Changes of placental syndecan-1 expression in preeclampsia and HELLP syndrome

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Abstract

Introduction—Preeclampsia is characterized by maternal systemic anti-angiogenic and pro-inflammatory states. Syndecan-1 is a cell surface proteoglycan expressed by the syncytiotrophoblast, which plays an important role in angiogenesis and resolution of inflammation. Our aim was to examine placental syndecan-1 expression in preeclampsia with or without HELLP syndrome.
Methods—Placentas were obtained from women in the following groups: (1) late-onset preeclampsia (n=8); (2) early-onset preeclampsia without (n=7) and (3) with HELLP syndrome (n=8); (4) preterm controls (n=5); and (5) term controls (n=9). Tissue microarrays (TMAs) were constructed from paraffin-embedded placentas. TMA slides were immunostained for syndecan-1 and evaluated using microscopy, virtual microscopy, and semi-automated image analysis. Maternal sera from patients with preeclampsia (n=49) and controls (n=32) were immunoassayed for syndecan-1. BeWo cells were treated with Forskolin or Latrunculin-B, or kept in ischemic conditions. SDC1 expression and syndecan-1 production were investigated with qRT-PCR, confocal microscopy, and immunoassays.

Results—Syndecan-1 was localized to the syncytiotrophoblast apical membrane in normal placentas. Syndecan-1 immunoscores were higher in late-onset preeclampsia (p=0.0001) and early-onset preeclampsia with or without HELLP syndrome (p=0.02 for both) than in controls. Maternal serum syndecan-1 concentration was lower in preeclampsia (median: 673ng/ml, interquartile range: 459-1161ng/ml) than in controls (1158ng/ml, 622-1480ng/ml). SDC1 expression and syndecan-1 immunostainings in BeWo cells and syndecan-1 concentrations in supernatants increased during cell differentiation. Disruption of the actin cytoskeleton with Latrunculin-B decreased syndecan-1 release, while ischemic conditions increased it.

Conclusions—Syncytiotrophoblastic syndecan-1 expression depends on the differentiation of villous trophoblasts, and trophoblastic syndecan-1 release is decreased in preeclampsia and HELLP syndrome. This phenomenon may be related to the disturbed syncytiotrophoblastic cortical actin cytoskeleton, and associated with maternal anti-angiogenic and pro-inflammatory states in these syndromes.

Keywords
cell signaling; pregnancy; proteoglycan; syncytiotrophoblast; systemic inflammation; virtual microscopy

Introduction
Preeclampsia is one of the ‘Great Obstetrical Syndromes’ [1] that affects 3%-5% of human pregnancies and is a major cause of maternal, perinatal, and neonatal mortality and morbidity worldwide.[2] Preeclampsia is diagnosed by the presence of proteinuria and new-onset hypertension after 20 weeks of gestation, and may cause maternal multi-organ damage affecting the kidneys, liver, and central nervous system.[2, 3] Preeclampsia has two major subtypes: early-onset preeclampsia (which develops before 34-35 weeks of gestation) and late-onset preeclampsia (which develops later during gestation).[2-5] Late-onset preeclampsia represents the majority of cases, is more frequent among healthy nulliparous women, is often mild, and primarily results in maternal consequences. Early-onset preeclampsia is a disease of multiparous women, is more severe, and is often associated with life-threatening HELLP (Hemolysis, Elevated Liver enzymes, and Low Platelet count) syndrome.[2-5] This syndrome is a consequence of microangiopathic haemolytic anaemia, hepatocellular injury, and consumptive thrombocytopenia, leading to characteristic changes in laboratory findings associated with hemolysis, abnormal liver function, and thrombocytopenia.[3, 6, 7] Originally, HELLP syndrome was thought to be a severe variant of preeclampsia, but subsequent studies suggested that it is a separate clinical entity.[3, 6] Indeed, one-fifth of HELLP syndrome patients do not have hypertension and/or proteinuria, which are necessary conditions for the diagnosis of preeclampsia.[3, 6, 7]

Although the exact pathophysologies of preeclampsia and HELLP syndrome are still incompletely understood, it is evident that the placenta plays an important role in their pathogeneses, as their only current specific therapy is the delivery of the fetus and placenta.
However, placental involvement in the pathogenesis of preeclampsia varies in extent as there is a high frequency of placental histopathological lesions observed in cases of early-onset preeclampsia and HELLP syndrome, while placental histopathological abnormalities are less frequent in cases of late-onset preeclampsia.[2, 3, 8-11] The extent of placental transcriptomics changes in late-onset and early-onset preeclampsia is also different when compared to gestational age-matched controls, but similar between early-onset preeclampsia and HELLP syndrome.[12-14] Irrespective of the observed differences, various subforms of preeclampsia and HELLP syndrome are associated with an increased release of anti-angiogenic molecules and trophoblastic debris from the chorionic villi, which lead to generalized maternal endothelial cell dysfunction and increased systemic inflammation, a common terminal pathway in these syndromes.[2-4, 15-23]

Syndecans are transmembrane proteoglycans that regulate various physiological processes including angiogenesis and inflammation.[24-26] The syndecan family consists of four members that evolved by gene duplication.[24, 25] Syndecans contain heparan sulfate and chondroitin sulfate chains that allow for specific interactions with a variety of ligands, including vascular endothelial growth factor, transforming growth factor-beta, and fibronectin, which all have important functions in the placenta.[27] Syndecan-1 is a cell-surface heparan-sulfate proteoglycan highly expressed in the syncytiotrophoblast layer of the chorionic villi in the human placenta.[28-31] It is involved in the regulation of cell behavior through its abilities to bind growth factors and to interact with components of the extracellular matrix, playing an important role in cell-cell and cell-extracellular matrix interactions, and in cell signaling. [24-26]

The exact placental functions of syndecan-1 and its possible involvement in the development of placental pathologies are still obscure.[29] Two recent publications noted that preeclampsia is accompanied by the decreased syncytiotrophoblastic expression of syndecan-1,[28, 30] while another study described increased syncytiotrophoblastic expression of this proteoglycan in cases of preeclampsia.[31] Thus far, no data have been published on the placental behavior of this proteoglycan for patients with HELLP syndrome. The aims of this study were 1) to examine the changes in the expression of syndecan-1 in the chorionic villi of the placenta, particularly in the syncytiotrophoblast, in various subforms of preeclampsia and HELLP syndrome; and 2) to model villous trophoblastic expression of syndecan-1 under normal conditions and in preeclampsia in trophoblast-like BeWo cells.

