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Decidual and placental NOD1 is associated with inflammation in normal and preeclamptic pregnancies

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ABSTRACT

Introduction: Inflammation is a normal physiological process that increases to harmful levels in preeclampsia. It affects the interaction between maternal immune cells and fetal trophoblasts at both sites of the maternal-fetal interface; decidua and placenta. The pattern recognition receptor nucleotide-binding oligomerization domain-containing protein (NOD)1 is expressed at both sites. This study aimed to characterize the cellular expression and functionality of NOD1 at the maternal-fetal interface of normal and preeclamptic pregnancies.

Methods: Women with normal or preeclamptic pregnancies delivered by caesarean section were included. Decidual (n = 90) and placental (n = 91) samples were analyzed for NOD1 expression by immunohistochemistry and an automated image-based quantification method. Decidual and placental explants were incubated with or without the NOD1-agonist iE-DAP and cytokine responses measured by ELISA.

Results: NOD1 was markedly expressed by maternal cells in the decidua and by fetal trophoblasts in both decidua and placenta, with trophoblasts showing the highest NOD1 expression. Preeclampsia with normal fetal growth was associated with a trophoblast-dependent increase in decidual NOD1 expression density. Compared to normal pregnancies, preeclampsia demonstrated stronger correlation between decidual and placental NOD1 expression levels. Increased production of interleukin (IL)-6 or IL-8 after *in vitro* explant stimulation confirmed NOD1 functionality.

Discussion: These findings suggest that NOD1 contributes to inflammation at the maternal-fetal interface in normal pregnancies and preeclampsia and indicate a role in direct maternal-fetal communication. The strong expression of NOD1 by all trophoblast types highlights the importance of combined assessment of decidua and placenta for overall understanding of pathophysiological processes at the maternal-fetal interface.

1. Introduction

Direct maternal-fetal interaction during pregnancy occurs in the uterine wall decidua and at the placental surface facing maternal blood. These sites are closely interconnected and adapted to allow maternal cells to directly interact with specialized fetal trophoblasts. In the decidua, extravillous trophoblasts facilitate remodeling of the spiral arteries and remain in close interaction with tissue resident maternal

Abbreviations: CK7, cytokeratin 7; CS, caesarean section; FGR, fetal growth restriction; iE-DAP, γ-D-glutamyl-meso-diaminopimelic acid; IL, interleukin; NOD1, nucleotide-binding oligomerization domain-containing protein 1; PRR, pattern recognition receptor; ER, endoplasmic reticulum; IP-10, interferon-γ-inducible protein-10.

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immune cells throughout pregnancy [1,2]. In the placenta, the multinucleated syncytiotrophoblast layer that covers the fetal placental structures is directly exposed to immune cells in the circulating maternal blood [3]. These two delicate maternal-fetal interaction sites are sensitive to perturbations of both exogenous and endogenous origin, and untimely inflammatory triggers can induce a harmful immune activation during pregnancy [3,4].

A low-grade inflammation at the maternal-fetal interface represents the body's physiological adaptation to pregnancy and is characterized by controlled production of cytokines and angiogenic factors by trophoblasts and maternal immune cells [5–7]. The corresponding maternal systemic inflammation is associated with leukocytosis and enhanced cytokine production by circulating maternal immune cells upon their interaction with the placental syncytiotrophoblast and activators released from the placenta [3]. The inflammatory pregnancy disorder preeclampsia manifests as maternal hypertension with proteinuria and/or organ dysfunction after 20 weeks of gestation. It affects 2–8% of pregnancies and can be further complicated by fetal growth restriction (FGR) [8,9]. Preeclampsia is characterized by exaggerated inflammation with increased production of inflammatory cytokines both at the maternal-fetal interface and in the maternal circulation [6,10]. This harmful inflammation is associated with deficient spiral artery remodeling in the decidua and subsequent altered uteroplacental perfusion causing hemodynamic, oxidative, and endoplasmic reticulum (ER) stress in the placenta. As the pregnancy progresses, the dysfunctional placenta releases increasing amounts of danger signals to the maternal circulation, thereby eliciting maternal systemic inflammation, endothelial dysfunction and symptomatic preeclampsia [11,12].

Inflammation is initiated when the innate immune system recognizes danger signals through pattern recognition receptors (PRRs). Both maternal immune cells and trophoblasts express PRRs, enabling them to communicate with each other and respond to their surroundings [5]. When dysregulated at the maternal-fetal interface, PRR activation may disrupt the delicate immunological environment, and we have previously identified a role for Toll-like receptor 3 and 4 and Nod-like receptor protein (NLRP)3 in the development of preeclampsia [13–16]. Nucleotide-binding oligomerization domain-containing protein (NOD) 1 is an intracellular PRR that responds to endogenous stress signals or invading pathogens [17]. Aberrant NOD1 expression and function are associated with inflammatory bowel disease, metabolic syndrome and cancer [18–20]. The receptor is ubiquitously expressed [21,22] and was first established as a sensor of bacterial peptidoglycan (γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP)) [23–25]. More recent studies have shown that endogenous danger signals derived from ER stress and saturated fatty acids also can elicit NOD1 signaling, but the exact mechanisms of activation have not been determined [26–28]. Activation of NOD1 culminates in the production of inflammatory cytokines through NF-κB and MAPK signaling pathways [29,30].

