Endothelial pathology in preeclampsia

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Chapter 3

Loss of Thrombomodulin in Placental Dysfunction in Preeclampsia

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ABSTRACT

Objective

Preeclampsia is a pregnancy-specific syndrome characterized by placental dysfunction and an angiogenic imbalance. Systemically, levels of thrombomodulin, an endothelium- and syncytiotrophoblast-bound protein that regulates coagulation, inflammation, apoptosis, and tissue remodeling, are increased. We aimed to investigate placental thrombomodulin dysregulation and consequent downstream effects in the pathogenesis of preeclampsia.

Approach and Results

Placentas from 28 preeclampsia pregnancies, 30 uncomplicated pregnancies and 21 pregnancies complicated by growth restriction as extra controls were included. Immunohistochemical staining of thrombomodulin, caspase-3, and fibrin was performed. Placental mRNA expression of thrombomodulin, inflammatory markers, matrix metalloproteinases 2 and 9, and soluble Flt-1 were measured with qPCR. Thrombomodulin mRNA expression was determined in vascular endothelial growth factor-transfected trophoblast cell lines.

Thrombomodulin protein and mRNA expression were decreased in preeclampsia as compared to both control groups ($P=0.001$). Thrombomodulin mRNA expression correlated with maternal body mass index ($P< 0.01$), and diastolic blood pressure ($P<0.05$) in preeclampsia. An increase in placental apoptotic cells was associated with preeclampsia ($P<0.001$). Thrombomodulin expression correlated positively with matrix metalloproteinase expression ($P<0.01$) in preeclampsia, but not with fibrin deposits or inflammatory markers Placental soluble Flt-1 expression correlated with decreased thrombomodulin expression. Vascular endothelial growth factor induced upregulation of thrombomodulin expression in trophoblast cells.

Conclusions

Decreased thrombomodulin expression in preeclampsia may play a role in placental dysfunction in preeclampsia and is possibly caused by an angiogenic imbalance. Hypertension and obesity are associated with thrombomodulin downregulation. These results set the stage for further basic and clinical

research on thrombomodulin in the pathogenesis of preeclampsia and other syndromes characterized by endothelial dysfunction.

NONSTANDARD ABBREVIATIONS

sFlt-1, soluble Flt-1
APC, activated protein C
IUGR, intra-uterine growth restriction
VEGF, vascular endothelial growth factor
MMP, matrix metalloproteinase

INTRODUCTION

Preeclampsia complicates 2–8% of all pregnancies and is a leading cause of maternal and fetal morbidity and mortality.[1, 2] The exact etiology of the syndrome is unknown, but impaired placentation plays a key role in its pathogenesis.[3] In preeclampsia, placental production of anti-angiogenic factors such as soluble Flt-1 (sFlt-1) and soluble endoglin is increased, which results in deprivation of essential survival factors in the endothelium and syncytiotrophoblast.[3] This contributes to the development of endothelial dysfunction, causing increased systemic vascular resistance, high blood pressure, and hypercoagulability. [3] Further, a hyper-inflammatory state develops, characterized for example by complement deposits in the kidneys and in the placenta. [4, 5]

Thrombomodulin is essential for the maintenance of endothelium; it is a transmembrane glycoprotein found on the endothelium of arteries, venules, and capillaries, and on the syncytiotrophoblast in the placenta.[6] Inflammation results in the release of thrombomodulin from endothelial cells into the circulation, leading to decreased expression of thrombomodulin on the endothelial surface.[7] Whether this cleaved form of thrombomodulin is functional remains unclear, but soluble thrombomodulin concentrations can be used to monitor inflammatory conditions.[8] In women with preeclampsia, maternal serum levels of cleaved soluble thrombomodulin in serum are elevated.[9–11] The placenta is a possible source of this soluble thrombomodulin, which could result in decreased available thrombomodulin on the syncytiotrophoblast.[12] Vascular endothelial growth factor stimulates thrombomodulin expression,[8] so the angiogenic imbalance in the placenta

in preeclampsia could lead to a decrease in placental thrombomodulin expression.

One of the pathways through which thrombomodulin exerts its protective effects on endothelium is by activating protein C. In mice, loss of thrombomodulin from the endothelium results in disruption of the activated protein C (APC) pathway, which leads to lethal massive thrombosis.[13] APC has an anticoagulant function by cleaving activated cofactors FV and FVIII. In addition to its anticoagulant effects, APC has an important cytoprotective function for endothelium through the endothelial protein C receptor: namely, direct anti-inflammatory and anti-apoptotic effects on endothelial cells.[14] In an experimental mouse model, this anti-apoptotic effect protected against diabetic nephropathy by inhibiting endothelial apoptosis.[15] Furthermore, APC plays a role in tissue remodeling by activating matrix metalloproteinase (MMP) 2 and 9, which are involved in placental development and are dysregulated in preeclampsia.[16-18]

We set out to investigate whether placental thrombomodulin dysregulation plays a role in the pathogenesis of preeclampsia. Therefore, we measured thrombomodulin expression in placentas from women with preeclampsia and compared this with thrombomodulin expression in placentas from control subjects with uncomplicated pregnancies, and in placentas from control subjects with pregnancies complicated by intra-uterine growth restriction (IUGR) to confirm that our findings were preeclampsia-specific rather than a consequence of lower gestational age or birth weight in our case group. Further, we investigated whether thrombomodulin expression in the placenta is correlated with its downstream effects; fibrin depositions, cell survival, inflammation, and MMP expression in the placenta, and if the angiogenic imbalance of preeclampsia is associated with a decrease in thrombomodulin expression.