**Materials and Methods**

**Patient groups, clinical samples, and definitions**

The Health Science Board (Budapest, Hungary) and the Institutional Review Boards of Wayne State University (Detroit, Michigan, USA) and the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD), National Institutes of Health (NIH), U.S. Department of Health and Human Services (DHHS) (Bethesda, Maryland, and Detroit, Michigan, USA) approved the collection and use of materials for research purposes. Informed consent was obtained from all women prior to sample collection. Specimens and data were collected and stored anonymously.

Placental specimens were collected at the First Department of Obstetrics and Gynecology, Semmelweis University (Budapest, Hungary). Women were enrolled in the following groups: (1) late-onset preeclampsia (*n*=8); (2) early-onset preeclampsia (*n*=7); (3) early-onset preeclampsia with HELLP syndrome (*n*=8); (4) preterm controls (*n*=5); and (5) term controls (*n*=9) (Table 1). Maternal blood specimens (*n*=81) were selected from the Bank of Biological Materials of the Perinatology Research Branch, NICHD/NIDH/DHHS (Detroit, Michigan). Patients were included in the following groups: (1) preterm and term
preeclampsia (n=49) and (2) gestational age-matched controls (n=32) (Table 2). Patients with fetal congenital anomalies and multiple gestations at each site were excluded from the study. Blood specimens were collected within a week before delivery, and sera were collected by centrifuging blood at 1,300×g for 10min at 4°C, and then stored at -70°C.

Preeclampsia was defined as new-onset hypertension (systolic or diastolic blood pressure ≥140 or ≥90mmHg, respectively, measured at two time points, 4h to 1 week apart) developing after 20 weeks of gestation along with proteinuria (≥300mg during a 24h urine collection, or two random urine specimens obtained 4h to 1 week apart containing ≥1+ by dipstick, or one dipstick of ≥2+ protein).[2, 32, 33] HELLP syndrome was defined by the presence of hemolysis (serum LDH >600IU/l; bilirubin >1.2mg/dl; presence of schistocytes in peripheral blood), elevated liver enzymes (serum ALT and/or AST >70IU/l), and thrombocytopenia (platelet count <100,000/mm³), developing after 20 weeks of gestation. [34] The term “small-for-gestational–age” (SGA) was defined as neonatal birth weight below the 10th percentile for gestational age according to the national birth weight distribution curves.[35, 36] No medical or obstetrical complications were detected in term controls who delivered a neonate with a birth weight appropriate for gestational age. Preterm controls had preterm deliveries with no clinical or histological signs of chorioamnionitis, and delivered a neonate with a birth weight appropriate for gestational age.

**Placental specimens and histopathological evaluations**

Placentas were obtained following Caesarean deliveries. For microarray studies, villous tissue samples were excised from central cotyledons, and microarray analysis was performed as previously described.[13] Placentas were examined according to a standard histopathological protocol that describes the topography and size of macroscopic lesions, [13, 37] and then were fixed in formalin. For tissue microarray, five representative histological blocks from each placenta were paraffin-embedded to include central and peripheral cotyledons and the maternal side of the placenta. For microscopic examinations, 4μm sections were cut from these blocks and mounted on SuperFrost/Plus slides (Gerhard Menzel GmbH, Braunschweig, Germany). After deparaffinization, slides were rehydrated, stained with hematoxylin-eosin, and evaluated in 10 randomly selected microscopic fields. Macroscopic and microscopic lesions were defined according to published criteria.[38, 39]

**Placental tissue microarray construction and syndecan-1 immunostaining**

Representative cores of 2mm in diameter were collected from all tissue blocks of each placenta and placed into recipient blocks. Five-micrometer-thick tissue sections were cut and mounted on SuperFrost/Plus slides and stored at +4°C until the staining. Immunostaining was carried out using a Leica BOND-MAX™ autostainer (Leica GmbH, Nussloch, Germany), according to the manufacturer’s protocol. Slides were dewaxed in Bond™ Dewax Solution (Leica Microsystems) and rehydrated in Bond Wash Solution (Leica Microsystems). Antigen retrieval was performed at pH 6 using Bond Epitope Retrieval 1 Solution (Leica Microsystems) for 30min at 100°C. Slides were incubated for 20min at room temperature with a mouse monoclonal anti-syndecan-1 antibody (clone MI15; DakoCytomation, Glostrup, Denmark; 1:50). Primary antibody binding to tissue sections were visualized using biotin-free Bond Polymer Refine Detection (Leica Microsystems). After post-primary amplification (30min) and detection with the Novolink Polymer Detection System using 3,3’-Diaminobenzidine (DAB, Novocastra Laboratories; 1:50), the slides were counterstained with hematoxylin.

**Immunostaining evaluation**

Syndecan-1 immunostaining in the syncytiotrophoblast of the chorionic villi was quantified by the Pannoramic Viewer v1.15 of MembraneQuant software (3DHISTECH Ltd.,
Budapest, Hungary), which is suitable for unbiased semi-automated analysis of digital image objects based on color, intensity, and size. After the detection algorithm was calibrated, MembraneQuant found approximately 90% of the villi with a diameter of 20μm–150μm in each core. Staining intensities (+1, +2, or +3) of all scored villi in a given core were averaged, and this score was used as the representative data for that core.