NOD1 is expressed in the decidua and placenta and mounts an inflammatory response to specific danger signals [31–39]. Aberrant NOD1 expression has been associated with the pregnancy complications miscarriage, premature rupture of membranes, preterm labor and gestational diabetes [31,34,38–40] and we have previously shown differential decidual gene expression of NOD1 in preeclampsia [41]. In mice, activation of NOD1 has been associated with preterm labor, FGR and intrauterine fetal death [36,42], as well as augmented inflammation in the mother and fetus [36]. The full role of NOD1 in the delicate interplay between maternal and fetal cells at the maternal-fetal interface remains largely unknown as previous studies have been small, focused on infections, and separately assessed either the decidua or the placenta. In this study, we aimed to determine the inflammatory role of NOD1 at the maternal-fetal interface in normal and preeclamptic pregnancies through characterization of the cellular expression and functionality of NOD1 in the decidua and placenta.

2. Methods

2.1. Ethical approval

This study was approved by the Norwegian Regional Committee for Medical and Health Research Ethics as part of the Preeclampsia Study (approval no. 2012/1040) and the First and Third Trimester Study (approval no. 2009/3). Informed written consent was obtained from all participants.

2.2. Study population

The Preeclampsia Study includes healthy and preeclamptic singleton pregnancies delivered by caesarean section (CS) in absence of labor at St. Olavs and Haukeland University Hospitals between 2002 and 2012. Pregnant women diagnosed with preeclampsia with or without FGR were included as cases. Healthy normotensive pregnant women with no previous history of preeclampsia or FGR were included as normal pregnant controls. Decidua basalis tissue was collected by vacuum aspiration of the placental bed during CS [43,44], and a placental biopsy was taken tangentially from the central part of the maternal side of the placenta shortly after delivery in a standardized manner. The tissue samples were fixed in 10% neutral-buffered formalin and embedded in paraffin for immunohistochemical analyses.

Preeclampsia was defined as persistent hypertension (systolic/diastolic blood pressure $>$ 140/90 mmHg) and proteinuria ($>$300mg/24 h or $>$ 1+ by dipstick) developing after 20 weeks of gestation. Women with pre-gestational hypertension developing proteinuria in the second half of pregnancy were diagnosed with superimposed preeclampsia and included in the preeclampsia group. FGR was determined by serial ultrasound measurements showing reduced intrauterine growth or, for neonates small for gestational age (n = 1), birth weight $<$ 5th percentile of Norwegian reference curves [45] combined with clinically and sonographically suspected FGR and/or postpartum defined placental pathology.

The First and Third Trimester Study includes women with normal singleton pregnancies delivered by CS at St. Olavs University Hospital between 2015 and 2017. Third trimester placentas were collected after delivery for immediate isolation of decidual and placental explants and used for stimulation assays.

2.3. Immunohistochemistry and quantitative protein expression

Serial decidual and placental tissue sections of 3 μm were pre-treated in PT link (#PT101, Dako) using target retrieval solution (#K8005 or #K8004, Dako) at 97 °C for 20 min, and next treated with peroxidase blocking solution (#K4007 or #K5361, Dako). The tissue sections were incubated with primary antibodies for NOD1 (decidua 1:50, placenta 1:150, #MAB7090, R&D Systems, overnight at 4 °C); cytokeratin 7 (CK7) (decidua 1:300 for 45 min, placenta 1:800 for 40 min, #M0851, Dako, room temperature); CD31 (1:50, #M0823, Dako, room temperature for 40 min); or CD45 (1:150, #M0701, Dako, room temperature for 40 min). All sections were incubated for 30 min with HRP-labeled polymer (#K4007, Dako) and for 10 min with DAB+ as chromogen (1:50, #K4007 or K5361, Dako). Decidual CK7 sections were double stained with smooth muscle actin antibodies (1:300, #M0851, Dako) using EnVision G|2 Doublestain System Rabbit/Mouse (DAB+/Permanent Red) Kit system (K#5361, Dako). Stainings were performed by Autostainer Plus (#S3800, Dako) or manually for overnight stainings. Sections were counterstained with hematoxylin. Negative isotype controls for NOD1 were included (decidua 1:1.25, placenta 1:5, Mouse IgG1 #349040, BD Pharmigen). Additional routine staining with hematoxylin (75290, Chemi-Teknik AS), erythrosine 239 (720–0179, VWR) and saffron (75100, Chemi-Teknik AS) (HES) was performed using a Sakura Tissue-Tek © Prisma StainerTM (Sakura Finetek).

To ensure a representative analysis, large tissue section scans were

obtained by the EVOS™ FL Auto Imaging System (Thermo Fisher Scientific) by combining between 4 and 81 (decidua) and 9–36 (placenta) adjacent bright field TIFF images for each donor (20X magnification, 2048× 1536 pixels) depending on the amount of available tissue. A custom ImageJ script was used to perform background correction and tile stitching [46–48]. The large scans were further analyzed by tissue-specific MATLAB scripts (version 2017b, The MathWorks, Inc.) developed for identification and automated quantification of staining density and intensity, as previously described by Gierman et al. [13] and Silva et al. [16]. Protein expression was quantified in one large tissue section scan for each decidua and placenta with the examiner blinded to pregnancy outcome.

2.3.1. Quantitative decidual protein expression

Decidual areas with smooth muscle tissue, placental tissue, blood vessels, endometrial glands and tissue with poor aberrant morphology were excluded by manually defining regions of disinterest. A mask of square patches (100 x 100 pixels, 1325 μm × 1325 μm) defining trophoblasts and maternal tissue without trophoblasts was created for each decidua based on CK7 positive staining. The created masks were used to relate NOD1 expression to trophoblasts and maternal tissue in the spatially aligned NOD1 tissue scans. The *decidual NOD1 expression density* was calculated as the total number of NOD1 positive pixels divided by the total amount of tissue pixels analyzed (pixels per patch x number of patches) to account for varying amounts of tissue between the samples. The *decidual NOD1 expression intensity* was calculated as the average intensity value of all positive patches using a color deconvolution algorithm based on DAB-specific RGB absorption [49]. NOD1 expression intensity was further analyzed according to the presence of trophoblasts in the decidua, ranging from 0% to 100% trophoblast coverage (based on the percentage of CK7 positive pixels). Trophoblasts were automatically counted, and the trophoblast density was calculated as the total number of trophoblasts divided by the total area of tissue (mm^2).

