


Placental inflammation in pre-eclampsia by Nod-like receptor protein (NLRP)3 inflammasome activation in trophoblasts

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Introduction

Increased inflammation, marked by changes in cytokines and C-reactive protein (CRP), is part of the physiological adaptation of a normal pregnancy [1,2]. Disturbance of this inflammatory state characterizes the development of pregnancy complications such as pre-eclampsia and fetal growth restriction (FGR), which are major causes of maternal and fetal morbidity and mortality [2–4]. Pre-eclampsia affects 2–8% of pregnancies and is associated with 12% of infants with FGR and approximately 20% of preterm deliveries [5]. Pre-eclampsia often starts with local placental dysfunction and develops into a maternal disease identified by maternal hypertension and proteinuria. Insufficient placental development creates a

Summary

Pre-eclampsia is associated with increased levels of cholesterol and uric acid and an inflamed placenta expressing danger-sensing pattern recognition receptors (PRRs). Crystalline cholesterol and uric acid activate the PRR Nod-like receptor protein (NLRP)3 inflammasome to release interleukin (IL)-1 β and result in vigorous inflammation. We aimed to characterize crystal-induced NLRP3 activation in placental inflammation and examine its role in pre-eclampsia. We confirmed that serum total cholesterol and uric acid were elevated in pre-eclamptic compared to healthy pregnancies and correlated positively to high sensitivity C-reactive protein (hsCRP) and the pre-eclampsia marker soluble fms-like tyrosine kinase-1 (sFlt-1). The NLRP3 inflammasome pathway components (NLRP3, caspase-1, IL-1 β) and priming factors [complement component 5a (C5a) and terminal complement complex (TCC)] were co-expressed by the syncytiotrophoblast layer which covers the placental surface and interacts with maternal blood. The expression of IL-1 β and TCC was increased significantly and C5a-positive regions in the syncytiotrophoblast layer appeared more frequent in pre-eclamptic compared to normal pregnancies. *In-vitro* activation of placental explants and trophoblasts confirmed NLRP3 inflammasome pathway functionality by complement-primed crystal-induced release of IL-1 β . This study confirms crystal-induced NLRP3 inflammasome activation located at the syncytiotrophoblast layer as a mechanism of placental inflammation and suggests contribution of enhanced NLRP3 activation to the harmful placental inflammation in pre-eclampsia.

Keywords: cholesterol, inflammation, NLRP3, placenta, pre-eclampsia

stressed and inflamed organ not able to meet the increasing demands of the growing fetus. The cellular interplay and underlying inflammatory mechanisms in the placenta are only partially understood. The main predictive marker for pre-eclampsia, soluble fms-like tyrosine kinase-1 (sFlt-1), is derived mainly from placental trophoblasts, and points to a central role for these specialized fetal cells in the pre-eclampsia pathogenesis [6,7]. This role is particularly important for the cytotrophoblasts, which fuse to form a multi-nucleated cell layer called the syncytium that covers the fetal structures in the placenta and interacts directly with maternal blood. This is the main maternal–fetal interaction site. Trophoblasts are immune-competent cells [8] and identification of their

inflammatory properties is required to understand the placental inflammation underlying the development of pre-eclampsia.

An inflammatory response can be initiated when danger signals from pathogens or damaged tissue are recognized by pattern recognition receptors (PRRs), and a cell's potential for inflammatory involvement is defined by its PRR repertoire. Endogenous danger signals initiate and maintain inflammatory responses through activation of PRRs such as nucleotide-binding domain leucine-rich repeat containing (NLR) proteins [9]. Members of the NLR protein family form intracellular multi-protein structures called inflammasomes [10], which are involved in inflammatory diseases such as atherosclerosis, gout and diabetes mellitus [11]. Activation of the Nod-like receptor protein (NLRP3) inflammasome by a wide variety of danger signals, including crystalline forms of cholesterol and uric acid [11–13], leads to activation of caspase-1 and cleavage of pro-interleukin (IL)-1 β to its biologically active form [10]. Mature IL-1 β has potent proinflammatory characteristics, and plays an important role in atherosclerosis development through NLRP3 activation [13,14]. Complement factors, cytokines or other danger signals are needed to induce a priming signal to render cells responsive to NLRP3 activation [15]. The complement system is involved in the pathology of several inflammatory diseases [16,17], and activation involves generation of the potent anaphylatoxin complement component 5a (C5a) and the terminal complement complex (TCC). Combined C5a and tumour necrosis factor (TNF)- α exposure is a known priming signal for cholesterol and uric acid crystal-induced NLRP3 activation [18,19].