In addition, visual evaluation of immunostaining was also performed by three examiners who were blinded to the clinical information and used an Axioskop 2 plus light microscope (Carl Zeiss MicroImaging GmbH, Gottingen, Germany). Subsequently, TMA slides were digitally scanned with a high-resolution scanner (Pannoramic Scan, 3DHISTECH Ltd.), and image data were deposited into a virtual laboratory (www.pathonet.org) and used for virtual microscopic evaluation with the Pannoramic Viewer 1.15 (3DHISTECH Ltd.).[37] Virtual slides were independently evaluated by the same examiners. In both cases, immunostaining of the syncytiotrophoblast brush border membrane was semi-quantitatively scored using an immunoreactive score modified from that previously described.[37] Briefly, at least 25 terminal or intermediate villi with a diameter of 20μm-150μm were evaluated in each core. Staining intensities (0, +1, +2, or +3) of the villi in a given core were averaged, and this score was used as the representative data for that core.

**BeWo cell cultures**

BeWo cells (American Type Culture Collection, Manassas, VA, USA) were cultured with F12 medium (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin (P/S). Cells were either plated on 6-well plates (0.5×10^6/plate) or on Nunc® Lab-Tek II® chambered coverglass slides (0.3×10^6/chamber) (Thermo Scientific, Wilmington, DE, USA), and were used for various experiments: (1) to test the effect of trophoblast differentiation on syndecan-1 expression, cells were treated either with Forskolin (25μM; Sigma-Aldrich Co. LLC, St. Louis, MO, USA) or with a vehicle (dimethyl sulfoxide, DMSO, Sigma-Aldrich) for three days; (2) to test the effect of actin cytoskeleton disruption on syndecan-1 expression and release from the trophoblast, cells were treated with 25μM Forskolin for three days, and one-half of the samples were treated with 25μM Latrunculin between hours 60-72; and (3) to test the effect of ischemic stress on syndecan-1 expression and release, cells were treated with 25μM Forskolin for three days, and one-half of the samples was kept in 20% O_2, while the other one-half was kept in alternating O_2 concentrations (20% for 6h, 1% for 6h; 4 cycles) in an OxyCycler C42 (Biospherix Ltd., Lacona, NY, USA) between hours 48-72.

Cell culture supernatants were collected from 6-well plates on each day and used for immunoassays. Total RNA was isolated from cells in 6-well plates on each day with the RNeasy Mini Kit and RNase-Free DNase Set (Qiagen, Valencia, CA, USA). The RNA concentrations were measured with the NanoDrop1000 Spectrophotometer (Thermo Scientific). Total RNA (500ng) was reverse-transcribed with the SuperScript® III First-Strand Synthesis System (Invitrogen). TaqMan® Assays (Applied Biosystems, Life Technologies Corporation) for SDC1 (Hs00896423_m1) and RPLP0 (large ribosomal protein; Endogenous Control; 4326314E) were used for gene expression profiling on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Cells in chambered coverglass slides were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA), blocked with Protein Block (Dako North America, Inc., Carpinteria, CA, USA), and stained with a mouse monoclonal anti-human syndecan-1 antibody (clone MI15; 5μg/ml; Novus Biologicals, LLC, Littleton, CO, USA) overnight at 4°C. After repeated washes, Alexa Fluor® 488 goat anti-mouse IgG (Invitrogen) was added at 1:1000 dilution in PBS with 10% normal Goat Serum (MP Biomedicals, LLC, Santa Ana, CA, USA). Cells were
mounted with ProLong Gold® antifade reagents and 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) and followed by confocal microscopy using a Zeiss LSM 780 spectral confocal system (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA).

**Syndecan-1 immunoassays**

Maternal serum and cell culture supernatant syndecan-1 concentrations were measured with a human syndecan-1 ELISA Kit (Cell Sciences, Canton, MA, USA), according to the manufacturer’s instructions. The sensitivity of the assay was <2.56 ng/ml, and the coefficients of intra-assay variation and inter-assay variation were 7.6% and 6.8%, respectively, according to the manufacturer.

**Statistical analysis**

Demographic data were analyzed using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). Comparisons among the groups were performed using Chi-square and Fisher’s exact tests for proportions, Kruskal-Wallis and Mann-Whitney tests for non-normally distributed continuous variables, and the t-test for normally distributed continuous variables. Pearson coefficients and associated p-values were calculated for the correlation between maternal serum syndecan-1 concentrations and clinical/demographic parameters using R (www.r-project.org). A linear model was used to reveal group differences in maternal serum syndecan-1 concentrations after adjusting for gestational age and birth weight. The level of significance was set to 5%.

**Results**

**Demographic and clinical data**

Demographic and clinical characteristics of the two study populations are displayed in Tables 1 and 2. In the placental study (Table 1), peak systolic and diastolic blood pressures were significantly higher in all patient, but not control, groups. Proteinuria was detected in all cases except those in the control groups. Although term and preterm controls were matched to cases within two weeks of gestational age, the median gestational age of term controls was slightly higher than that of cases with late-onset preeclampsia. In the maternal serum study (Table 2), peak systolic and diastolic blood pressures were higher in women with preeclampsia compared to the controls. Proteinuria was present in all cases, and HELLP syndrome developed in 10 cases of preeclampsia. Primiparity was more frequent, and birth weight was lower, in patients with preeclampsia.

**Syndecan-1 expression is dependent on villous trophoblast differentiation**

In term control placentas, syndecan-1 immunostaining was confined to the syncytiotrophoblast apical membrane, displaying a linear granular structure. A moderate amount of these syndecan-1 immunopositive granules was also detected in the syncytiotrophoblast cytoplasm. No syndecan-1 immunostaining was detected in the cytotrophoblast or in other cell types of the chorionic villi (Figure 1A). These findings suggest that syndecan-1 expression is dependent on the differentiation of villous trophoblasts, which we aimed to model *in vitro* using BeWo trophoblast-like cells. Forskolin-treated BeWo cells expressed an increasing amount of SDC1 during morphological and biochemical differentiation (Day 1: no change; Day 2: 3.2-fold increase, \(p=8.3 \times 10^{-4}\); Day 3: 3.4-fold increase, \(p=5.3 \times 10^{-5}\)) (Figure 1B). Furthermore, differentiating BeWo cells released significantly higher amounts of syndecan-1 into the cell culture medium on Day 2 (4.3±0.5ng/ml, \(p=0.028\)) and Day 3 (15.2±2.0ng/ml, \(p=0.008\)) than on Day 1 (under the detection limit of 2.56ng/ml) (Figure 1C). When compared to non-treated BeWo cells, Forskolin-treated BeWo cells, especially multinucleated fused cells, had strong
cell membrane syndecan-1 immunostaining on Day 3 of the cultures. In accord with placental findings, we also detected a punctuate pattern of cell membrane and cytoplasmic syndecan-1 immunostaining in these BeWo cells (Figure 1D).