2.3.2. Quantitative placental protein expression

Placental areas with stem villi, decidual tissue and tissue with poor aberrant morphology were excluded by defining regions of disinterest. The images were analyzed by binary masks created by segmentation based on RGB color values using the color threshold app in MATLAB. The cells positively stained for NOD1 were used as reference points to create a mask selecting only NOD1 positive pixels, and the *NOD1 expression density* in placental villi was quantified as the total number of NOD1 positive pixels divided by the total number of tissue image pixels. The *NOD1 expression intensity* in placental villi was measured as the average intensity in a mask selecting only the tissue and not the intervillous space. Placental NOD1 expression intensity was further quantified and related to trophoblasts by creating a mask selecting the syncytiotrophoblast layer and underlying cytotrophoblasts by using the syncytiotrophoblast layer of each individual placenta as reference point. The NOD1 expression intensity values were measured as gray-level intensity values ranging from 0 (absence of color, black) to 255 (presence of all colors, white) after conversion from RGB to grayscale images. Staining intensity is therefore inversely proportional to the protein expression level, and the intensity values were subtracted from 255 to obtain a direct proportional relation to protein expression.

2.4. Decidual and placental explants

Third trimester placentas were processed within 2 h and 45 min after delivery. Healthy-looking cotyledons were dissected from the central region of the maternal side of the placenta. From these cotyledons; decidual tissue was isolated by removing the fetal membranes and placental villous tissue; and placental tissue was isolated by removing the fetal membranes and decidual tissue. The resulting tissue was washed in sterile phosphate-buffered saline and cut into pieces (explants) of similar weight (26.3 ± 5.0 mg (decidua) and 24.7 ± 4.6 mg (placenta)). Explants were cultured in Ham's F12/Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 100 mg/mL penicillin-streptomycin (Sigma-Aldrich) and incubated for 24 h at 37 °C, 8% O_2 and 5% CO_2 [50]. Culture medium was then replaced with fresh culture medium with or without iE-DAP (NOD1-agonist, 10 μg/mL, #tlrl-dap, InvivoGen) or iE-lys (NOD1-antagonist, 10 μg/mL, #tlrl-lys, InvivoGen). Supernatants were collected after 24 h, centrifuged and stored at −80 °C. For further analysis, six technical replicates for each experimental condition were combined. Tissue viability was assessed by lactate dehydrogenase cytotoxicity assay (#04744926001, Roche), confirming that the stimuli had no toxic effect. Interleukin (IL)-6 and IL-8 in supernatants from decidual and placental explants (diluted 1:500 and 1:1000, respectively) and interferon-γ-inducible protein-10 (IP-10) from placental explants (diluted 1:10) were measured in duplicate using quantitative sandwich ELISA (#DY206, #DY208, #DY266, R&D Systems).

2.5. Statistical analyses

Statistical analyses were performed in SPSS (IBM SPSS Statistics 25) and GraphPad Prism (Prism8). For clinical data, one-way ANOVA or Kruskal-Wallis with Tukey's or Dunn's test, respectively, were used for comparisons of continuous variables, and Fisher's exact test for categorical variables. Protein measurements in supernatants were analyzed by one-way ANOVA with Dunnet's test or Kruskal-Wallis with Dunn's test. For the immunohistochemistry data, the amount and density of decidual trophoblasts were compared between study groups by one-way ANOVA with Tukey's test for pairwise comparisons. The overall protein expression of NOD1 in decidua and placenta was evaluated by a linear regression model with recruitment location and study group as additional covariates. For normal pregnancies, NOD1 expression in placental villi was compared to the NOD1 expression in the syncytiotrophoblast and cytotrophoblasts by a linear mixed model with expression location as fixed effect variable. In all linear mixed models, within-subject correlations were accounted for by including a subject-specific random intercept. To analyze decidual NOD1 expression levels according to the number of trophoblasts, average intensity values for each patch were included in the statistical analyses. Trophoblast coverage were either included as average percentage for each patch, ranging from 0% to 100% (continuous variable) or grouped into intervals of 0%, >0–50% and >50% trophoblast density (categorical variable). To compare NOD1 expression in maternal tissue (0% trophoblasts) and trophoblast-containing tissue (>0–50 or >50% trophoblasts), a linear mixed model with recruitment location and trophoblast density interval as fixed effects variables was used. NOD1 expression levels were further compared between the study groups using a linear mixed model with recruitment location, study group and the interaction between study group and percentage of trophoblast coverage and the percentage of trophoblast coverage in each patch implemented as fixed effects variables. The significance level was set to 0.05.

3. Results

3.1. Clinical characteristics of study subjects

Of the 122 pregnancies included from the Preeclampsia Study, two decidual and five placental samples were excluded due to poor tissue morphology or methodological problems (immunohistochemical staining or image analysis). The remaining 119 pregnancies were included with decidual (n = 90) and placental (n = 91) samples, some of which had both tissue types (n = 62). The decidual analyses included women with normal pregnancies (n = 44), preeclampsia without FGR (n = 19) and preeclampsia with FGR (n = 27); the placental analyses included women with normal pregnancies (n = 43), preeclampsia without FGR (n = 22) and preeclampsia with FGR (n = 26); and the combined decidual

and placental analyses included women with normal pregnancies (n = 29), preeclampsia without FGR (n = 16) and preeclampsia with FGR (n = 17). As expected, women who developed preeclampsia had higher systolic and diastolic blood pressure, more were primiparas, and their infants were delivered at earlier gestation with lower placenta and birth weights when compared to normal pregnancies (Table 1). The gestational age at delivery as well as placenta and birth weights were lower in preeclamptic pregnancies complicated with FGR compared to preeclamptic pregnancies without FGR (Table 1).

