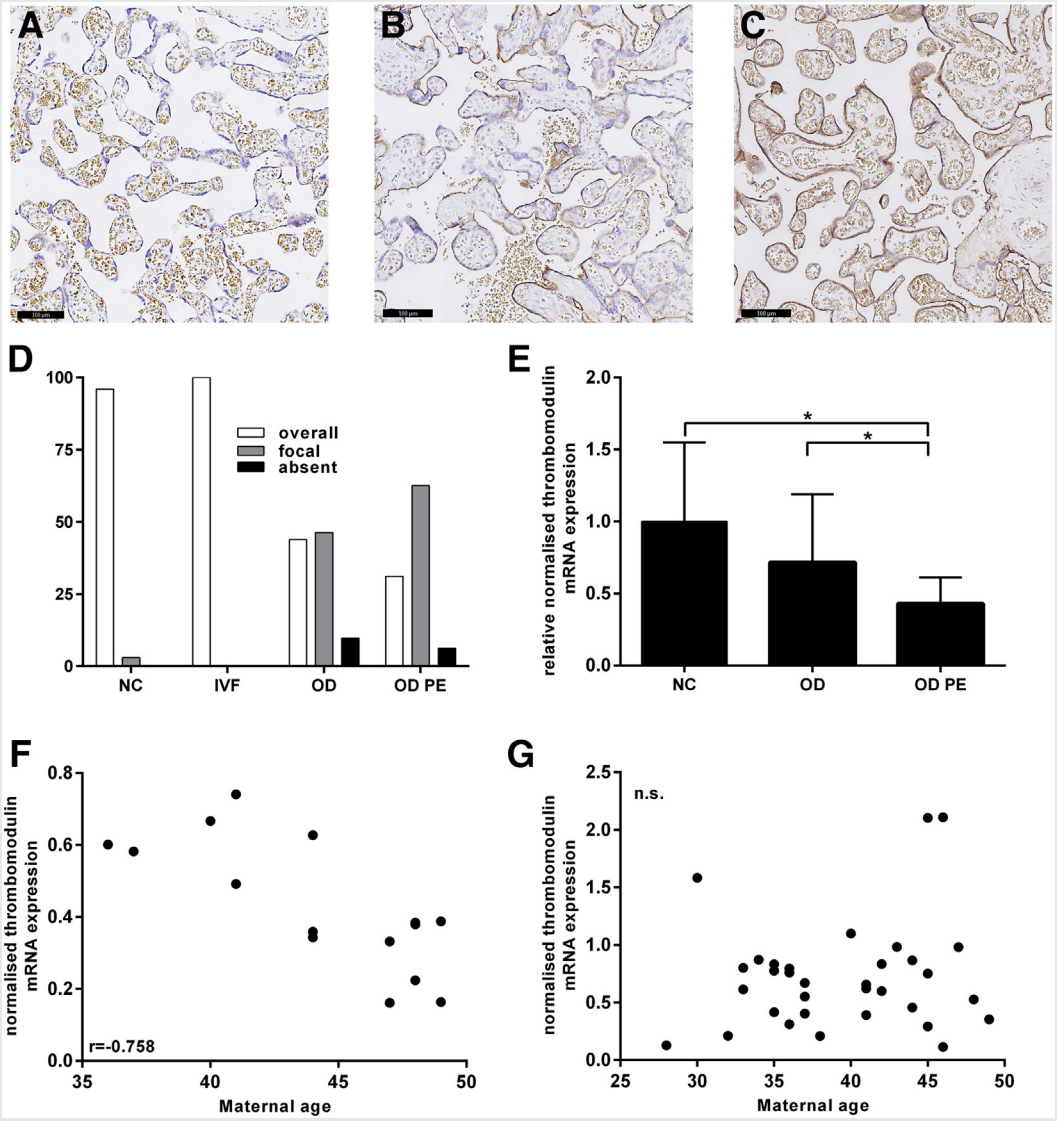
Thrombomodulin Protein Expression in Older Women

Since the maternal age of women who had an uncomplicated OD pregnancy was significantly higher than that of women who had a naturally conceived pregnancy or a pregnancy after IVF, we included an additional group of women >37 years who had an uncomplicated naturally conceived pregnancy to study the effect of maternal age on placental thrombomodulin expression. The mean maternal age of these women was 39.4 years; this was significantly higher compared with women who had an uncomplicated naturally conceived pregnancy; characteristics of this additional group are depicted in Supplemental Table 2. Placental thrombomodulin expression was diffuse in 19 out of 20 older women; this was similar as the placental thrombomodulin abundance in the group of uncomplicated naturally conceived control pregnancies in younger women.