MATERIALS AND METHODS

Samples

We studied 79 placentas obtained from women who delivered at the Leiden University Medical Center Department of Obstetrics between 2002 and 2011. After delivery all placentas were macroscopically examined and samples

from pre-defined locations on the placenta and fetal membranes were taken according to a standard protocol. For this study, tissue samples from lateral sites of the basal plate of the placenta were selected to study mature and terminal villi. The placentas were divided between a case group of 28 placentas obtained from women with mild or severe preeclampsia and a healthy control group of 30 placentas obtained from women with uncomplicated pregnancies that resulted in term livebirths. Preeclampsia were diagnosed according to ISSHP guidelines, with severe preeclampsia being defined as the presence of diastolic blood pressure >110 mmHg, proteinuria > 3 g / 24H, or HELLP syndrome. Because the majority of women with preeclampsia delivers by caesarean section, we aimed to collect control placentas from women who delivered by caesarean section as well. A second control group consisting of 21 placentas from pregnancies complicated by IUGR (defined as birth weight below fifth percentile for gestational age), but not by preeclampsia or hypertension, was included to confirm that our findings on thrombomodulin expression were preeclampsia-specific rather than a consequence of lower gestational age or birth weight in our case group. Paraffin-embedded samples were available for immunohistochemical staining from 11 patients in this control group; from the remaining ten patients, frozen samples were available for RNA isolation and PCR. This study was approved by the ethics committee of the Leiden University Medical Centre (P13.084) and subjects gave informed consent.

Histology

Specimens were stained with hematoxylin and eosin. 27 control samples and 21 preeclampsia samples were examined by a single pathologist blinded for cases and controls. The presence, extent, chronicity and composition of villous- and intervillous infiltrates were determined.

Immunohistochemistry

To investigate the placental protein expression of thrombomodulin and to investigate apoptosis and coagulation in the placentas, we performed immunohistochemical staining for thrombomodulin, caspase-3, and fibrin. Sections were deparaffinized, and antigen retrieval was performed. Sections were incubated with an anti-thrombomodulin mouse monoclonal antibody (1:200; Leica Biosystems, Danvers, MA, USA) or an anti-fibrin mouse

monoclonal antibody (1:200, Immunotech, Prague, Czech Republic) for 1 hour, or with an anti–cleaved caspase-3 rabbit antibody (1:300; Cell Signaling, Danvers, MA, USA) overnight at room temperature. Binding of the primary antibody was visualized with labelled anti-mouse IgG polymer or with labelled anti-rabbit IgG polymer (DAKO, Glostrup, Denmark) and diaminobenzidine as a chromogen.

Scoring of staining patterns

Slides were scored by two observers blinded with respect to cases and controls. The presence of thrombomodulin 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). Positivity for caspase-3 was scored quantitatively as the number of apoptotic cells per mm^2. Fibrin deposits were scored quantitatively as the number of villi with fibrin deposits divided by the total number of villi in ten 0.28 mm^2 sections per sample.

PCR

Quantitative PCR was performed to quantify placental mRNA expression of thrombomodulin, MMP 2 and 9, tumor necrosis factor alpha, intercellular adhesion molecule 1 and sFlt-1. RNA isolation was performed with TRIzol® (Lifetechnologies, San Francisco, CA, USA). Synthesis of cDNA was performed with AMV reverse transcriptase (Roche, Basel, Switzerland), and SYBR Green quantitative PCR was performed according to the manufacturer's protocol (Bio-Rad Laboratories Inc, Hercules, CA, USA). Primer sequences are described in Supplementary Table 1. Expression was measured by the comparative threshold cycle method and normalized to hypoxanthine phosphoribosyltransferase and GAPDH expression. A melting curve analysis was performed to verify the specificity of amplification.

In vitro experiments

Trophoblast cell lines BeWo (CCL-98, ATCC, Manassas, VA, USA) and Jeg-3 (HTB-36, ATCC, Manassas, VA, USA) were grown on 6-Multiwell plates. After 24 h, cells were transfected with a VEGF plasmid (pHIPPY-PGL3-VEGF). For transfection, plasmid DNA was diluted into growth medium at a concentration

of 1 g/L and X-tremeGene HP DNA Transfection Reagent (Roche, Basel, Switzerland) was added at a 3:1 ratio. 48 hours after transfection, RNA was isolated with TRIzol® (Lifetechnologies, San Francisco, CA, USA). cDNA synthesis was performed and expression of thrombomodulin, hypoxanthine phosphoribosyltransferase and GAPDH was determined with PCR as described above. The experiments were repeated three times.