Tissue from nine normal third trimester pregnancies with gestational age range 38–41 weeks was used for isolation of either decidual or placental explants.

3.2. Cellular composition at the maternal-fetal interface

The decidual tissue consisted of decidualized endometrial stroma cells, maternal leukocytes (CD45+) and extravillous trophoblasts (CK7+), as well as smooth muscle tissue (smooth muscle actin+) and extracellular matrix components (Fig. 1A–F). Trophoblasts were clustered together surrounded by fibrinoid tissue or appeared as single cells closely connected to maternal leukocytes and decidual stroma cells. Both mononucleated extravillous trophoblasts and multinucleated trophoblast giant cells were identified (Fig. 1A–F and [13]). The placental sections showed numerous villous structures, predominantly mature intermediate and terminal villi, but also some larger stem villi and a few immature villi (Fig. 1G-L). All villous structures were covered by the multinucleated syncytiotrophoblast layer and underlying cytotrophoblast cells (CK7+). The core of the villi contained fetal endothelial cells (CD31+), fibroblasts and leukocytes (CD45+) (Fig. 1G-L).

Table 1
Clinical characteristics of subjects included in third trimester decidual and placental analyses (n = 119).

	Normal pregnancies (n = 58)	PE[1] without FGR (n = 25)	PE with FGR (n = 36)
Baseline characteristics			
Maternal age, years	31.4 (±5.3)	29.8 (±4.9)	30.4 (±5.7)
Primiparas, n (%)[a]	11 (19)	15 (60)*	19 (53)*
BMI[b]	24.9 (±4.0)	26.4 (±6.1)	26.4 (±4.6)
Characteristics at time of delivery			
Systolic BP, mmHg[c]	119.3 (±11.1)	166.3 (±19.3)*	158.7 (±16.6)*
Diastolic BP, mmHg[c]	72.7 (±8.8)	101.6 (±12.3)*	98.8 (±9.2)*
Gestational age, weeks	38.6 (±0.8)	32.8 (±3.6) *	30.6 (±3.2) *,**
Severe PE[2], n (%)	n.a.	21 (84)	25 (69)
Early onset PE < 34 weeks, n (%)	n.a.	20 (80)	30 (83)
Placental weight, g[d]	610 (183)	420 (225)*	274 (100)*,**
Fetal birth weight, g	3490 (517)	2070 (820)*	1152 (698) *,**

PE; preeclampsia; FGR, fetal growth restriction; BMI, body mass index; BP, blood pressure; n.a., not applicable; n.s., not significant.
Continuous variables listed as means (±standard deviation) or median (interquartile range), assessed for differences between groups by one-way ANOVA with Tukey's post hoc test or Kruskal-Wallis with Dunn's post hoc test. Categorical variables listed as number (percent in column), assessed for differences between groups by Fisher's exact test.
*$P < 0.05$ vs normal pregnancies. **$P < 0.05$ vs PE without FGR.
[1] Six of the women included as preeclamptic were diagnosed with superimposed PE. [2] PE was sub-phenotyped as severe if diagnosed with one or more severe features [62].
[a] Information is missing from one woman.
[b] Maternal BMI in first trimester. Information is missing from four women.
[c] Blood pressure from last healthcare visit before delivery. Information is missing from two women.
[d] Information is missing from 13 women.

3.3. Decidual and placental expression and function of NOD1 in normal pregnancies

NOD1 was expressed by multiple cells of both fetal and maternal origin in the decidua (Fig. 1E). NOD1 was strongly expressed in the fetal mononucleated extravillous trophoblasts and multinucleated trophoblast giant cells. Maternal decidual stroma cells, leukocytes and smooth muscle cells showed a moderate expression of NOD1. When quantified according to fetal and maternal cells, the intensity of NOD1 expression increased with increasing presence of trophoblasts in the decidua (Fig. 2A). In the placenta, NOD1 was markedly expressed in the syncytiotrophoblast layer and cytotrophoblasts of placental villi (Fig. 1K) and a weaker expression of NOD1 was located to fetal leukocytes and around fetal vessels. The intensity of NOD1 expression was significantly higher in the syncytiotrophoblast layer and underlying cytotrophoblasts compared to in placental villi as a whole (Fig. 2B).

Explant stimulation confirmed NOD1 activity at the maternal-fetal interface. The NOD1-agonist iE-DAP induced a significantly increased release of IL-8, but not IL-6, in decidual explants (Fig. 3A and B). NOD1 activation of placental explants induced a significantly increased release of IL-6 and IL-8 (Fig. 3C and D), but not IP-10 (data not shown). Viability assessment confirmed that the stimuli had no toxic effects in any of the culture conditions (Fig. S1).

3.4. Overall NOD1 expression at the maternal-fetal interface comparing normal and preeclamptic pregnancies

The cellular localization of NOD1 was comparable and the amount and density of decidual trophoblasts did not differ between normal and preeclamptic pregnancies (data not shown). Quantification of the overall decidual NOD1 expression showed a significantly higher NOD1 density (Fig. 4A) ($P = 0.041$) and a tendency towards stronger NOD1 intensity (Fig. 4B) ($P = 0.079$) in preeclamptic pregnancies with normal fetal growth compared to normal pregnancies. The overall placental NOD1 expression did not differ between normal and preeclamptic pregnancies (Fig. 4C and D). Summary of the results are shown in Table S1 for decidua and Table S2 for placenta.