Detailed characterization of placental expression and activation of PRRs is needed to reveal the underlying mechanisms and cellular contributions to placental inflammation in pre-eclampsia [20]. PRR activation in pre-eclampsia may be induced by factors derived from maternal serum and/or within the stressed placental tissue. It has been reported that maternal serum levels of cholesterol and uric acid are elevated in pre-eclampsia and FGR [21–28], that cholesterol is accumulated in pre-eclamptic placentas [29] and that uric acid crystals activate the NLRP3 inflammasome in human placentas [30]. Trophoblast NLRP3 activation has been assigned a role in pre-eclampsia development [31–34]. Raised serum and placental levels of IL-1 β and excessive complement activation in women with pre-eclampsia [3,28,31,35–39] further indicates a role for placental NLRP3 inflammasome involvement in this disease, but a detailed mechanistic outline with defined cellular involvement is missing. We hypothesize that complement-primed crystal-mediated activation of the NLRP3 inflammasome in trophoblasts leads to exaggerated IL-1 β production and contributes to placental inflammation in pre-eclampsia.

Materials and methods

Study population

This study used placental and maternal serum samples from two collections approved by the Norwegian Regional Committee for Medical and Health Research Ethics; approval number 2012/1040 and 2009/03. All participants signed informed consent.

Third-trimester placental biopsies from healthy ($n = 13$) and pre-eclamptic singleton pregnancies with ($n = 12$) or without ($n = 11$) FGR, and maternal serum samples from healthy ($n = 43$) and pre-eclamptic ($n = 34$) pregnancies, were analysed. Serum samples from non-pregnant women in the follicular phase of the menstrual cycle ($n = 28$) were also included. Pregnant women were diagnosed with pre-eclampsia when developing persistent hypertension (blood pressure $\geq 140/90$ mmHg) plus proteinuria (≥ 0.3 g/24 h or reproducible $\geq 1+$ by dipstick) after 20 weeks of gestation [40]. FGR was established by serial ultrasound measurements or diagnosed if neonates had birth weight < 5 th percentile, according to Norwegian reference curves [41]. Healthy normotensive pregnant women with no previous pre-eclampsia or FGR were included as controls. Maternal venous blood was collected prior to delivery and placentas obtained directly after delivery by caesarean section without labour. Serum aliquots were stored at -80°C , and placental biopsies were cut tangentially from the central part of the maternal side before fixation in 10% neutral-buffered formalin and paraffin embedding.

First-trimester placental tissue was collected from surgical elective abortions at gestational age 7–12 weeks. Placental samples ($n = 10$) were snap-frozen and stored at -80°C until fixation and paraffin-embedding. A group of third-trimester placentas ($n = 10$) were collected from healthy women delivering by caesarean section for immediate isolation of chorionic villous explants.

Serum measurements

Total cholesterol and uric acid were measured in maternal serum. Esterified cholesterol was modified enzymatically by cholesterol esterase and cholesterol oxidase. The resulting hydrogen peroxide reacted with 4-aminoantipyrene and phenol in presence of peroxidase and the coloured product was measured at 505 nm. Uric acid was oxidized by uricase, and the resulting hydrogen peroxide reacted with N-ethyl-N-(2-hydroxy-3-sulphopropyl)-3-methylalanine and 4-aminopyrene in the presence of peroxidase, and the coloured product was measured at 545 nm. High-sensitivity (hs) CRP was measured by turbidimetric assay and measured at 571 nm. All analyses were performed according to clinically validated standards on a Roche Modular P analytical system at the Department of Clinical Chemistry at St Olav's Hospital, Trondheim, Norway. sFlt-1 was measured by enzyme-linked immunosorbent assay (ELISA) (no. DVR100B; R&D Systems, Minneapolis, MA, USA).

Immunohistochemical staining and quantification

Tissue sections (3 μm) were pretreated in PT link (no. PT101; Dako, Glostrup, Denmark) using target retrieval solution (no. K8005 or K8004; Dako) at 97°C for 20 min and peroxidase blocking solution for 5 min (no. K4007; Dako). Slides were incubated overnight at 4°C [IL-1 β (1 : 200, no. NB600–633; Novus Biologicals, Littleton, CO, USA), complement component 5a (C5a) (1 : 10, no. HM2079; Hycult Biotech, Plymouth Meeting, PA, USA) and terminal complement complex (TCC) (1 : 30, no. DIA011-01-02; Thermo Fisher, Waltham, MA, USA)]; or for 40 min at room temperature [NLRP3 (1 : 2000, no. 19771-1-AP; Proteintech, Chicago, IL, USA), caspase-1 (1 : 1200, no. Ab108362; Abcam, Cambridge, MA, USA), cytokeratin 7 (CK7) (1 : 800, no. M7018; Dako), CD31 (1 : 50, no. M0823; Dako) or CD45 (1 : 300, no. M0701; Dako)]. All slides were incubated for 30 min with horseradish peroxidase (HRP)-labelled polymer (no. K4007; Dako). Diaminobenzidine (DAB+) (1 : 50, no. K4007; Dako) was used as chromogen with two 5-min incubations and slides counterstained with haematoxylin. Rabbit linker (no. K8009; Dako) was used for NLRP3 staining and mouse linker (no. K8021; Dako) for cell markers.