**Placental syndecan-1 mRNA expression is not affected by preeclampsia**

Since it was previously hypothesized that preeclampsia may be related to the dysregulation of the differentiation of villous trophoblasts, we re-investigated our microarray data [13] on putative changes in SDC1 expression in preeclampsia. We did not find any significant difference in placental SDC1 expression for early-onset preeclampsia, either complicated with (1.43-fold down-regulation, adjusted p=0.74) or without (1.56-fold down-regulation, adjusted p=0.65) HELLP syndrome when compared to the control group (data not shown).

**Placental syndecan-1 immunoreactivity is increased in preeclampsia and HELLP syndrome**

Next, we evaluated placental syndecan-1 expression for cases of preeclampsia with or without HELLP syndrome. As shown in Figure 2, the most profound alteration was observed in cases with early-onset preeclampsia where the cytoplasm of the syncytiotrophoblast gave a strong immunoreaction, and the syncytiotrophoblast apical membrane was immunonegative for this proteoglycan in several cases. Similar but less intensive syndecan-1 immunostaining of the syncytiotrophoblast cytoplasm was observed in late-onset preeclampsia specimens. In cases of early-onset preeclampsia with HELLP syndrome, the syncytiotrophoblast apical membrane showed strong linear positivity. The syncytiotrophoblast also showed signs of syndecan-1 release to the intervillous space in 29%-55% of cases in all study groups, and to a somewhat larger extent in cases with early-onset preeclampsia and HELLP syndrome than in the control groups (Figure 2).

To objectively quantify placental syndecan-1 immunostaining on scanned TMA slides, we used the newly developed Pannoramic Viewer v1.15 of MembraneQuant and found that: (1) syndecan-1 immunostaining did not change with gestational age as immunoscores in preterm and term control groups were similar (2.33±0.33 versus 2.29±0.33, p=0.66); (2) syndecan-1 immunoscores were higher in cases of late-onset preeclampsia than in the term control group (2.59±0.26 versus 2.29±0.33; p=0.001); (3) syndecan-1 immunoscores were higher in cases of early-onset HELLP syndrome than in the preterm control group (2.63±0.23 versus 2.33±0.33; p=0.02); and (4) syndecan-1 immunoscores were higher in cases of early-onset preeclampsia than in the preterm control group (2.59±0.36 versus 2.33±0.33; p=0.02) (Figure 3). These findings were supported by microscopic and virtual microscopic evaluations and semi-quantitative TMA immunoscorings, although these latter scoring systems slightly differed (Supplementary Figures 1 and 2).

**Maternal serum syndecan-1 concentrations are decreased in preeclampsia**

Next, we investigated how maternal serum syndecan-1 concentrations changed in preeclampsia. Our study population consisted of early-onset and late-onset preeclampsia cases and gestational age-matched controls. Seven women with early-onset preeclampsia and three with late-onset preeclampsia also had HELLP syndrome. Maternal serum syndecan-1 concentrations positively correlated with birth weight ($R^2=0.25$, $p=1.7\times10^{-5}$) and gestational age ($R^2=0.22$, $p=1\times10^{-7}$) (Figure 4A), and negatively correlated with mean arterial pressure ($R^2=0.08$, $p=0.012$) and urine protein content ($R^2=0.06$, $p=0.039$). Indeed, maternal serum syndecan-1 concentration was lower in cases with preeclampsia (median: 673ng/ml, interquartile range (IQR): 459-1161ng/ml) than in the control group (median: 1158ng/ml, IQR: 622-1480ng/ml) (Figure 4B), even after adjusting for birth weight and gestational age ($p=0.03$). There was no difference in maternal serum syndecan-1 concentrations among cases of preeclampsia without HELLP syndrome (median: 718ng/ml, 673ng/ml, 1158ng/ml for preeclampsia, HELLP syndrome, and control group, respectively).
IQR: 460-1168ng/ml) and those with (median: 546ng/ml, IQR: 413-721ng/ml) this syndrome. Lower maternal serum syndecan-1 concentration among cases with lower birth weight was not due to the presence of SGA pregnancies, as there were no SGA neonates delivered in the control group (Table 2), and maternal serum syndecan-1 concentration was not different in cases of preeclampsia associated with or without SGA pregnancies (AGA: 703ng/ml, IQR: 497-1220ng/ml; SGA: 617ng/ml, IQR: 411-1035ng/ml; p=0.2) (Figure 4C).

Syndecan-1 release from BeWo cells is altered by ischemia and actin cytoskeleton disruption

To model syncytiotrophoblastic syndecan-1 release or retention in preeclampsia, we placed Forskolin-treated BeWo cells in ischemic conditions for 24 hours, or co-treated them for 12 hours with Latrunculin B, a disruptor of actin cytoskeleton.[40] We detected no difference in \textit{SDC1} expression for the BeWo cells kept in ischemic conditions (1.14-fold change, p=0.72) or for those treated with Latrunculin B (1.07-fold change, p=0.69) compared to the controls (Figure 5A). However, there was an increased release of syndecan-1 from BeWo cells into the culture medium in ischemic conditions (30±1.8ng/ml, p=0.042), and a decreased release after Latrunculin B treatment (17.4±3.3ng/ml, p=0.050) compared to the controls (24.0±4.2ng/ml) (Figure 5B). Immunofluorescence stainings for syndecan-1 and confocal imaging of Forskolin-treated control BeWo cells revealed the strongest cell membrane syndecan-1 immunostaining of multinucleated, fused cells (Figure 5C). BeWo cells kept in ischemic conditions had faint cell membrane staining of syndecan-1, suggesting an increased shedding of syndecan-1 from the trophoblast cell surface into the cell culture medium, similar to the increased shedding of syndecan-1 from cell surfaces in pro-inflammatory conditions [24, 26, 41] (Figure 5D). Latrunculin B-treated BeWo cells had no (or faint) cell membrane syndecan-1 immunoreactivity; however, there was increased granular syndecan-1 accumulation in their cytoplasm, suggesting a defect in the cytoplasmic and cell membrane transport of syndecan-1, leading to its decreased release into the cell culture medium upon the disruption of the actin cytoskeleton (Figure 5E).