We further investigated whether decidual and placental NOD1 expression intensity was regulated in the same manner within each pregnancy. Interestingly, the decidual-placental correlation of NOD1 expression intensity diverged across the diagnostic groups. NOD1 showed a weak decidual-placental correlation in normal pregnancies (r = 0.4), whereas this correlation was markedly stronger in preeclamptic pregnancies with (r = 0.6) and without (r = 0.7) FGR.

3.5. Cellular NOD1 expression in the decidua comparing normal and preeclamptic pregnancies

NOD1 expression intensity in the heterogenous decidual tissue was further related to trophoblasts and maternal cells. Decidual NOD1 intensity markedly increased with increasing presence of trophoblasts both in normal and preeclamptic pregnancies (Fig. 5). In decidual tissue with high amounts of trophoblasts (>48%), NOD1 intensity was significantly higher in preeclamptic pregnancies with normal fetal growth compared to normal pregnancies (Fig. 5). The NOD1 expression intensity did not differ between normal and preeclamptic pregnancies in decidual tissue with only maternal cells (0% trophoblasts) (Fig. 5).

4. Discussion

This study demonstrated that NOD1 is strongly expressed by all types of trophoblasts across the maternal-fetal interface and moderately expressed by maternal cells in the decidua. Preeclamptic pregnancies showed stronger correlation between decidual and placental NOD1 expression, and preeclampsia with normal fetal growth was associated with a trophoblast-dependent increase in decidual NOD1 expression

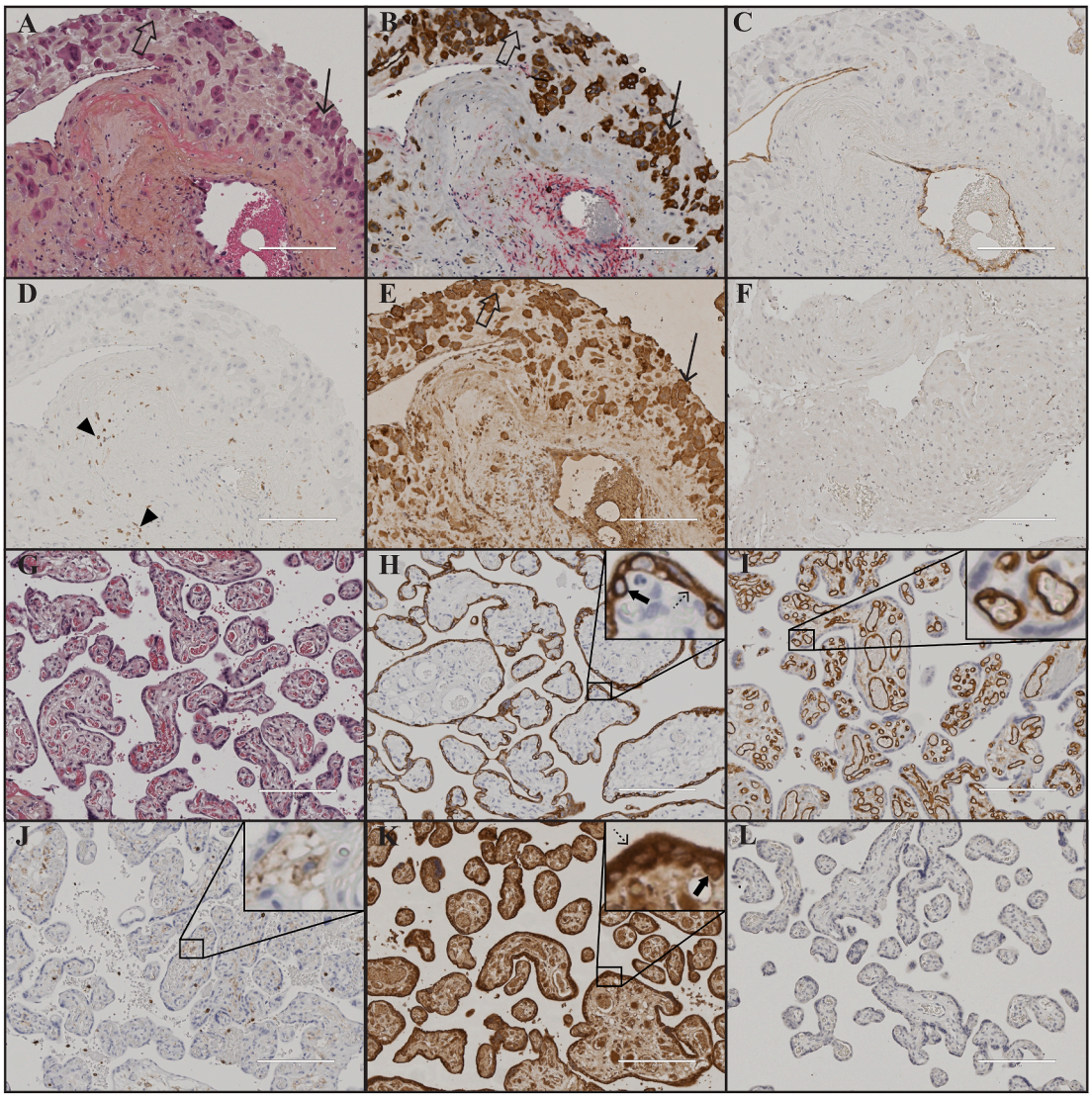

Fig. 1. Nucleotide-binding oligomerization domain-containing protein (NOD)1 protein expression and cell types in decidua (A–F) and placenta (G–L) at delivery. Representative images of decidual and placental tissue from normal pregnancies at gestational age 40 weeks and 39 weeks, respectively, are shown stained by (A,G) hematoxylin erythrosine saffron (HES), (B,H) the trophoblast marker cytokeratin 7 (CK7), (C,I) the endothelium marker CD31, (D,J) the leukocyte marker CD45, (E, K) NOD1, and (F,L) negative isotype control for NOD1. Black arrows indicate extravillous trophoblasts, transparent arrows indicate maternal decidual stroma cells, arrow heads indicate maternal leukocytes, dashed arrows indicate the syncytiotrophoblast layer and bold arrows indicate cytotrophoblasts. Scale bar 200 μM.