Thrombomodulin mRNA Expression

Placental thrombomodulin mRNA expression was decreased in women with preeclampsia after OD, compared with in uncomplicated OD pregnancies ($P<.001$, Fig. 1E). Placental thrombomodulin mRNA expression was similar in naturally conceived pregnancies and uncomplicated OD pregnancies. Thrombomodulin mRNA expression was slightly higher in samples with an overall thrombomodulin protein staining pattern (mean = 0.5415; SD = 0.1315) compared with samples with a focal or absent staining pattern (mean = 0.3891; SD = 0.1850) in OD pregnancies complicated by preeclampsia ($P=.138$).

FIGURE 1



Placental thrombomodulin expression. (**A**) Representative example of an absent (<10% of viable syncytiotrophoblast) placental thrombomodulin protein expression pattern. (**B**) Representative example of a focal (10%–50% of viable syncytiotrophoblast) placental thrombomodulin protein expression pattern. (**C**) Representative example of an overall (>50% of viable syncytiotrophoblast) placental thrombomodulin protein expression pattern. (**D**) Distribution of placental thrombomodulin protein abundance patterns in women with an OD pregnancy complicated by preeclampsia and control groups ($P<.001$ for overall χ^2 testing). (**E**) Relative normalized placental thrombomodulin mRNA expression in case and control groups (*$P<.05$, Mann-Whitney *U*-test). (**F**) Correlation of thrombomodulin mRNA expression and maternal age in OD pregnancies complicated by preeclampsia (r = −0.758, $P<.001$, Pearson's correlation test). (**G**) Correlation of thrombomodulin mRNA expression and maternal age in uncomplicated OD pregnancies ($P>.05$, Pearson's correlation test). NC = naturally conceived; OD PE = OD pregnancies complicated by preeclampsia; NS = not significant.

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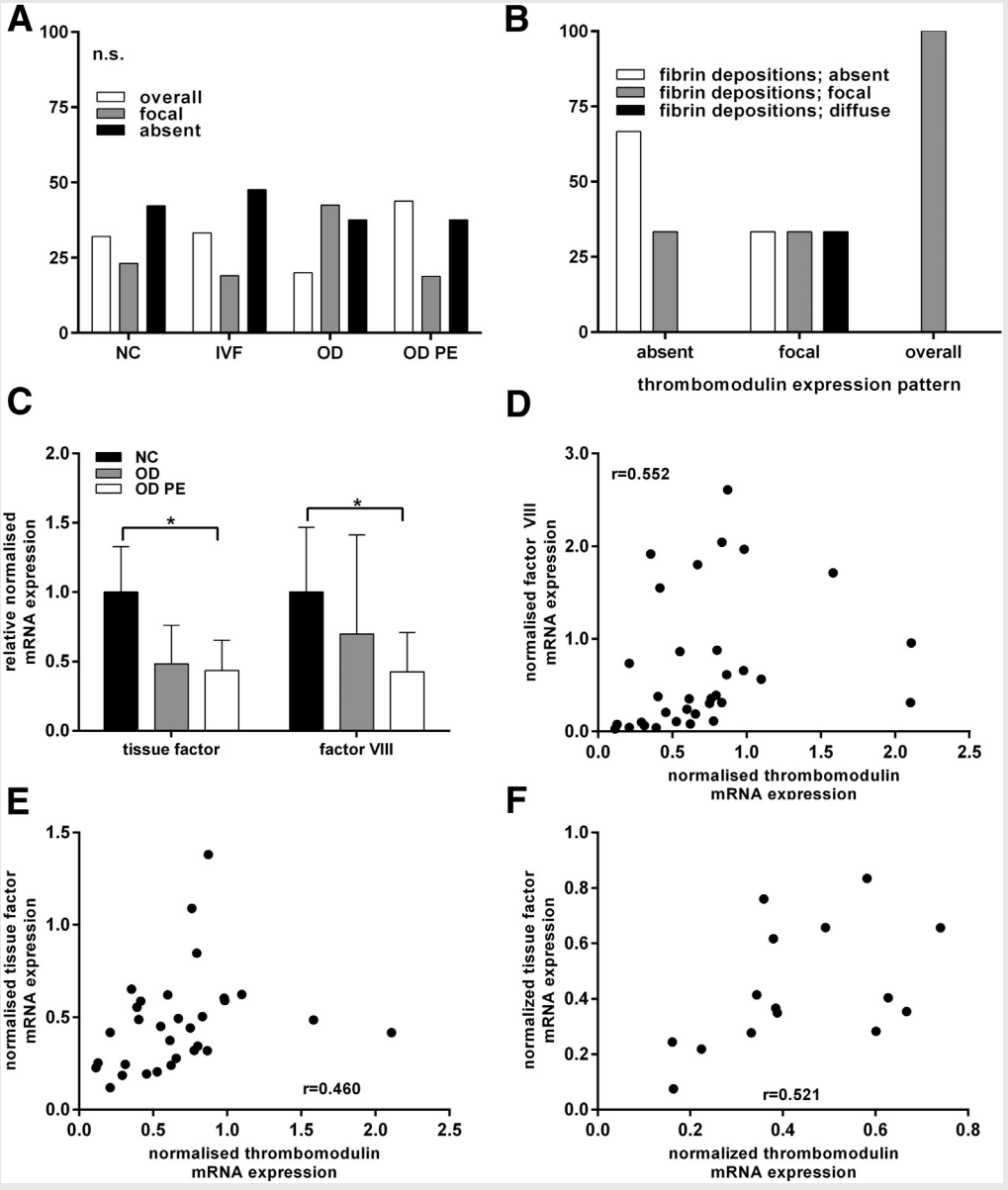
Thrombomodulin mRNA correlated inversely with maternal age in the preeclampsia OD group (r = −0.758, $P=.001$; Fig. 1F). This correlation was not present in uncomplicated OD pregnancies (Fig. 1G). Thrombomodulin mRNA levels were not associated with fetal characteristics nor patient characteristics. Furthermore, thrombomodulin mRNA levels were similar in multiplets and singletons in uncomplicated OD pregnancies and OD pregnancies complicated by preeclampsia (Supplemental Fig. 1).