Discussion

Principal findings of this study

(1) Syndecan-1 was mainly localized to the syncytiotrophoblast apical membrane in the chorionic villi of control placentas; (2) syndecan-1 immunostaining of the syncytiotrophoblast and its apical membrane did not change with gestational age in control placentas; (3) there was increased syndecan-1 immunostaining intensity of the syncytiotrophoblast in cases of late-onset preeclampsia and early-onset preeclampsia with or without HELLP syndrome; (4) maternal serum syndecan-1 concentration positively correlated with gestational age and birth weight, and negatively correlated with blood pressure and proteinuria; (5) maternal serum syndecan-1 concentration was lower in cases of preeclampsia than in gestational age-matched controls; (6) the presence of SGA neonates did not affect maternal serum syndecan-1 concentration in pregnancies with preeclampsia; and (7) the disruption of the actin cytoskeleton led to the accumulation of cytoplasmic syndecan-1 in BeWo cells, while ischemic stress caused increased release of syndecan-1 from BeWo cells.

Placental syndecan-1 expression increases during trophoblast differentiation

We found syndecan-1 immunostaining of the syncytiotrophoblast and its apical membrane, but other cells in the chorionic villi of the placenta were immunonegative for this proteoglycan. No changes were found in syndecan-1 immunostaining with increasing gestational age during the third trimester. These findings are consistent with those of Crescimanno et al [29] and Jokimaa et al [28, 30] who showed similar syndecan-1...
immunostaining of the syncytiotrophoblast in placentas obtained during the first and third trimesters of pregnancy, which are also consistent with previous reports describing placental syndecan-1 expression to be restricted to the syncytiotrophoblast [28-31, 42]. This suggests that syndecan-1 expression is dependent on the differentiation of the villous cytotrophoblasts to form the syncytiotrophoblast. As a confirmation, we observed increasing \textit{SDC1} expression and syndecan-1 release from BeWo trophoblastic-like cells during Forskolin-induced differentiation.

The remarkable structural, functional, and metabolic changes that the villous trophoblast undergoes during its morphological and biochemical differentiation, as well as fusion into the syncytiotrophoblast, are secondary to the categorical reprogramming of the villous trophoblastic transcriptional program.[43, 44] This temporal transcriptomic reprogramming involves the induction or repression of thousands of genes, leading to the gain of a multitude of functions, some of them unique to the placenta.[43] The fascinating output of this tightly regulated process is the multinucleated syncytiotrophoblast, which plays essential roles throughout pregnancy, including materno-fetal nutrients, gas and waste exchange, synthesis of placental hormones required for fetal development, and generation of an immunological barrier between the mother and her fetus.[45] Results for the high syndecan-1 expression in the syncytiotrophoblast suggest the importance of its function(s) at the maternal-fetal barrier. Since syndecan-1 expression is developmentally regulated [25] and can also be very high in various tumors [46-49], this proteoglycan may have oncodevelopmental significance.

**Placental syndecan-1 immunostaining intensity is increased in preeclampsia and HELLP syndrome**

While examining placental syndecan-1 expression in various disease groups, we found increased syndecan-1 immunostaining intensity of the syncytiotrophoblast apical membrane in both late-onset and early-onset preeclampsia and HELLP syndrome using microscopy, semi-automated image analysis, and virtual microscopy, which becomes more and more accepted in on-line evaluations and testings [50]. These findings are consistent with the results of Ogawa et al., who used a semi-quantitative immunoscoring system and showed that syndecan-1 immunostaining of the syncytiotrophoblast was increased in cases of early-onset preeclampsia when compared to the preterm controls.[31] Interestingly, two publications from Jokimaa et al. [28, 30] presented data on faint syndecan-1 immunostaining in placentas of patients with late-onset and early-onset preeclampsia compared to strong immunoreactivity for syndecan-1 in placentas taken from preterm and term controls. The discrepancy between these studies may lie in critical differences in the applied methodologies. All three previous studies utilized the same mouse monoclonal IgG1 antibody (clone B-B4) that binds to a linear epitope between residues 90 to 95 of the core protein on human syndecan-1.[51] This B-B4 antibody reacts with the same or closely related epitope on the syndecan-1 core as the MI15 antibody used in our study.[52] The important difference among the four studies is that, similar to Ogawa et al,[31] we used antigen retrieval by heating the paraffin sections. On the other hand, there was no mention of antigen retrieval in the publications of Jokimaa et al. [28, 30] even though it is mandatory for syndecan-1 immunostaining of paraffin sections (DakoCytomation recommendation). Thus, a possible explanation is that the lack of antigen retrieval kept the B-B4 antibody-reactive epitope(s) masked by formalin-induced cross-links on syndecan-1 core protein, which we observed to be accumulated in the syncytiotrophoblast in preeclampsia.