density. Functional NOD1 activity was confirmed by cytokine response in *in vitro* stimulated decidual and placental explants.

We present the first large and comprehensive study of cellular NOD1 expression across the two sites of maternal-fetal interaction at delivery. In accordance with our findings, NOD1 expression and functionality have been shown in separate and smaller studies and mainly located to the syncytiotrophoblast and cytotrophoblasts in placental villi [33,36, 38] and cells within the decidua [33,34]. Together with reports of first trimester NOD1 expression and activation in trophoblasts and placental villi [31,35,37] and in the decidua [31,32,39], our data suggest that NOD1 mediated inflammation plays a role at the maternal-fetal interface throughout pregnancy. The universal expression of NOD1 in all trophoblast types at the maternal-fetal interface shown here points to importance of NOD1 mediated inflammation in fetal cells with diverse biological functions, such as migration, tissue preservation and immunity. This further supports that NOD1 is involved in maternal-fetal interaction through the decidual changes in cellular phenotype and composition observed in the latter half of pregnancy [51,52], but the nature of these changes needs further investigation.

The NOD1 expression markedly increased with increasing aggregation of decidual trophoblasts and was particularly strong in the placental syncytiotrophoblast layer and underlying cytotrophoblasts. Supportive of our findings, first trimester studies show higher levels of NOD1 in isolated primary trophoblasts compared to placental tissue [35], and in placental villi compared to decidua [31]. The different trophoblast types are located at the sites of direct maternal-fetal interaction and the close proximity to maternal cells and associated danger signals may facilitate the strong NOD1 expression in trophoblasts.

Preeclampsia was associated with higher density of decidual NOD1 expression. This may represent one inflammatory mechanism contributing to the dysregulated immune activation at the maternal-fetal interface and the consecutive change in immune cell composition in decidua to the more inflammatory phenotype seen in preeclampsia [53, 54]. Supporting this is that potential NOD1 ligands are produced from ER stress and lipid deposits at the maternal-fetal interface, both processes that are at play in normal pregnancies [55,56] and elevated in preeclampsia [56,57]. Although the underlying placental pathology is partly shared in preeclampsia with or without FGR, and normotensive

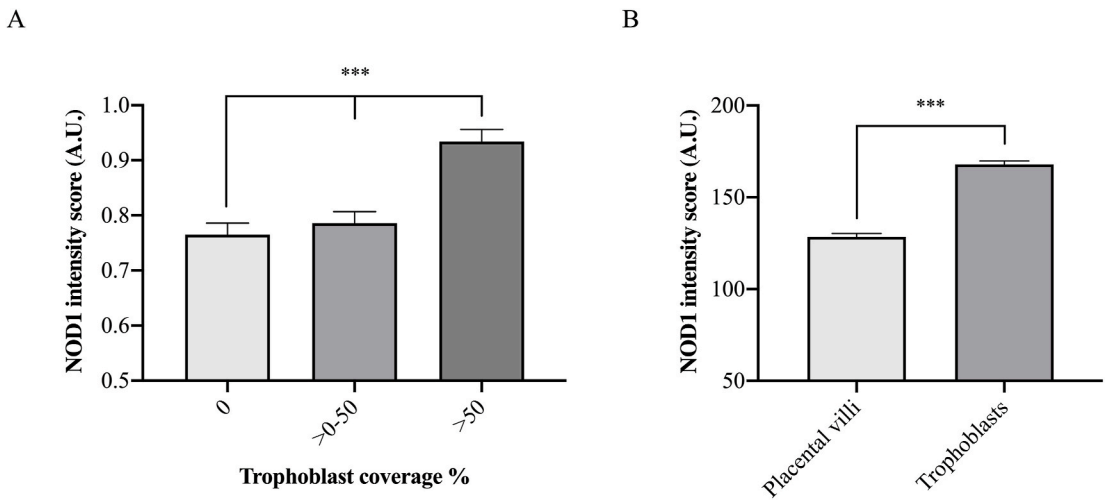

Fig. 2. Protein expression levels of Nucleotide-binding oligomerization domain-containing protein (NOD)1 in decidua and placenta at delivery in normal pregnancies. NOD1 expression intensity in normal pregnancies was quantified (A) according to the number of fetal trophoblasts (0%, >0–50% and >50% trophoblasts) in decidual tissue (n = 44), and (B) overall in placental villi and specifically in the placental syncytiotrophoblast layer and underlying cytotrophoblasts (n = 13). Data were analyzed by a linear mixed model and expression levels are shown as estimated means with standard error of mean. ***$P < 0.0001$. A.U., arbitrary units.