Downstream Effects of Thrombomodulin: Coagulation

Fibrin depositions were evenly distributed over all groups (Fig. 2A). Increasing thrombomodulin protein abundance was associated with decreasing presence of fibrin deposits in women with OD pregnancies complicated by preeclampsia ($P=.019$; Fig. 2B). This association was not present between thrombomodulin mRNA levels and fibrin deposits.

FIGURE 2



Downstream effects of thrombomodulin: coagulation and inflammation. (**A**) Presence of fibrin deposits as detected with histological PTAH staining in placentas from OD pregnancies complicated by preeclampsia and control pregnancies (naturally conceived, IVF and OD; $P>.05$ with overall χ^2 testing). (**B**) The association between placental thrombomodulin expression pattern and the amount of fibrin deposits in OD pregnancies complicated by preeclampsia ($P=.019$, with overall χ^2 testing). (**C**) Relative normalized tissue factor and factor VIII mRNA expression in OD pregnancies complicated by preeclampsia and control groups (*$P<.05$, Mann-Whitney *U*-test). (**D**) Correlation of factor VIII and thrombomodulin mRNA expression in the uncomplicated OD control group (r = 0.552, $P<.001$). (**E**) Correlation of normalized tissue factor mRNA and thrombomodulin mRNA expression in the uncomplicated OD control group (r = 0.460, $P=.009$). (**F**) Correlation of normalized tissue factor mRNA and thrombomodulin mRNA expression in OD pregnancies complicated by preeclampsia (r = 0.521, $P=.046$).

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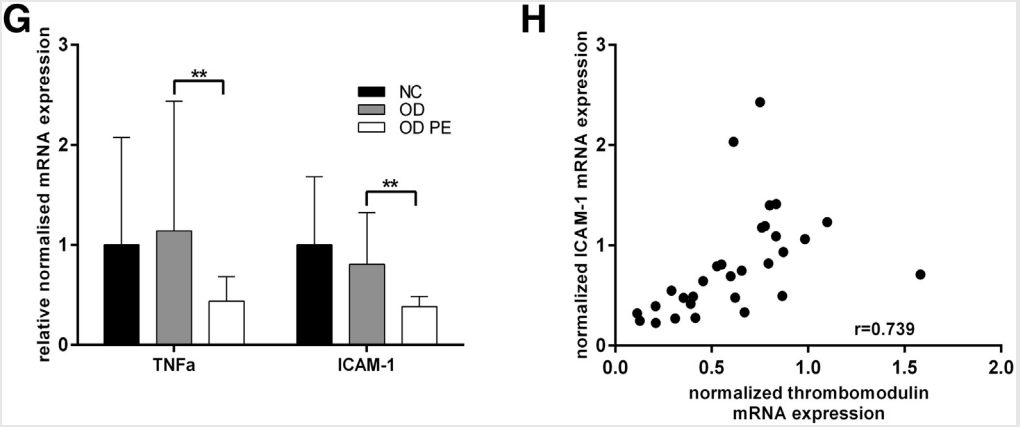
FIGURE 2 Continued



(**G**) Relative normalized tumor necrosis factor-alpha and intercellular adhesion molecule-1 mRNA expression in placentas of women with preeclampsia after OD and control groups (**P<.001; Mann-Whitney U-test). (**H**) Correlation of intercellular adhesion molecule 1 and thrombomodulin mRNA expression in uncomplicated OD control pregnancies r = 0.739, P<.001; Pearson's correlation test). ICAM-1 = intercellular adhesion molecule-1; NC = naturally conceived; OD PE = OD pregnancies complicated by preeclampsia; TNFa = tumor necrosis factor-alpha; NS = not significant.