Statistical analysis

Mean differences in normalized mRNA expression levels between two groups were analyzed with the unpaired *t*-test for normally distributed data or with the Mann–Whitney U test for skewed distributions. The distributions of staining patterns of thrombomodulin and histological scores in the preeclampsia and control groups were analyzed with the chi-square test or Fisher's exact test. Correlations between thrombomodulin mRNA levels, clinical parameters or mRNA levels of other proteins were calculated with the Pearson test or Spearman test. A *P* value of <0.05 was considered statistically significant. Mean differences in fibrin deposits and in caspase-3 positive cells between more than two groups were analyzed with the one-way ANOVA. Posthoc tests, Bonferroni and LSD, were subsequently performed to investigate differences between the preeclampsia group and the control groups. Standard deviations depict variance in the figures. All analyses were performed with the IBM SPSS statistics software package (version 20; IBM, Armonk, NY, USA).

RESULTS

Patient characteristics

Diastolic blood pressure and proteinuria were significantly higher in the preeclampsia group as compared to both control groups. The mean gestational age in healthy control subjects was 39 weeks and 4 days; in IUGR control subjects and in preeclampsia patients the mean gestational ages were was 33 weeks and 2 days and 30 weeks and 4 days, respectively, which were both significantly lower as compared to healthy control subjects. The mean birth weight in the healthy control group was 3609 g. Mean birth weights in the IUGR control group and in the preeclampsia group were 1521 g and 1167 g, respectively, which were both significantly lower compared to the mean birth weight in the healthy control group. The mean placenta weight was

significantly lower in preeclampsia patients and in growth-restricted control subjects as compared to healthy control subjects. Patient characteristics are illustrated in Table 1.

Immunohistochemical staining for thrombomodulin

In 29 of 30 placentas from healthy controls, thrombomodulin was observed in an overall staining pattern, with thrombomodulin present on the syncytiotrophoblast of >50% of villi, and in 1 control placenta a focal staining pattern was observed, with thrombomodulin being present on the syncytiotrophoblast of <50% of villi. In the IUGR group, thrombomodulin was present on the syncytiotrophoblast of >50% of villi in all 11 cases. In 12 of 28 placentas from patients with preeclampsia, thrombomodulin protein expression was decreased. In ten cases, we found a focal staining pattern, with thrombomodulin present on the syncytiotrophoblast of <50% of villi, and in two cases, thrombomodulin was nearly absent (present on the syncytiotrophoblast of <10% of villi). In the remaining 16 placentas from the preeclampsia group, thrombomodulin staining was present in an overall staining pattern. These findings are illustrated in Figure 1. Chi-square analysis indicated a strong association between decreased thrombomodulin expression and preeclampsia ($P = 0.001$). Thrombomodulin staining on fetal vessel endothelium was present in a similar pattern in preeclampsia and control groups .Supplemental Figure I shows thrombomodulin staining on fetal vessel endothelium in placentas from preeclampsia and control cases with most reduced thrombomodulin staining on the syncytiotrophoblast; fetal vessel thrombomodulin staining was present in these samples and consequently serves as an internal control for the adequacy of the staining procedure.

Placental mRNA expression of thrombomodulin

Placental mRNA levels of thrombomodulin were on average 3-fold lower in preeclamptic women compared to control subjects ($P = 0.001$), whereas in women with IUGR, the relative mRNA expression of thrombomodulin was not significantly different compared to healthy control placentas. These results are illustrated in Figure 2. In placenta samples where thrombomodulin protein expression was absent, thrombomodulin mRNA levels were 4-fold lower than

in placentas with a focal or overall staining pattern for thrombomodulin at the syncytiotrophoblast in the preeclampsia group (Supplemental Figure II).

Placental mRNA expression of thrombomodulin and clinical parameters in pre-eclampsia

Placental thrombomodulin mRNA levels were significantly lower in patients with mild preeclampsia and in patients with severe preeclampsia compared to healthy control subjects; levels were not significantly different between patients with mild and severe preeclampsia. Placental thrombomodulin mRNA levels were negatively correlated with maternal diastolic blood pressure and maternal body mass index in patients with preeclampsia. In controls, this correlation was not present. There were no correlations between gestational age or placental weight and thrombomodulin mRNA levels. These correlations are illustrated in Figure 2. Thrombomodulin mRNA levels were not associated with parity ($P>0.05$). When exclusively primiparous cases were analyzed, thrombomodulin mRNA levels were still significantly lower in preeclampsia cases compared to placentas from control cases.

Fibrin depositions

In placentas from healthy control subjects, villous fibrin deposits were present on 13 percent of the villi, and in IUGR control placentas on 7 percent. In pregnancies complicated by preeclampsia, fibrin depositions were present on 17 percent of villi, on average. One-way ANOVA analyses indicated an association between increased fibrin deposits and preeclampsia, but post-hoc analyses did not reveal any significant differences between pre-eclampsia cases and healthy or growth-restricted control cases. These data are shown in Figure 3. There was no significant association between fibrin deposits and thrombomodulin mRNA, or between fibrin deposits and the thrombomodulin protein staining pattern.

Immunohistochemical staining of cleaved caspase-3

In placentas from healthy subjects and IUGR control subjects, there were four and three apoptotic cells per mm^2, on average. In placentas from preeclamptic patients, there were on average 16 apoptotic cells per mm^2, which was significantly different from both control groups. These data are shown in Figure 3. There was no significant association between

thrombomodulin mRNA and the amount of apoptotic cells and a focal or absent thrombomodulin staining pattern was not associated with an increase in apoptotic cells.