It has been well-established by placental morphometrical studies that placental development is altered and placental morphology is abnormal in cases of early-onset preeclampsia complicated by intrauterine growth restriction (IUGR), but these parameters are not affected.
in late-onset preeclampsia not complicated by IUGR.[53] It was also previously shown that the placental transcriptome is quite dissimilar in cases of late-onset and early-onset preeclampsia complicated with IUGR,[12] and that the placental gene expression signature in early-onset preeclampsia and early-onset HELLP syndrome is similar.[13] Many other characteristics and parameters observed in different subforms of preeclampsia, including the differences we found in the frequency of placental lesions consistent with maternal underperfusion [10, 13, 37] also underline the heterogeneity of this syndrome.[3-5] In this regard, it is remarkable that we found a general increase in syncytiotrophoblastic syndecan-1 immunostaining in various subforms of preeclampsia and HELLP syndrome, suggesting that the dysregulation of this proteoglycan in the syncytiotrophoblast may be part of the common terminal pathway of these syndromes. Nevertheless, some heterogeneity was also observed in the syncytiotrophoblastic staining pattern of this proteoglycan, and as were remarkable changes in its cellular distribution in preeclampsia and HELLP syndrome. There were cases with very intense immunostaining in or below the apical membrane region, especially for cases with early-onset HELLP syndrome. There were placenta for which strong cytoplasmic immunostaining was also revealed, suggesting the retention of the protein in the cytoplasm. However, in some cases, the apical membrane was completely immunonegative for syndecan-1, especially in early-onset preeclampsia, suggesting the decreased transport of syndecan-1 to the cell surface and its increased shedding from that in these cases. Indeed, syncytiotrophoblastic syndecan-1 release into the intervillous space in 29%-55% of the cases in all study groups was observed.

**Maternal serum syndecan-1 concentrations are decreased in preeclampsia**

In accord with this latter observation, we detected a large amount of syndecan-1 in the maternal sera between 24 and 40 weeks of gestation. Our observations supported the findings of a previous study [54] in that maternal serum syndecan-1 concentrations continuously rise during pregnancy, and these can be more than 100-fold higher than in non-pregnant women.[54] Since syndecan-1 immunostaining of the syncytiotrophoblast does not change with gestational age,[28-30] and birth weight was positively associated with maternal serum syndecan-1 concentration in this study, we propose that maternal serum syndecan-1 concentration increases with advancing gestational age due to the increase in trophoblast cell volumes and surface areas as shown by stereological methods.[55]

This is the first study to evaluate maternal serum syndecan-1 concentration in preeclampsia. We found that syndecan-1 concentration was decreased in women with preeclampsia (both early- and late-onset) compared to gestational-age-matched controls at the time of the onset of clinical symptoms, in spite of the stronger placental syndecan-1 immunostaining detected in these syndromes. Since our microarray data [13] showed no change in SDC1 expression in early-onset preeclampsia or early-onset HELLP syndrome, the increased placental syndecan-1 content and the decreased maternal serum syndecan-1 concentrations suggest that there is an alteration in syncytiotrophoblastic transport of syndecan-1 in these syndromes.

**Disrupted actin cytoskeleton may inhibit syncytiotrophoblastic syndecan-1 transport**

It has previously been shown that syndecan-1 co-localizes with actin filaments and that cellular syndecan-1 transport is dependent on the intactness of the actin cytoskeleton.[24, 25, 56] In this context, one study revealed that the expression of a set of genes involved in the regulation of the actin cytoskeleton is characteristically down-regulated in the placenta in cases of preeclampsia.[57] Another study showed that actin and ezrin, proteins important to the structural integrity of the brush border membrane, have decreased expression in the villous cytotrophoblast in preeclampsia.[58] It was also shown that the network of cytoskeletal proteins in the apical syncytiotrophoblast membrane is significantly altered in
preeclampsia,[59] and this may have a significant impact on the deformation of the
syncytiotrophoblast brush border membrane in preeclampsia, as observed during an electron
microscopic study.[60] Our findings on the increased retention of syndecan-1 in BeWo cells
upon the disruption of the actin cytoskeleton with Latrunculin B support the hypothesis that
alterations of the cortical cytoskeletal actin network in the syncytiotrophoblast in
preeclampsia and HELLP syndrome will lead to the accumulation of syndecan-1 below the
apical membrane and, ultimately, in the cytoplasm, also leading to decreased syndecan-1
maternal serum concentration (Figure 6).

Another relevant finding from previous studies has shown that the integrity of lipid rafts is
essential for the clustering and biological activity of syndecan-1 on cell surfaces.[61, 62] We
found a granular staining pattern of syndecan-1 in the syncytiotrophoblast of the control
placentas, which may be the consequence of the localization of syndecan-1 in distinct lipid
raft domains of the apical brush border membrane of the syncytiotrophoblast. Interestingly,
studies by Riquelme et al. found that two different lipid raft structures are distinctly
associated with the classical “microvillous membrane” (MVM) and the “light microvillous
membrane” (LMVM) on the syncytiotrophoblast brush border surface,[63] and that there is
only one type of lipid raft in the MVM and LMVM membranes in preeclampsia, suggesting
considerable differences in lipid raft structures of the syncytiotrophoblast for this syndrome.
[59] Our observation on the change from a granular to a more linear membrane staining
pattern of syndecan-1 in disease samples suggests that this phenomenon may be related to
the altered lipid raft structures. Collectively, these results suggested that changes of the actin
cytoskeleton and lipid raft structures may play a role in the altered cellular transport and
localization of syndecan-1 for the syncytiotrophoblast in preeclampsia in a different manner
as in the case of galectin-13.[40]

**Possible consequences of altered syndecan-1 release from the trophoblast**

Syndecan-1 is a transmembrane protein with heparan sulfate chains attached to its
extracellular domain, and has a wide variety of functions on epithelial surfaces through the
binding and regulating of heparan-sulfate and heparan-binding molecules.[24-26, 41, 64, 65] The
physiological and pathophysiological processes that involve syndecan-1 include host
defense against pathogens, leukocyte recruitment, resolution of inflammation, extracellular
matrix remodeling, angiogenesis, cell signaling, proliferation and apoptosis, cell invasion,
and metastasis.[24-26, 41, 48, 49, 64-68] Although the physiological functions of
syndecan-1 in the chorionic villi of the placenta are still obscure, we propose that
syndecan-1 may play an important role during pregnancy based on its high expression in this
fetal tissue and its unique localization to the maternal-fetal barrier where the fetus comes
into direct contact with maternal blood and the immune system.