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Placental mRNA expression of tissue factor and factor VIII was slightly lower in uncomplicated OD pregnancies compared with in naturally conceived pregnancies and were lower in OD pregnancies complicated by preeclampsia compared with naturally conceived pregnancies (P<.05 for both; Fig. 2C). However, placental mRNA levels of tissue factor and factor VIII were not different between OD pregnancies complicated by preeclampsia and uncomplicated OD pregnancies.

Placental thrombomodulin mRNA expression and factor VIII mRNA expression were correlated in uncomplicated OD pregnancies (r = 0.552, P<.001; Fig. 2D). Placental thrombomodulin mRNA expression correlated with tissue factor mRNA expression in uncomplicated OD pregnancies (r = 0.460, P=.009; Fig. 2E) and OD pregnancies complicated by preeclampsia (r = 0.521, P=.046; Fig. 2F).

Downstream Effects of Thrombomodulin: Inflammation

Placental tumor necrosis factor-alpha and intercellular adhesion molecule-1 mRNA expression were similar in uncomplicated OD pregnancies and naturally conceived pregnancies. Remarkably, tumor necrosis factor-alpha and intercellular adhesion molecule-1 mRNA levels were lower in OD pregnancies complicated by preeclampsia compared with in uncomplicated OD pregnancies (P<.001 for both; Fig. 2G). Placental thrombomodulin mRNA expression correlated positively with intercellular adhesion molecule-1 mRNA expression in uncomplicated OD pregnancies (r = 0.739, P<.001; Fig. 2H).

Regulation of Thrombomodulin: Angiogenic Factors

Placental vascular endothelial growth factor mRNA expression was higher in uncomplicated naturally conceived pregnancies compared with in uncomplicated OD pregnancies and OD pregnancies complicated by preeclampsia (P=.038 and P=.013; Supplemental Fig. 2). Placental soluble Flt-1 mRNA expression was higher in complicated OD pregnancies compared with in uncomplicated OD pregnancies (P=.006; Supplemental Fig. 2). Thrombomodulin mRNA expression and protein abundance were not associated with vascular endothelial growth factor or soluble Flt-1 mRNA expression.

Regulation of Thrombomodulin: Vitamin D

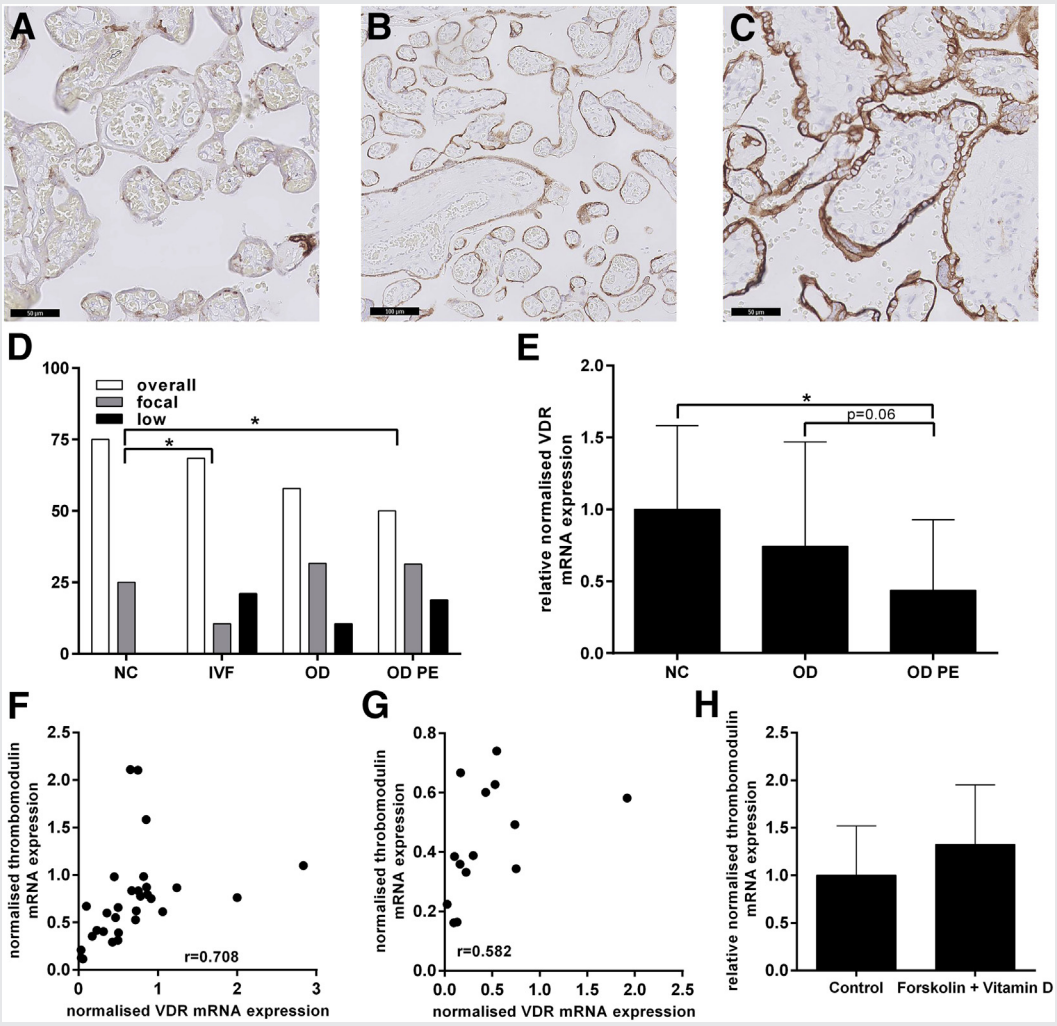
Placental VDR protein abundance was observed in three distinct patterns; representative examples of these patterns can be found in Figure 3A–3C. VDR abundance differed between groups, with OD pregnancies complicated by preeclampsia tending to be associated with decreased VDR abundance (P<.05; Fig. 3D). No seasonal changes of VDR abundance were observed in this cohort.