Placental inflammation

Villitis was present in 14.8% of the control placentas and in 42.9% of the preeclampsia cases; this difference was significant ($P<0.05$). In all villitis cases, the infiltrate was multifocal and composed of mononuclear cells. Intervillositis occurred in 7.4% of control placentas and in 19% of preeclampsia placentas; the infiltrate consisted of histiocytes in the majority of cases. Neither villitis nor intervillositis was associated with a decreased thrombomodulin staining pattern or with decreased thrombomodulin mRNA expression. Placental mRNA levels of tumor necrosis factor alpha and intercellular adhesion molecule 1 were lower in placentas from preeclampsia patients as compared to healthy controls ($P < 0.001$ for both, Figure 3). There was no correlation between thrombomodulin mRNA expression and tumor necrosis factor alpha or intercellular adhesion molecule 1 expression in placentas from preeclamptic patients (Supplemental Figure III).

Placental mRNA expression of MMPs

On average, MMP2 mRNA levels were 2-fold lower in placentas from preeclamptic women compared to placentas from both control groups ($P < 0.01$). MMP9 mRNA levels were 5-fold lower in placentas from preeclamptic women compared to control subjects ($P < 0.01$). In IUGR control placentas, MMP2 and MMP9 mRNA levels were not significantly different compared to control placentas. In preeclamptic patients, MMP2 expression was positively correlated with thrombomodulin mRNA expression. The correlation between MMP9 and thrombomodulin did not reach statistical significance ($P = 0.095$). These findings are illustrated in Figure 3.

Placental expression of sFlt-1 and thrombomodulin

An inverse correlation between thrombomodulin protein expression and sFlt-1 expression was observed; in placentas from preeclampsia patients with a focal syncytiotrophoblast thrombomodulin staining pattern, mRNA expression of sFlt-1 was 3-fold higher compared to the expression in placentas with an overall thrombomodulin staining pattern at the syncytiotrophoblast

($P < 0.05$, Figure 4). This inverse correlation was not observed between thrombomodulin mRNA and sFlt-1 mRNA.

***In vitro* experiments**

In BeWo cells, vascular endothelial growth factor (VEGF) transfection resulted in a 1.5-fold upregulation of thrombomodulin mRNA expression. This increase was significant ($P<0.05$) compared to untreated control cells and control cells that had received the transfection reagent without plasmid DNA. In Jeg-3 cells, VEGF transfection was associated with a 1.3-fold upregulation of thrombomodulin mRNA expression, but this was not significantly different compared to both control cell groups. These results are illustrated in Figure 5.

DISCUSSION

Increasing evidence suggests that preeclampsia is caused by deprivation of factors essential for the placenta and endothelium, with consequent placental and endothelial dysfunction. This study demonstrates that thrombomodulin mRNA and protein expression are decreased in placentas of mildly and severely preeclamptic patients, and that this decrease correlates negatively with maternal body mass index and diastolic blood pressure. Additionally, thrombomodulin mRNA expression correlates directly with placental expression of MMPs and the decrease in placental thrombomodulin is accompanied by impaired villous cell survival.

In the preeclampsia group, parity, birth weight, placenta weight and duration of pregnancy were significantly different compared to the term control group. Therefore, we added an extra control group to our study, consisting of placentas from pregnancies complicated by growth restriction and not by hypertensive disorders. In this group, parity, birth weight, placenta weight and duration of pregnancy were comparable to the preeclampsia group. Thrombomodulin expression in the growth-restriction control group was not different from the expression in the term control group; therefore, we conclude that our findings on placental thrombomodulin loss in the preeclampsia group cannot be explained by, for example, the lower gestational age in the preeclampsia group.

A significant correlation was present between placental thrombomodulin expression and diastolic blood pressure in preeclampsia cases. This indicates that thrombomodulin expression is connected to the degree of endothelial

dysfunction in preeclampsia, which could be upstream, in the pathogenesis of endothelial dysfunction, or downstream, as a consequence of endothelial dysfunction. However, thrombomodulin expression was not significantly different between mild and severe preeclampsia cases, so placental loss of thrombomodulin alone does not seem to be a major contributor to the development of end-organ involvement in preeclampsia.

If thrombomodulin is involved in the pathogenesis of preeclampsia, one would expect mutations in the thrombomodulin gene to be associated with the syndrome. For example, hemolytic uremic syndrome, a disease also characterized by endothelial dysfunction and complement activation, is associated with mutations in the thrombomodulin gene.[19] However, a recent meta-analysis of genetic variants in preeclampsia revealed that thrombomodulin mutations are not associated with preeclampsia.[20] Our study shows that a decreased thrombomodulin protein expression pattern in the placenta is accompanied by an increase in placental expression of sFlt-1, and that VEGF transfection is associated with thrombomodulin upregulation in trophoblast cells *in vitro*. These results strengthen the hypothesis that the angiogenic imbalance of preeclampsia could cause the decreased thrombomodulin expression in the placenta that we found in our cohort.