Since syndecan-1 has anti-inflammatory roles by binding to and removing chemokines from
cell surfaces after shedding,[24, 26, 41, 65, 67] it is possible that the decreased shedding /
secretion of syndecan-1 from the syncytiotrophoblast in preeclampsia and HELLP syndrome
may promote the exaggeration of the maternal systemic inflammation found in these
syndromes. Syndecan-1 also has a central role in angiogenesis as it can bind to many growth
factors (e.g. fibroblast growth factor 2, hepatocyte growth factor, transforming growth factor
beta, and vascular endothelial growth factor), activate them, and promote angiogenesis.[24,
26, 65] Therefore, it is possible that there may be a link between decreased maternal serum
syndecan-1 concentrations and the anti-angiogenic state in preeclampsia.[16, 18, 20, 21]
Considering the important role of this proteoglycan in cell signaling,[24, 25, 65] the changes
in syncytiotrophoblastic syndecan-1 transport and localization may lead to altered signaling
and pro-inflammatory changes in the trophoblast, which have been observed in
preeclampsia.[13, 23]
Conclusions

Syncytiotrophoblastic syndecan-1 expression is dependent on trophoblast differentiation, and trophoblastic syndecan-1 release is decreased in preeclampsia and HELLP syndrome. This phenomenon may be related to the disturbed cortical actin cytoskeleton in the syncytiotrophoblast, and associated with maternal anti-angiogenic and pro-inflammatory states in these syndromes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References


Figure 1. Syndecan-1 expression is dependent on villous trophoblast differentiation

A) In term control placentas, a linear granular syndecan-1 immunostaining was confined to the syncytiotrophoblast apical cell membrane, and moderate amounts of these immunopositive granules were also detected in the cytoplasm. No syndecan-1 immunostaining was detected in other cell types of the villi (hematoxylin counterstain, 400× magnification).

B) $SDC1$ expression significantly increased from the second day of differentiation in Forskolin-treated BeWo cells (Day 2: 3.2-fold, $p=8.3\times10^{-4}$; Day 3: 3.4-fold, $p=5.3\times10^{-5}$).

C) Syndecan-1 release into the cell culture medium significantly increased from the second day of differentiation in Forskolin-treated BeWo cells (Day 2: 4.3ng/ml, $p=0.028$; Day 3: 15.2ng/ml, $p=0.008$). D) Forskolin-treated BeWo cells had strong cell membrane syndecan-1 immunostaining on Day 3 of culture compared to non-treated
BeWo cells. A punctuate cell membrane and cytoplasmic syndecan-1 staining could also be detected in BeWo cells (DAPI counterstain, 1500x magnification).
Figure 2. Placental syndecan-1 immunostaining is increased in preeclampsia and HELLP syndrome
A-C) Syndecan-1 immunostaining was similar in preterm controls (A: GW26+6, B: GW35+2) and term controls (C: GW39+5), showing a linear granular pattern, mainly confined to the syncytiotrophoblast apical membrane and moderately to the syncytiotrophoblast cytoplasm. D) In early-onset preeclampsia complicated with HELLP syndrome (GW27), the syncytiotrophoblast apical membrane showed strong linear positivity. E) In early-onset preeclampsia (GW34+6), the cytoplasm of the syncytiotrophoblast gave strong immunoreaction. Some areas in the syncytiotrophoblast showed signs of syndecan-1 release, and the apical membrane was focally immunonegative. F) Similar but less intensive cytoplasmic syndecan-1 immunostaining was observed in late-onset preeclampsia (GW40+3) specimens. The syncytiotrophoblast apical membrane area is shown in larger magnification in a case of a term control (G) and early-onset preeclampsia (H). Arrows show immunonegative apical membrane regions of the syncytiotrophoblast in preeclampsia. Hematoxylin counterstain, 400× (A-F) or 600× (G-H) magnifications.
Figure 3. MembraneQuant evaluation of placental syndecan-1 immunoscores
A) There was a larger proportion of 3+ villi in all disease groups than in the corresponding controls. The number of placenta and investigated villi are shown below the stacked bars.
B) The same data are represented in immunoscores (mean±SD). Syndecan-1 immunoscores did not change with gestational age (preterm controls: 2.33±0.33 versus term controls 2.29±0.33, p=0.66). Syndecan-1 immunoscores were higher in cases of early-onset HELLP syndrome (2.63±0.23, p=0.02) and early-onset preeclampsia (2.59±0.36, p=0.02) than in preterm controls (2.33±0.33), and these were higher in cases of late-onset preeclampsia (2.59±0.26, p=0.0001) than in term controls (2.29±0.33).
Figure 4. Maternal serum syndecan-1 concentrations are decreased in preeclampsia
A) Maternal serum syndecan-1 concentrations correlated with gestational age in both groups (gray: control, black: preeclampsia; regression lines are provided for controls (gray), preeclampsia (black), and all cases combined (dotted line, $R^2=0.22$, $p=1\times10^{-5}$)). B) Median maternal serum syndecan-1 concentration was lower in preeclampsia (673ng/ml, interquartile range (IQR): 459-1161ng/ml) than in the controls (1158ng/ml, IQR: 622-1480ng/ml). C) Maternal serum syndecan-1 concentrations were not different ($p=0.2$) among cases with preeclampsia who had small-for-gestational-age neonates (filled circles; median: 617ng/ml, IQR: 411-1035ng/ml) and those who had appropriate-for-gestational-age neonates (open circles; median: 703ng/ml, IQR: 497-1220ng/ml). GW: gestational week.
Figure 5. Syndecan-1 release from BeWo cells is altered by ischemic stress and the disruption of the actin cytoskeleton

A) SDC1 expression was not changed in ischemic conditions (1.14-fold change, p=0.72) or after treatment with Latrunculin B (1.07-fold change, p=0.69) when compared to Forskolin-treated control cells. B) Syndecan-1 release into the cell culture medium increased in BeWo cells kept in ischemic conditions (30±1.8ng/ml, p=0.042) and decreased in cells treated with Latrunculin B (17.4±3.3ng/ml, p=0.050) when compared to the controls (24.0±4.2ng/ml). C) Multinucleated, fused BeWo cells had the strongest cell membrane syndecan-1 immunostaining. D) BeWo cells kept in ischemic conditions had faint cell membrane syndecan-1 immunostaining. E) Latrunculin B-treated BeWo cells had no or faint cell membrane syndecan-1 immunostaining, while there was an increased granular syndecan-1 accumulation in their cytoplasm. C-E) 3D reconstruction of stack images; DAPI counterstain; 1 unit=10.65μm.
Figure 6. A possible explanation on the altered trophoblastic transport and localization of syndecan-1 in preeclampsia