Placental VDR mRNA expression was lower in OD pregnancies complicated by preeclampsia compared with in naturally conceived pregnancies (P=.016; Fig. 3E). Placental thrombomodulin mRNA expression was positively correlated with placental VDR mRNA expression in uncomplicated OD pregnancies (r = 0.704, P<.001; Fig. 3F) and OD pregnancies complicated by preeclampsia (r = 0.582, P=.029; Fig. 3G). Placental VDR mRNA expression was significantly higher in women pregnant after OD when the child was born in the spring compared with children born in the autumn (P<.05). Other associations with seasonality and VDR mRNA expression levels could not be found (Supplemental Fig. 3).

In Vitro Experiments on Vitamin D Signaling and Thrombomodulin

Adding forskolin to cell culture medium led to increased VDR mRNA expression compared with cells cultured in control medium, as described elsewhere (29). Cells cultured with 50 μM forskolin and 100 nM vitamin D expressed higher

FIGURE 3



Regulation of thrombomodulin: vitamin D. (**A**) Representative example of a low (<50%) placental VDR expression pattern. (**B**) Representative example of a decreased/focal (50%–90%) placental VDR expression pattern. (**C**) Representative example of a diffuse (>90%) placental VDR expression pattern. (**D**) Placental VDR protein abundance patterns in women with an OD pregnancy complicated by preeclampsia and control groups (*P<.05, χ^2 testing). (**E**) Relative normalized placental VDR mRNA expression in OD pregnancies complicated by preeclampsia and control groups (*P<.05; Mann-Whitney U-test). (**F**) Correlation of VDR mRNA expression and thrombomodulin mRNA expression in uncomplicated OD pregnancies (r = 0.708, P<.001; Pearson's correlation test). (**G**) Correlation of VDR mRNA expression and thrombomodulin mRNA expression in OD pregnancies complicated by preeclampsia (r = 0.582, P=.029; Pearson's correlation test). (**H**) Relative normalized thrombomodulin mRNA expression in BeWo cells cultured with forskolin and vitamin D and in cells cultured with control medium. NC = naturally conceived; OD PE = OD pregnancies complicated by preeclampsia.

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levels of thrombomodulin mRNA compared with cells cultured with control medium (Fig. 3H).

DISCUSSION

Compelling evidence indicates that pregnancies after OD are subject to a vast risk of preeclampsia (1–8), but the underlying pathophysiology of this phenomenon remains uncertain. Similar to what has been described in naturally conceived pregnancies complicated by preeclampsia (20), we show that placental thrombomodulin mRNA expression is decreased in OD pregnancies complicated by preeclampsia. Furthermore, thrombomodulin protein abundance is decreased in uncomplicated OD pregnancies and OD pregnancies complicated by preeclampsia; this could indicate that loss of thrombomodulin, due to loss of endothelial protection, plays a role in the development of more pregnancy complications in OD pregnancies. Decreased thrombomodulin expression is associated with changes in coagulation pathways and inflammatory pathways. Furthermore, this study demonstrates that disturbances in the vitamin D metabolism possibly contribute to placental thrombomodulin loss during OD pregnancies. We found that decreased VDR expression is associated with loss of

thrombomodulin in OD pregnancies and that vitamin D increases thrombomodulin expression in vitro.