The decreased thrombomodulin expression in the placenta in preeclampsia could have unfavorable downstream effects on the placenta through at least four pathways, which are illustrated in a hypothetical scheme in Figure 6. First, thrombomodulin inhibits coagulation through the APC pathway. Systemically, increased levels of the thrombin–antithrombin complex in the presence of increased thrombomodulin cleavage product have been reported in preeclampsia, [21] but we did not find an increase in placental fibrin deposits in preeclampsia or a correlation between thrombomodulin expression and placental villous fibrin deposits in our study; apparently, the placental decrease in thrombomodulin we observed in our cohort did not lead to increased fibrin at the fetomaternal interface. Second, we found that decreased placental thrombomodulin expression was accompanied by impaired placental cell survival in preeclampsia; these changes could also contribute to the impaired placentation seen in preeclampsia. However, a direct correlation between the amount of apoptotic cells detected with immunohistochemical staining and thrombomodulin expression was not present, possibly caused by sampling error due to the heterogeneous

nature of placental lesions. *In vitro* studies investigating the effects of thrombomodulin depletion on trophoblast cells would shed some light on this area. Third, although preeclampsia itself is associated with a hyper-inflammatory state,[4, 5] we found no increased mRNA expression of ICAM1 and TNFA in placentas from preeclamptic patients, and there was no correlation between thrombomodulin expression and these parameters. Further, thrombomodulin expression was not associated with villous- or intervillous infiltrates. Last, we found a positive correlation between thrombomodulin expression and expression of MMP2 and MMP9. These results indicate that thrombomodulin expression could lead to decreased MMP2 and MMP9 activity in the placenta, which is associated with impaired trophoblast invasion and preeclampsia.[22] Since our cohort contained exclusively third-trimester placentas obtained after delivery, early stages of placental development, and trophoblast invasion, were not studied. Further studies on thrombomodulin expression in the placenta at earlier stages of placental development are warranted.

In our cohort, we found a negative correlation between body mass index and placental thrombomodulin expression. Obesity is associated with endothelial dysfunction and increased levels of circulating thrombomodulin, suggestive for loss of endothelial thrombomodulin.[23] Obese pregnant women show increased levels of circulating markers of endothelial dysfunction and have an increased risk of developing preeclampsia. [24] Possibly, the highly inflammatory and hypoxic vascular environment of obesity leads to decreased expression of vasculoprotective factors, such as thrombomodulin, both on the fetomaternal interface and in the systemic vasculature, and contributes to the development of systemic endothelial dysfunction and preeclampsia. However, obesity in healthy control pregnancies was not associated with thrombomodulin loss. Apparently additional endothelial- or syncytiotrophoblast damaging factors are needed to cause placental thrombomodulin loss in preeclampsia.

Thrombomodulin has raised interest as a possible target in the treatment of preeclampsia; it could act beneficially by reducing both endothelial and placental dysfunction. Recently, administration of recombinant thrombomodulin was shown to rescue fetal tissue oxygenation and growth in a rat model of preeclampsia.[25] However, in rats, thrombomodulin did not improve the maternal systemic symptoms of preeclampsia such as hypertension. This could indicate that thrombomodulin administration

rescues fetal growth by attenuating placental dysfunction. Our findings, which link decreased thrombomodulin expression to impaired placental cell function, support this hypothesis. Further research exploring the safety and efficacy of thrombomodulin as a target for the prevention and treatment of preeclampsia are of interest.

To conclude, preeclampsia is characterized by placental and endothelial dysfunction, but the exact etiology is not completely understood. Our study shows that thrombomodulin is a new candidate role-player in the pathogenesis of preeclampsia that is associated with both clinical parameters and dysregulation of factors essential for placental function. These insights set the stage for further basic and clinical research on placental development, the development of placental pathology and on thrombomodulin as a target for the prevention and treatment of preeclampsia.

SUPPLEMENTAL MATERIAL

Supplemental material is available online at http://atvb.ahajournals.org/.