During normal pregnancy, syndecan-1 is transported to the apical membrane of the syncytiotrophoblast, from where it is continuously shed into the maternal circulation. For preeclampsia, the alterations in the cortical cytoskeletal actin network lead to the accumulation of syndecan-1 below the apical membrane and, ultimately, in the cytoplasm. For early-onset preeclampsia, there may be an increased shedding of syndecan-1 from the cell surface because of increased pro-inflammatory conditions, which may explain our findings on the immunonegativity of the apical membrane for syndecan-1 in several cases. Still, the net amount of syndecan-1 shed into the maternal circulation is decreased compared to normal pregnancies. Parts of the figure were adapted from Riquelme et al.[59], © Springer.
Table 1

Demographic and clinical characteristics of the placental study groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Preterm controls</th>
<th>Early-onset preeclampsia</th>
<th>Early-onset HELLP syndrome</th>
<th>Term controls</th>
<th>Late-onset preeclampsia</th>
</tr>
</thead>
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<tr>
<td>Number of cases(^a)</td>
<td>5</td>
<td>7</td>
<td>8</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Maternal age (years)(^b)</td>
<td>31.6 (31.1-34.3)</td>
<td>34.0 (27.6-35.0)</td>
<td>29.4 (26.1-30.2)</td>
<td>30.8 (30.1-34.2)</td>
<td>31.3 (25.2-34.8)</td>
</tr>
<tr>
<td>Gestational age at delivery (weeks)(^b)</td>
<td>31.7 (31.0-34.0)</td>
<td>32.6 (31.2-34.4)</td>
<td>29.4 (28.0-32.6)</td>
<td>38.9 (38.7-39.7)</td>
<td>37.4 (36.8-38.1) (^#)</td>
</tr>
<tr>
<td>Primiparity(^c)</td>
<td>60</td>
<td>71</td>
<td>50</td>
<td>11</td>
<td>50</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)(^b)</td>
<td>120 (120-133)</td>
<td>160 (155-160) (^*)</td>
<td>165 (145-170) (^*)</td>
<td>130 (125-135)</td>
<td>156 (150-170) (^##)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)(^b)</td>
<td>80 (70-80)</td>
<td>100 (100-100) (^*)</td>
<td>100 (95-110) (^*)</td>
<td>80 (78-85)</td>
<td>95 (90-100) (^##)</td>
</tr>
<tr>
<td>Proteinuria(^c)</td>
<td>0</td>
<td>100 (^**)</td>
<td>100 (^**)</td>
<td>0</td>
<td>100 (^**)</td>
</tr>
<tr>
<td>Maternal BMI (kg/m(^2))(^b)</td>
<td>23.4 (20.1-24.6)</td>
<td>24.4 (23.4-25.2)</td>
<td>24.7 (21.3-26.9)</td>
<td>26.7 (23.1-28.0)</td>
<td>22.0 (19.2-23.2)</td>
</tr>
<tr>
<td>Neonatal birth weight (g)(^b)</td>
<td>1990 (910-2210)</td>
<td>1100 (1010-1280)</td>
<td>965 (840-1545)</td>
<td>3470 (3400-4030)</td>
<td>2955 (2555-3175) (^##)</td>
</tr>
<tr>
<td>Cesarean section(^c)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

All women were Caucasian.

\(^*\) p<0.05 compared to gestational-age-matched, preterm controls

\(^**\) p<0.01;

\(^#\) p<0.05 compared to gestational-age-matched, term controls

\(^##\) p<0.01;

\(^\text{Values are presented as number}\)

\(^\text{Values are presented as median (interquartile (IQR) range)}\)

\(^\text{Values are presented as percentage}\)
Table 2

Demographic and clinical characteristics of the maternal serum study groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Controls</th>
<th>Preeclampsia</th>
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<tr>
<td>Number of cases&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32</td>
<td>40</td>
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<tr>
<td>Maternal age (years)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25.5 (20-31)</td>
<td>23 (19.5-28.5)</td>
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<tr>
<td>African-American race&lt;sup&gt;c&lt;/sup&gt;</td>
<td>84</td>
<td>94</td>
</tr>
<tr>
<td>Gestational age at delivery (weeks)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>36.8 (29.9-38.8)</td>
<td>34.6 (30.1-37.7)</td>
</tr>
<tr>
<td>Primiparity&lt;sup&gt;c&lt;/sup&gt;</td>
<td>25</td>
<td>51&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>118 (114-128)</td>
<td>176 (166-190)&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>67 (62-73)</td>
<td>106 (101-112)&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>Proteinuria&lt;sup&gt;c&lt;/sup&gt;</td>
<td>12.5</td>
<td>100&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>HELLP syndrome&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0</td>
<td>20&lt;sup&gt;**&lt;/sup&gt;</td>
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<tr>
<td>Maternal BMI (kg/m&lt;sup&gt;2&lt;/sup&gt;)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26.4 (21.7-32.8)</td>
<td>26.8 (23-32.6)</td>
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<tr>
<td>Neonatal birth weight (g)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3073 (1258-3268)</td>
<td>1825 (1070-2635)&lt;sup&gt;**&lt;/sup&gt;</td>
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<tr>
<td>Small-for-gestational-age neonates&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0</td>
<td>47&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>Caesarean section&lt;sup&gt;c&lt;/sup&gt;</td>
<td>50</td>
<td>59</td>
</tr>
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</table>

<sup>**</sup>p<0.01; <sup>*</sup>p<0.05

<sup>a</sup>Values are presented as number
<sup>b</sup>Values are presented as median (interquartile (IQR) range)
<sup>c</sup>Values are presented as percentage