Loss of placental thrombomodulin in OD pregnancies calls into question whether thrombomodulin loss in OD pregnancies lies in the causal pathway of the development of preeclampsia or whether this loss is merely a result of the OD and subsequent immunological disturbances. Thrombomodulin loss may be caused by specific maternal characteristics typical for endothelial dysfunction. For example, we previously showed that thrombomodulin mRNA expression correlated significantly with maternal BMI and diastolic blood pressure in preeclampsia in naturally conceived pregnancies (20). Furthermore, in this study we showed that maternal age correlated significantly with thrombomodulin mRNA in OD pregnancies complicated by preeclampsia. Because of this correlation, and because increasing age is associated with loss of endothelial protection, the older age of the OD group could explain the thrombomodulin loss seen in this group. However, placental thrombomodulin protein expression was not decreased in older women who had an uncomplicated naturally conceived pregnancy. Also, no correlations between thrombomodulin expression and maternal characteristics such as BMI or hypertension were present. Therefore, it seems less likely that loss of placental thrombomodulin is part of the causal pathway of the development of preeclampsia after OD. Logically, thrombomodulin loss could be a result of OD, since thrombomodulin can be downregulated by inflammatory factors, such as matrix metalloproteinases and tumor necrosis factor-alpha (21). An OD pregnancy is a challenging state for the mother's immune system; a regulated immune response to the allogeneic fetus has to be established. OD pregnancies are characterized by more human leukocyte antigen (HLA) mismatches, more T-helper cells are found in the peripheral blood (18, 30), and the number of activated regulatory T-cells in the parietal decidua correlates with the number of HLA mismatches (31). Therefore, thrombomodulin loss in uncomplicated OD pregnancies could be caused by OD-specific immune regulation.

On the contrary, thrombomodulin exerts cytoprotective effects through activation of anti-inflammatory pathways. Immune dysregulation is a distinct pathogenic pathway in preeclampsia. Preeclampsia is associated with placental dysregulation of intercellular adhesion molecule-1 and tumor necrosis factor-alpha (32–34). Indeed, we found an upregulation of tumor necrosis factor-alpha and intracellular adhesion molecule-1 in OD pregnancies complicated by preeclampsia and a significant correlation between placental thrombomodulin and intercellular adhesion molecule-1. An association between preeclampsia and hypercoagulation is also well established; it is associated with mutations in the prothrombin and factor V Leiden genes, and the use of the anticoagulant drugs decreases development, mortality, and morbidity of preeclampsia (35–37). Loss of thrombomodulin protein abundance in OD pregnancies could result in reduced anticoagulation and thereby contribute to symptoms of preeclampsia. In this study, thrombomodulin protein abundance was inversely associated with fibrin deposits in women with an OD pregnancy complicated by preeclampsia. Furthermore, significant correlations of mRNA expression of tissue factor and factor VIII with thrombomodulin mRNA expression were found in OD pregnancies. Together these results indicate that loss of thrombomodulin could contribute to the pathogenesis of preeclampsia after OD through its distinct effects on inflammation and coagulation.

In this study, lower VDR expression on the syncytiotrophoblast of women with OD pregnancies complicated by preeclampsia compared with naturally conceived pregnancies was found. Decreased VDR expression is known to be associated with disturbances in vitamin D signaling, subsequent placental disorders, and growth restriction (27, 28). In our cohort, a decrease in VDR mRNA expression was associated with a decrease in thrombomodulin mRNA expression. Moreover, addition of vitamin D to cell culture medium increased thrombomodulin mRNA expression in vitro. These results are suggestive of vitamin D as a regulator of thrombomodulin in placental cells. Perhaps increasing levels of thrombomodulin through increasing vitamin D serum levels could restore syncytiotrophoblast maintenance in OD pregnancies. Vitamin D serum levels can be increased via vitamin D supplementation (38). Since thrombomodulin protein expression decreases in uncomplicated OD pregnancies, and thrombomodulin mRNA is not upregulated in this group, loss of thrombomodulin on the syncytiotrophoblast is not compensated by an increased production of thrombomodulin mRNA in uncomplicated OD pregnancies. This is suggestive for a role of a diminished vitamin D metabolism in the development of preeclampsia after OD. An interesting point for further research would be to investigate the effect of maternal vitamin D status on placental cytoprotection through thrombomodulin.

It is difficult to define a proper control group for OD pregnancies. Conception through assisted reproductive techniques on its own is associated with distinct maternal characteristics such as older age and premature ovarian failure (14) and an increased risk of pregnancy complications due solely to OD (15). In our cohort, uncomplicated OD pregnancies were significantly different from uncomplicated naturally conceived pregnancies with respect to maternal age, maternal BMI, gravidity, parity, and the number of twin or triplet pregnancies. However, IVF-induced pregnancies were only significantly different from uncomplicated OD pregnancies regarding maternal age and could therefore serve as a proper control group. Additionally, placentas from older women with an uncomplicated naturally conceived pregnancy were included to investigate the effect of maternal age on thrombomodulin protein expression. Thrombomodulin expression was mainly diffuse in these samples. Furthermore, no correlation was found between maternal age and thrombomodulin mRNA in uncomplicated OD pregnancies. Therefore, maternal age can probably not elucidate thrombomodulin downregulation in uncomplicated OD pregnancies. Multiplets occur more often in pregnancies induced by OD and IVF compared with in naturally conceived pregnancies (39, 40). This might theoretically influence thrombomodulin expression. To our knowledge, no current literature on the influence of multiplet pregnancies on placental thrombomodulin expression exists. In our study, thrombomodulin levels were similar in placentas from singleton and multiplet pregnancies; thrombomodulin mRNA appears not to be affected by twin pregnancy.