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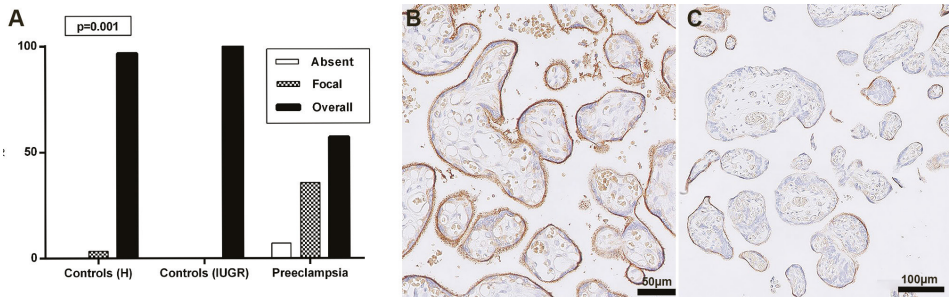
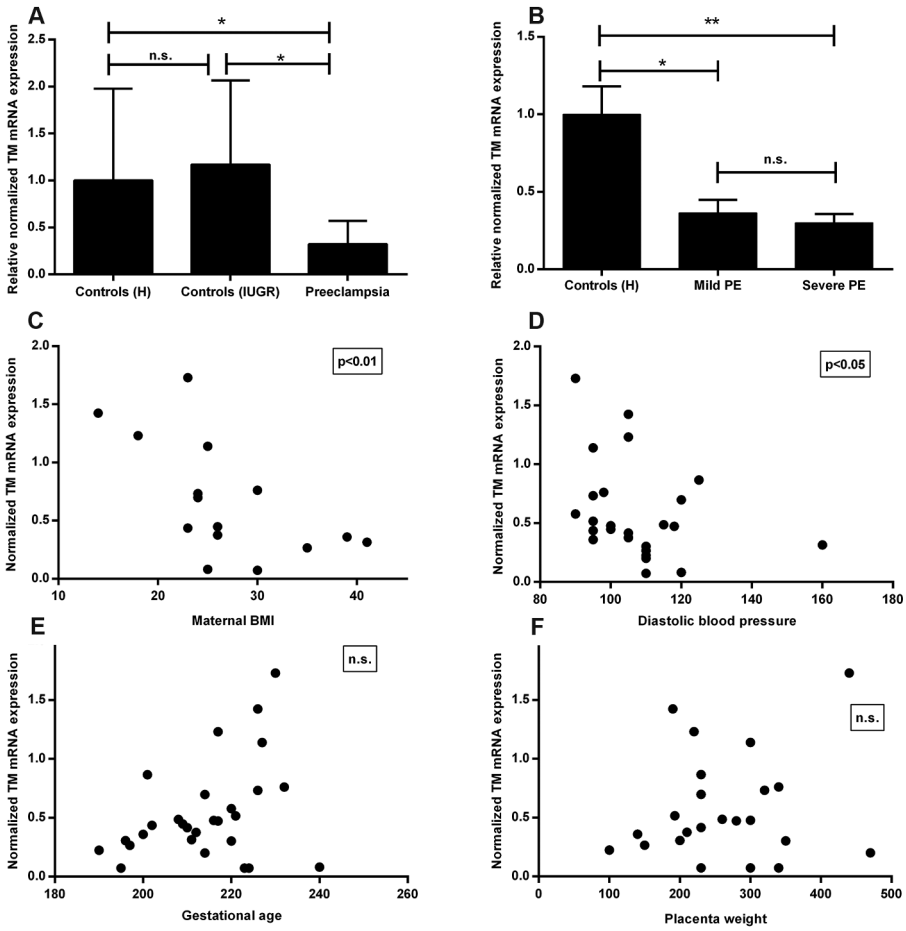
FIGURES

Figure 1 Placental thrombomodulin protein expression



Results of immunohistochemical and histological staining of placentas from preeclamptic patients and control subjects **A** Abundance of thrombomodulin protein expression in placentas from 30 placentas from healthy controls, from 11 control pregnancies complicated by IUGR and from 28 preeclampsia patients. ($P = 0.001$, Controls (H), healthy control subjects; Controls (IUGR), control subjects with pregnancies complicated by intra-uterine growth restriction). **B** Example of an overall staining pattern of thrombomodulin on the syncytiotrophoblast in control placenta. **C** Example of a focal staining pattern of thrombomodulin in a placenta from the preeclampsia group.

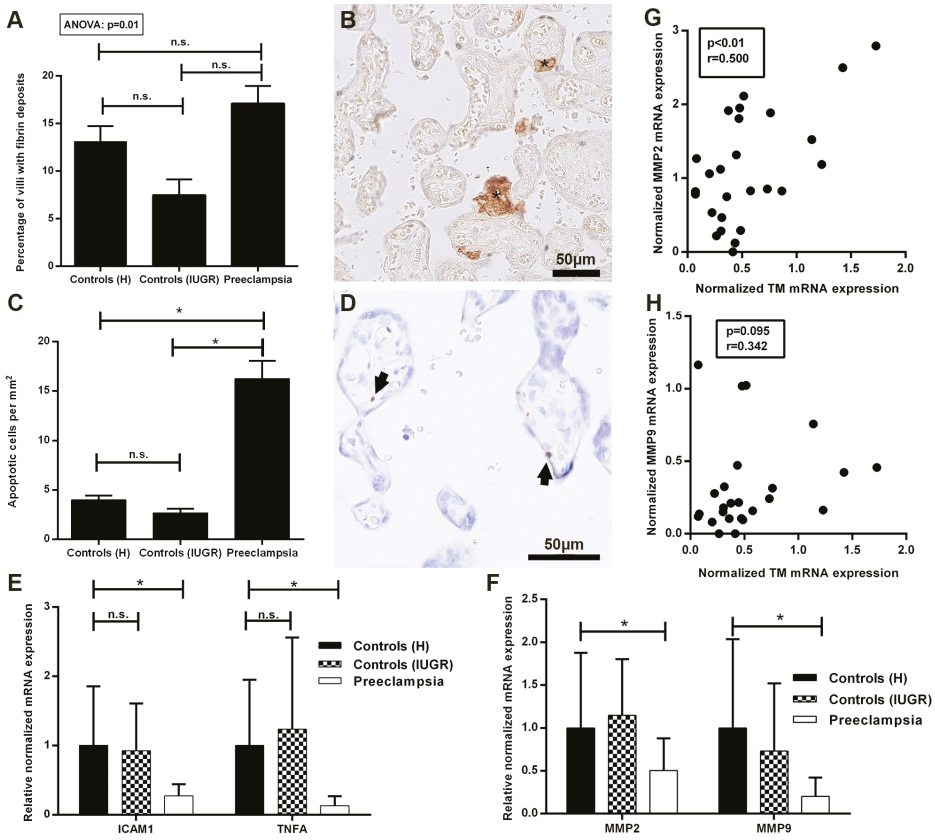
Figure 2 Placental mRNA expression of thrombomodulin