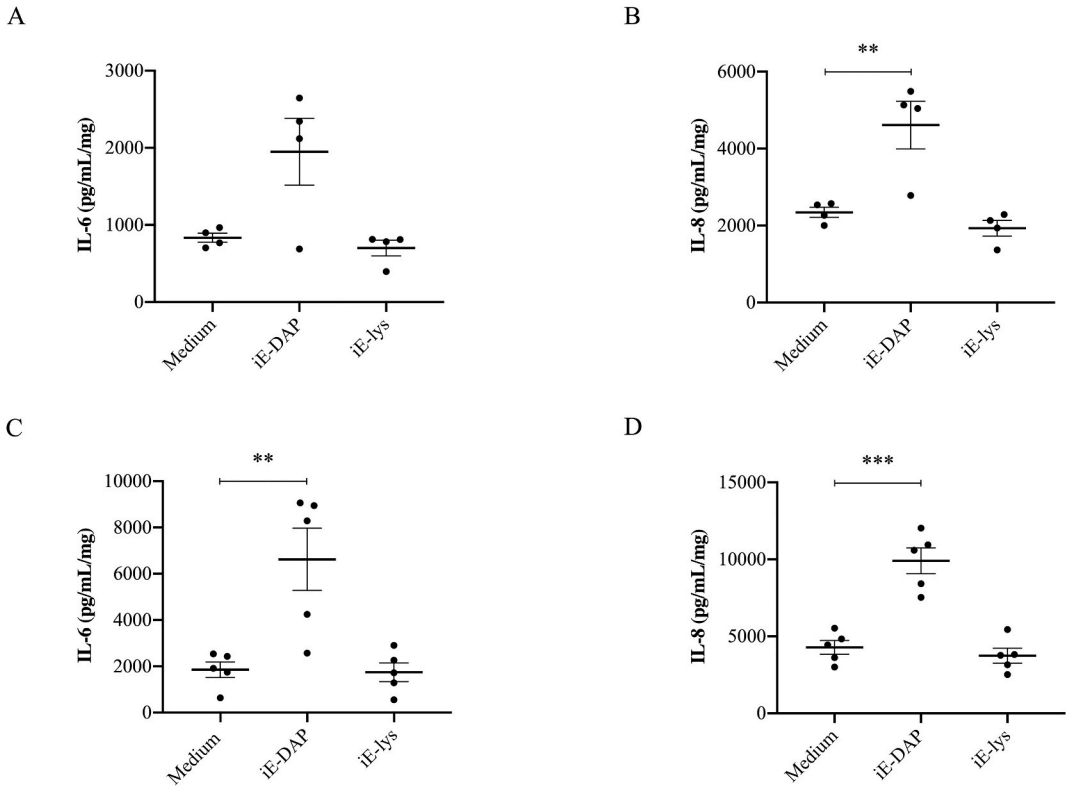

Fig. 3. Cytokine response following Nucleotide-binding oligomerization domain-containing protein (NOD)1 stimulation of decidual and placental explants. Explants from (A,B) decidua (n = 4) and (C,D) placenta (n = 5) from normal pregnancies at delivery were incubated for 24 h with or without iE-DAP (NOD1-agonist) or iE-lys (NOD1-antagonist). Six technical replicates were included for each experimental condition. Release of (A,C) interleukin (IL)-6 and (B,D) IL-8 was measured by ELISA and is presented as mean ± standard error of the mean relative to explant weight (mg). Data were analyzed using one-way ANOVA or Kruskal-Wallis with Dunnett's and Dunn's multiple comparison post-hoc test, respectively. **$P < 0.01$, ***$P < 0.001$.

pregnancies with FGR [58,59], the maternal systemic inflammatory activation is more vigorous in isolated preeclampsia [60,61]. The elevated NOD1 expression in the decidua from preeclamptic women without FGR may point to significant influences from the systemic maternal inflammatory state on the decidua and reflect a stronger maternal contribution to the disease. The pronounced systemic inflammation in preeclampsia may augment local inflammatory responses at the maternal-fetal interface and vice versa. This interactive inflammatory activation clearly supports the strong correlation found for decidual-placental regulation of NOD1 expression associated with preeclampsia. Our findings suggest that NOD1 mediated inflammation in normal pregnancies is regulated locally, while it in preeclampsia may be speculated that the inflammatory regulation at the two aligned sites of the maternal-fetal interface reflects a more widespread and coordinated