A limitation of this study is that only nine patients are included in the group of OD pregnancies complicated by preeclampsia. In the Netherlands, anonymous and commercial OD is not allowed (41). Hence, the number of women who receive OD is relatively low in the Netherlands.

In summary, in this study we showed that placental thrombomodulin protein expression is lower after uncomplicated OD pregnancies and OD pregnancies complicated by preeclampsia, which might contribute to a higher susceptibility for the development of preeclampsia after OD. Thrombomodulin expression is associated with parameters of inflammation and coagulation. Furthermore, downregulation of the VDR in OD pregnancies complicated by preeclampsia might contribute to loss of thrombomodulin on the syncytiotrophoblast. More research is needed to understand thrombomodulin regulation and other pathways contributing to the maintenance of endothelium in women with preeclampsia to find possible targets to treat endothelial dysfunction in women susceptible to preeclampsia. Although a very specific patient group was studied, our results provide new insights into the pathogenesis of preeclampsia but also set the stage for further research into the role of endothelium in the regulation of inflammation and coagulation.

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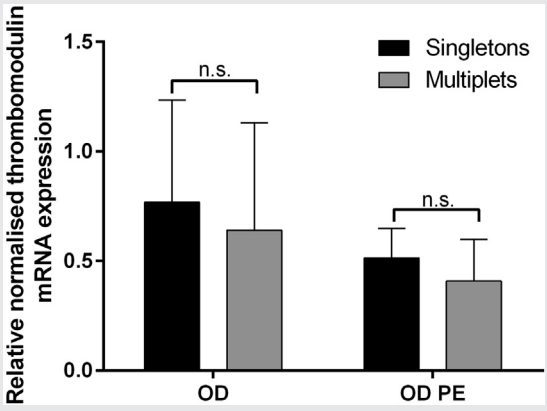
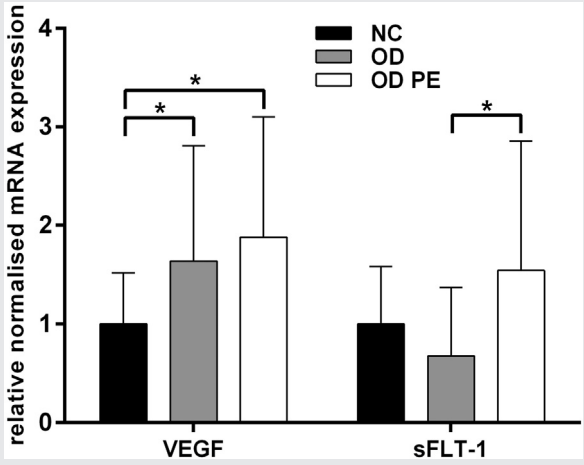
SUPPLEMENTAL FIGURE 1



Thrombomodulin mRNA expression in singletons and multiplets. Relative normalized placental thrombomodulin mRNA expression in uncomplicated OD pregnancies and OD pregnancies complicated by preeclampsia (not significant, independent *t*-test). NS = not significant; OD PE = OD pregnancies complicated by preeclampsia.

Bos. Thrombomodulin in oocyte donation. Fertil Steril 2016.

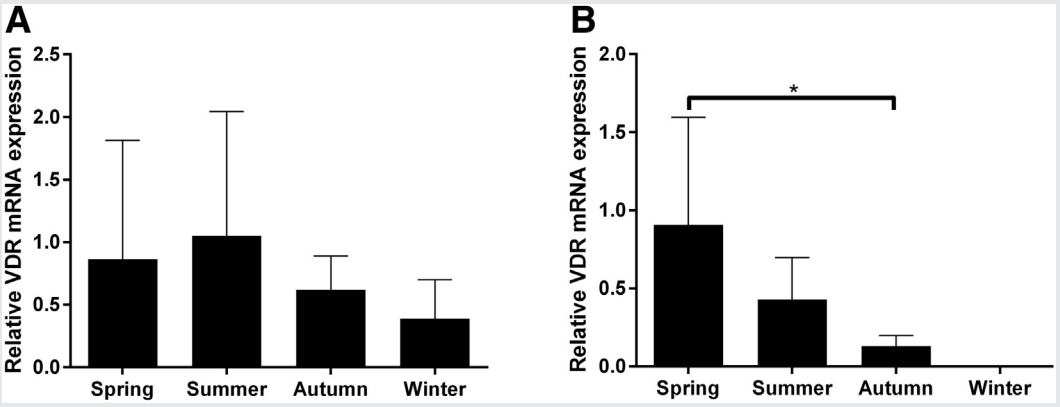
SUPPLEMENTAL FIGURE 2



Regulation of thrombomodulin: angiogenic factors. Relative normalized placental vascular endothelial growth factor and soluble Flt-1 mRNA expression in OD pregnancies complicated by preeclampsia and control groups (*$P<.05$, Mann-Whitney *U*-test). NC = naturally conceived; OD PE = OD pregnancies complicated by preeclampsia; sFlt-1 = soluble Flt-1; VEGF = vascular endothelial growth factor.