A Relative normalized placental mRNA expression of thrombomodulin in preeclampsia cases and controls; *, $P = 0.001$ Mann-Whitney U test comparing preeclampsia and growth-restricted or healthy control placentas. **B** Normalized placental thrombomodulin mRNA expression in healthy control subjects and patients with mild or severe preeclampsia. Expression was significantly lower in patients with mild or severe preeclampsia compared to healthy control placentas. Expression was not significantly different between patients with mild and severe preeclampsia ($p>0.05$, Mann-Whitney U test). *, $P < 0.05$; **, $P < 0.01$, calculated with Mann-Whitney U testing. **C** Maternal BMI and normalized placental thrombomodulin mRNA levels in the preeclampsia group; Spearman's rho=-0.671,

p<0.01. **D** Diastolic blood pressure and normalized placental thrombomodulin mRNA levels in the preeclampsia group; Spearman's rho=-0.431, $P < 0.05$. **E** Gestational age and normalized placental thrombomodulin mRNA levels in the preeclampsia group; Spearman's rho=0.361, $P > 0.05$. **F** Placenta weight and normalized placental thrombomodulin mRNA levels in the preeclampsia group; Spearman's rho=0.033, $P > 0.05$.
TM, thrombomodulin; Controls (H), healthy controls; Controls (IUGR), control subjects with pregnancies complicated by intra-uterine growth restriction; PE, preeclampsia.

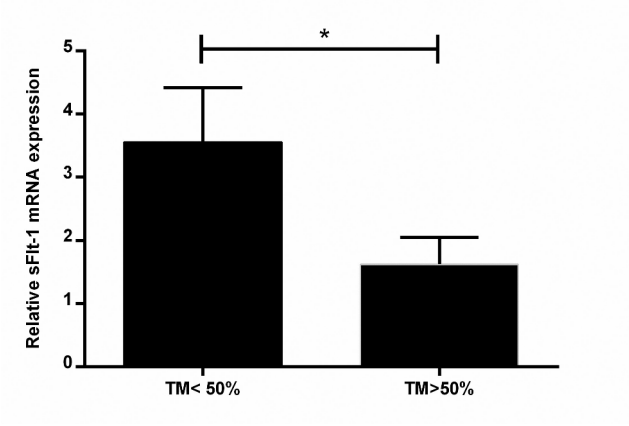
Figure 3 Downstream effects of thrombomodulin in placentas



A Percentage of villi with fibrin deposits as detected with immunohistological fibrin staining in placentas from preeclamptic patients compared to control subjects; $P = 0.01$, One-way ANOVA. **B** Placenta from a preeclamptic patient with

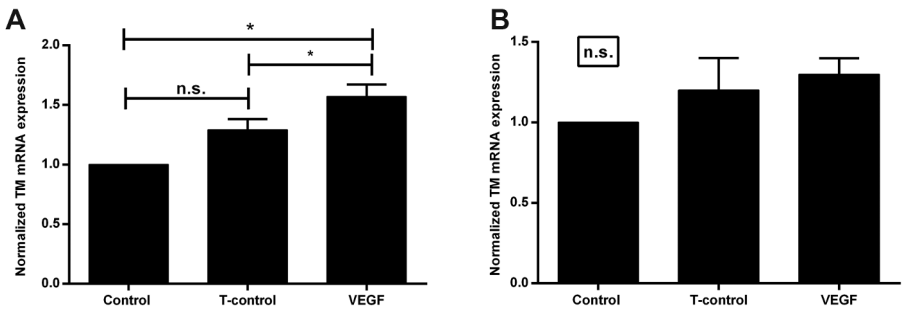
fibrin deposits. Asterisks indicate villous fibrin deposits. **C** Average number of apoptotic cells per mm^2 in placentas from preeclamptic patients and control subjects. *, $P < 0.001$. **D** Example of caspase-3 staining in a placenta from a patient with preeclampsia. Arrows indicate cells positive for caspase-3. **E** Relative mRNA expression of tumor necrosis factor alpha and intercellular adhesion molecule 1 in placentas from preeclamptic patients and control subjects. *, $P < 0.001$; n.s., not significant. **F** Relative mRNA expression of MMP2 and MMP9 in placentas from preeclamptic patients and control subjects; *, $P < 0.01$, Mann-Whitney U tests. **G** Correlation between thrombomodulin and MMP2 expression in placentas from the preeclampsia group; Spearman's rho=0.500, $P < 0.01$. **H** Correlation between thrombomodulin and MMP9 expression in placentas from the preeclampsia group; Spearman's rho=0.342, $P = 0.095$.
Controls (H), healthy controls; Controls (IUGR), control subjects with pregnancies complicated by intra-uterine growth restriction; TNFA, tumor necrosis factor alpha; TM, thrombomodulin; ICAM1, intercellular adhesion molecule 1; MMP, matrix metalloproteinase.