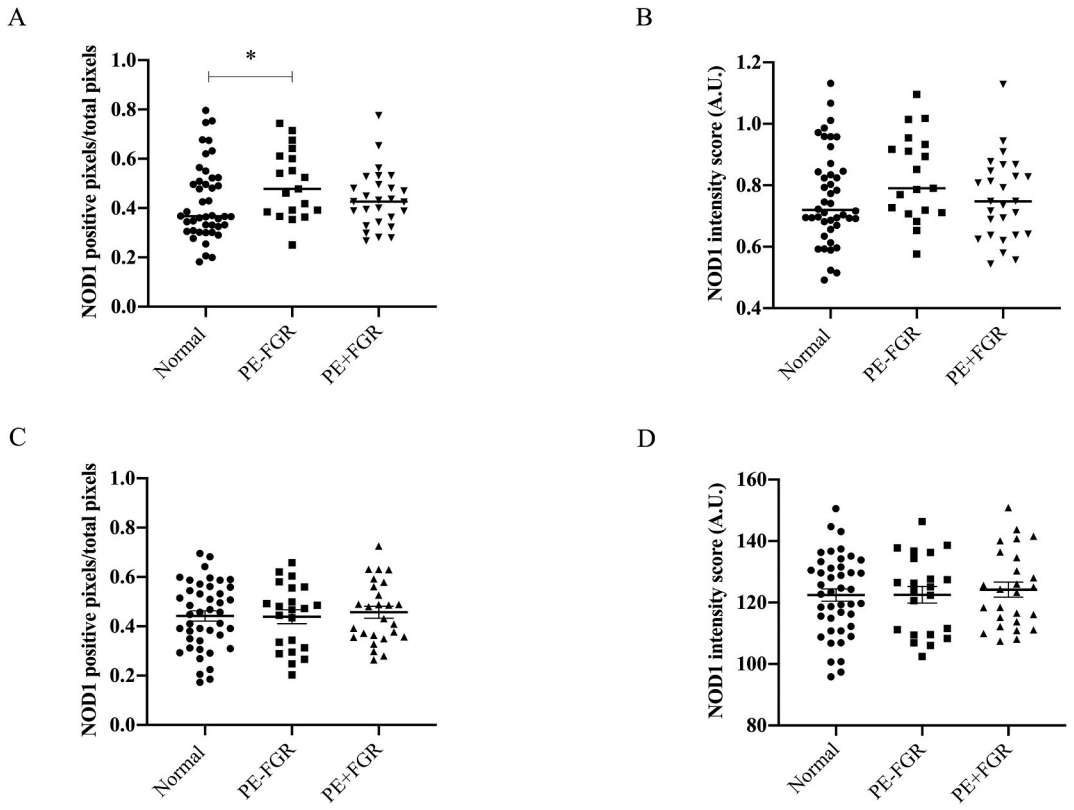

Fig. 4. Protein expression levels of Nucleotide-binding oligomerization domain-containing protein (NOD)1 in decidua and placenta at delivery in normal pregnancies and pregnancies with preeclampsia (PE) with or without fetal growth restriction (FGR). NOD1 expression was quantified as (A,C) NOD1 density and (B,D) NOD1 intensity in (A,B) decidual tissue from normal pregnancies (n = 44), PE without FGR (PE-FGR, n = 19) and PE with FGR (PE + FGR, n = 27), and (C,D) placental tissue from normal pregnancies (n = 43), PE-FGR (n = 22) and PE + FGR (n = 26). Data were analyzed by a linear regression model. *$P < 0.05$. A.U., arbitrary units.

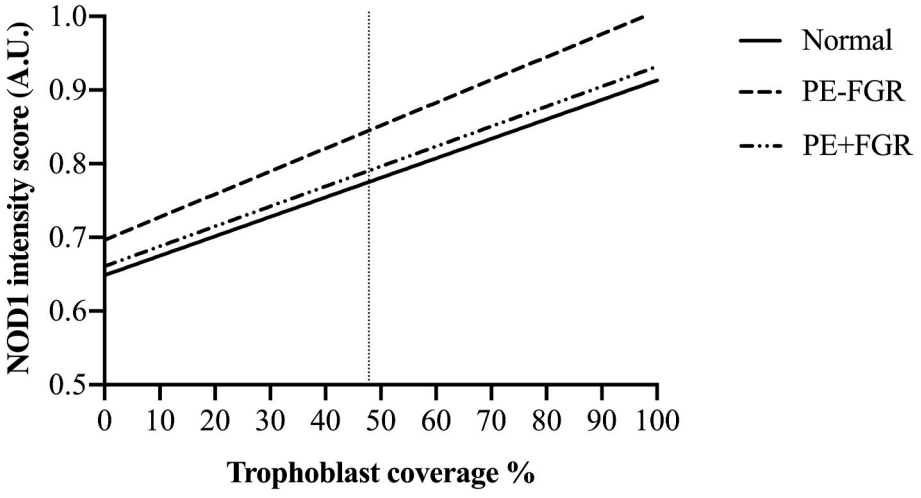

Fig. 5. Cellular expression of Nucleotide-binding oligomerization domain-containing protein (NOD)1 related to presence of fetal trophoblasts in decidua at delivery. NOD1 expression was quantified and related to presence of fetal trophoblasts in third trimester decidual tissue from normal pregnancies (n = 44), preeclampsia (PE) without fetal growth restriction (FGR) (PE-FGR, n = 19) and PE with FGR (PE + FGR, n = 27) as indicated. NOD1 expression was significantly increased in PE with normal fetal growth when compared to normal pregnancies for presence of fetal trophoblasts above 48% (vertical line). Data were analyzed by a linear mixed model. A.U., arbitrary units.

inflammation. This important finding results from studying more severe phenotypes of preeclampsia. Still, it can be expected that NOD1 mediated inflammation plays a possible coordinated systemic and local role at the maternal-fetal interface also in less severe preeclampsia phenotypes, but this needs to be further investigated. Our results clearly highlight the importance of combined understanding of pathological processes at the two interconnected sites of the maternal-fetal interface. The limitation of analyzing single tissue samples from the decidua and placenta was sought to be overcome by investigating large tissue scans by a novel automated cellular quantification method.

This study suggests that activation of NOD1 may be involved in the physiological inflammation occurring in pregnancy through mechanisms of cellular communication at the maternal-fetal interface. The universal expression of NOD1 by trophoblasts across the two sites of the maternal-fetal interface points to a particular role for NOD1 in the diverse trophoblast activities. The augmented expression of NOD1 in preeclampsia with normal fetal growth and the enhanced decidual-placental correlation of NOD1 expression in preeclampsia, suggest added involvement and regulation of NOD1 mediated inflammation at the maternal-fetal interface in preeclampsia. By combined assessment of

both sites of the maternal-fetal interface in normal pregnancies and in distinct subtypes of preeclampsia, this work contributes to the understanding of underlying inflammatory processes at the maternal-fetal interface and how these processes are interconnected with and influenced by the maternal systemic response. Such comprehensive approaches are needed to establish a solid fundament for biomarker selection and identification of treatment strategies for the complex pregnancy disorder preeclampsia.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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