Bos. Thrombomodulin in oocyte donation. Fertil Steril 2016.

SUPPLEMENTAL FIGURE 3



Seasonality of placental VDR mRNA expression. (**A**) Relative placental VDR mRNA expression of children born in spring (March 21–June 20), summer (June 21–September 20), autumn (September 21–December 20), or winter (December 21–March 20) after an uncomplicated OD pregnancy. (**B**) Relative placental VDR mRNA expression of children born in spring, summer, or autumn after OD pregnancies complicated by preeclampsia. Placental VDR mRNA expression is significantly lower in children born in autumn compared with children born in spring (*$P<.05$, Mann-Whitney *U*-test).

Bos. Thrombomodulin in oocyte donation. Fertil Steril 2016.

SUPPLEMENTAL TABLE 1

Primer sequences, forward (F) or reverse (R), used for quantitative PCR.

Gene	F or R	Primer sequence
Factor VIII	F	GCTTCCCATCCTGTCAGTCT
	R	CAGAGGCCATTGGACCATTCT
GAPDH	F	TTCCAGGAGCGAGATCCCT
	R	CACCCATGACGAACATGGG
HPRT	F	TGACACTGGCAAAACAATGCA
	R	GGTCCTTTTCACCAGCAAGCT
ICAM-1	F	ACCATCTACAGCTTTCCGGC
	R	TCAGCGTCACCTTGGCTCTA
sFLT-1	F	CATTCAGGCCGAGGGGGCTG
	R	TGCACCCCTGGGGCCCATTT
Tissue factor	F	GGGAACCCAAACCCGTCAAT
	R	GTCGGTGAGGTCACACTCTG
Thrombomodulin	F	ACATCCTGGACGACGGTTTC
	R	CGCAGATGCACTCGAAGGTA
TNFa	F	CCCGAGTGACAAGCCTGTAG
	R	TGAGGTACAGGCCCTCTGAT
VDR	F	GCCCAACTCCAGACACACTC
	R	GGGTCACAGAAGGGTCATCT
VEGF	F	TGTGCCCCTGATGCGATGCG
	R	TCCTTCCTCCTGCCCGGCTC

Note: HPRT = hypoxanthine phosphoribosyltransferase; ICAM-1 = intercellular adhesion molecule 1; sFlt-1 = soluble Flt-1; TNFa = tumor necrosis factor-alpha; VEGF = vascular endothelial growth factor.

Bos. Thrombomodulin in oocyte donation. Fertil Steril 2016.

SUPPLEMENTAL TABLE 2

Patient characteristics of older age group.

Characteristic	NC (n = 28)	Older age (n = 20)
Maternal		
Age (y), mean (SD)	33.7 (4.0)	39.4 (1.5)[a]
BMI (kg/m^2), mean (SD)	25.81 (4.9)	25.67 (5.6)
Smoking (%)		
Yes	1 (3.6)	2 (12.6)
No	27 (96.4)	14 (87.6)
Gravidity, mean (range)	2.9 (1–7)	3.6 (1–8)
Parity, mean (range)	1.4 (0–5)	1.9 (0–5)
Diastole (mmHg), highest (SD)	75 (70–80)	78 (70–85)
Proteinuria, n (%)		
Yes	0 (0)	0 (0)
No	28 (100)	7 (100)
Prior history of hypertension or preeclampsia, n (%)		
Yes	0 (0)	0 (0)
No	28 (100)	20 (100)
Pregnancy		
Gestational age at delivery, wk + d (SD [d])	39 + 4 (9)	39 + 1 (9)
Term, n (%)		
A term	28 (100)	20 (100)
Preterm	0 (0)	0 (0)
Mode of delivery, n (%)		
Cesarean section	16 (63.2)	12 (63.2)
Vaginal delivery	12 (36.8)	12 (36.8)
Twin or triplet, n (%)		
Yes	0 (0)	0 (0)
No	28 (100)	20 (100)
Fetal	n = 28	n = 20
Sex of child, n (%)		
Male	12 (42.9)	13 (65)
Female	16 (57.1)	7 (35.0)
Birth weight, g (SD)	3,597 (435)	3,563 (564)
Small for gestational age, n (%)		
Yes	2 (7.1)	1 (5)
No	26 (92.9)	19 (95)
Placenta weight, g (SD)	638 (140)	611 (119)

Note: NC = naturally conceived.
[a] $P < .05$ compared with uncomplicated NC pregnancies.

Bos. Thrombomodulin in oocyte donation. Fertil Steril 2016.