Figure 4 Placental sFlt-1 expression in preeclampsia cases with and without decreased thrombomodulin expression



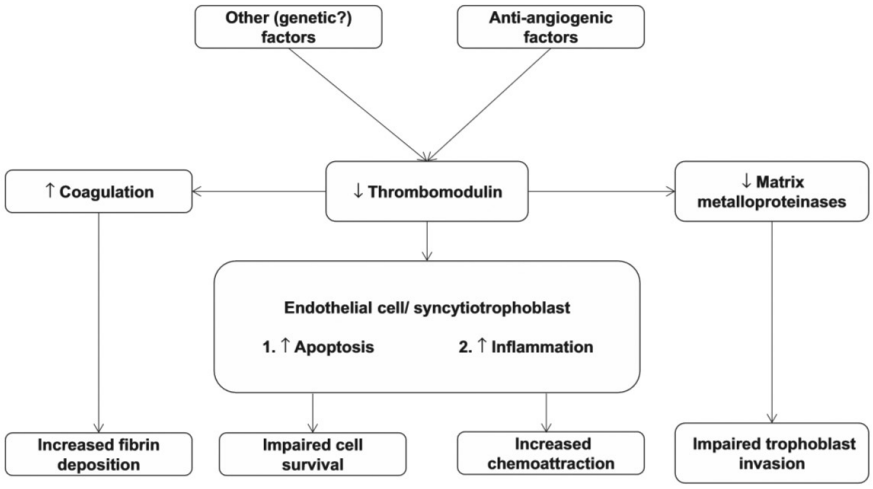
Relative mRNA expression levels of sFlt-1 in placenta samples from the preeclampsia group with decreased thrombomodulin protein expression (TM<50%) or without decreased thrombomodulin protein expression (TM>50%) are depicted. *, $P < 0.05$, Mann-Whitney U test.

Figure 5 Thrombomodulin expression in VEGF-transfected trophoblast cells



A Normalized relative thrombomodulin mRNA expression in BeWo control cells, cells treated with transfection reagent and cells treated with VEGF plasmid DNA. Thrombomodulin expression was significantly higher in the VEGF-transfected cells compared to both control groups ($P < 0.05$). **B** Normalized relative thrombomodulin mRNA expression in Jeg-3 control cells, cells treated with transfection reagent and cells treated with VEGF plasmid DNA. There were no significant differences between the three groups.
T-control, control cells treated with transfection reagent; VEGF, vascular endothelial growth factor; *, $P < 0.05$; n.s., not significant. .

Figure 6 Hypothetical scheme of thrombomodulin in the pathogenesis of preeclampsia



This figure shows the hypothetical role of thrombomodulin in the placenta in preeclampsia. Anti-angiogenic and possibly other (genetic) factors lead to thrombomodulin downregulation in the placenta, both on mRNA and protein levels. Since thrombomodulin inhibits coagulation, this downregulation could lead to increased fibrin deposition. Further, downregulation could increase apoptosis and inflammatory processes in endothelial cells and/ or syncytiotrophoblast. Lastly, thrombomodulin plays a role in activating matrix metalloproteinases, so its downregulation could result in impaired trophoblast invasion.

TABLES

Table 1 Patient characteristics

Characteristics	Healthy controls n=30	IUGR controls n=21	Preeclampsia n=28
Mean maternal age in years (SD)	33.9 (4.0)	31.4 (6.0)	31.6 (7.2)
Mean maternal BMI (SD)**	25.3 (4.2)	n.a.	26.9 (7.2)
Mean gravidity (SD)	2.9 (1.2)	1.9 (0.8)*	2.1 (1.9)*
Mean parity (SD)	1.3 (1.0)	0.5 (0.7)*	0.8 (1.2)*
Highest diastole (mmHg) (SD)	76.4 (5.1)	81.0 (8.3)*	107.5 (13.9)*†
Proteinuria (g/24h) (SD)	0.0 (0.0)	0.0 (0.0)	5.2 (5.1) *†
Severe preeclampsia (%)‡	0 (0)	0 (0)	19 (67.9)
Birth weight (g) (SD)	3608.5 (429.5)	1520.7 (854.0)*	1167.0 (320.0)*
Placenta weight (g) (SD)	645.7 (137.3)	302.4 (118.1)*	261.9 (90.2)*
Mode of delivery‡			
Caesarean section (%)	19 (63.3)	14 (66.7)	28 (100)
Vaginal delivery (%)	11 (36.7)	7 (33.3)	0 (0)
Gestational age at delivery (weeks + days; SD in days)	39 + 4 (9)	33 + 2 (35)*	30 + 4 (12)*

*, P < 0.05 compared to healthy controls; †, P < 0.05 compared to IUGR controls, ‡, P < 0.05 for overall comparison with Chi-square testing, **, data were available from 15 patients from the preeclampsia group.