Immunohistochemistry was performed in an Autostainer Plus (no. S3800; Dako). Negative isotype control staining was performed for all antibodies using normal rabbit serum (no. 011-000-120; Jackson ImmunoResearch, West Grove, PA, USA), rabbit monoclonal immunoglobulin (Ig)G (no. 3900; Cell Signaling Technology, Danvers, MA, USA), mouse monoclonal IgG1 (no. 349040; BD Biosciences, Franklin Lakes, MA, USA) and mouse monoclonal IgG2a κ (no. 553454; BD Pharmingen) (Supporting information, Fig. S1). Bright-field images were obtained with an Eclipse E400 microscope and DS-Fi1 camera (Nikon, Melville, NY, USA) or the EVOSTM FL Auto Imaging System (Invitrogen, Carlsbad, CA, USA), with defined microscope settings. For quantitative image analysis, the staining intensity of the syncytiotrophoblast cytoplasm was assessed automatically using the NIS-Elements BR 4.0 software (Nikon). The syncytiotrophoblast cytoplasm was delineated in a binary layer by manual adjustment. This binary layer defined the area included in automatic assessment of intensity values (the statistical mean of intensity pixel values). Protein expression was quantified blinded to pregnancy outcomes. IL-1 β and NLRP3 expression was quantified in three images per placenta from 13 healthy and 23 pre-eclamptic pregnancies, while TCC quantification was performed in two images from four healthy and four pre-eclamptic placentas.

Placental explants and trophoblasts

A cotyledon was dissected from the central region of fresh third-trimester placentas, fetal membranes and decidua basalis were removed, and chorionic villous tissue washed in sterile phosphate-buffered saline (PBS) and cut into

pieces (24.0 ± 1.3 mg). Explants were cultured in Ham's F12/Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 100 mg/ml penicillin–streptomycin (Sigma-Aldrich, St Louis, MO, USA) and incubated overnight at 37°C, 8% O₂ and 5% CO₂ [42]. The trophoblast cell line SGHPL-5, generously provided by Professor Whitley (London, UK) [43], was cultured in Ham's F12 medium with 10% FBS, 2 mM L-glutamine and 100 mg/ml penicillin–streptomycin. SGHPL-5 cells (passage 19–20) were seeded at 1×10^4 cells/well and incubated overnight at 37°C, 20% O₂ and 5% CO₂. Culture medium was then replaced by fresh culture medium with or without priming; either 500 pg/ml lipopolysaccharide (LPS) (no. tlr1-3pelps; InvivoGen, San Diego, CA, USA) or a combination of 1 $\mu\text{g}/\text{ml}$ C5a (no. hc2101; Hycult Biotech) and 10 ng/ml tumour necrosis factor (TNF)- α (no. ct3011; GIBCO, Carlsbad, CA, USA) [19]. After 2 h, the medium was replaced by fresh culture medium with or without stimuli; 200 or 2000 $\mu\text{g}/\text{ml}$ cholesterol crystals (no. C3045; Sigma-Aldrich), 100 or 200 $\mu\text{g}/\text{ml}$ uric acid crystals (no. tlr1-msu; InvivoGen) or the positive control 3 mM adenosine triphosphate (ATP) (no. A7699; Sigma Aldrich). 100 nM of the NLRP3 inflammasome inhibitor MCC950 (no. PZ0280; Sigma [44]) was added 30 min prior to the stimuli. Supernatants were collected after 24 h, centrifuged and stored at -80°C . Viability was assessed by the lactate dehydrogenase cytotoxicity assay (no. 04744926001; Roche, Basel, Switzerland).

IL-1 β levels in supernatant from third-trimester placental explants and SGHPL-5 cells were measured undiluted in duplicate using ELISA (no. 557953; BD Biosciences). For explants, six technical replicates for each experimental condition were fused before analysis.

Statistical analyses

Statistical analyses were performed in GraphPad Prism version 6.0 or SPSS version 21, with $P < 0.05$ considered statistically significant. Protein measurements in serum and supernatant were analysed by Kruskal–Wallis test with Dunn's multiple comparison *post-hoc* test, and quantified immunohistochemistry data by one-way analysis of variance (ANOVA) with Tukey's multiple comparison test or two-tailed Mann–Whitney *U*-test. Correlation between variables was calculated using the Spearman's rank test.

Results

Clinical characteristics of study subjects

The clinical characteristics of the 36 subjects included in protein expression analyses of third-trimester placentas are shown in Supporting information, Table S1. Among the pre-eclamptic pregnancies, 52% were additionally complicated by FGR and 70% experienced severe pre-eclampsia. In line

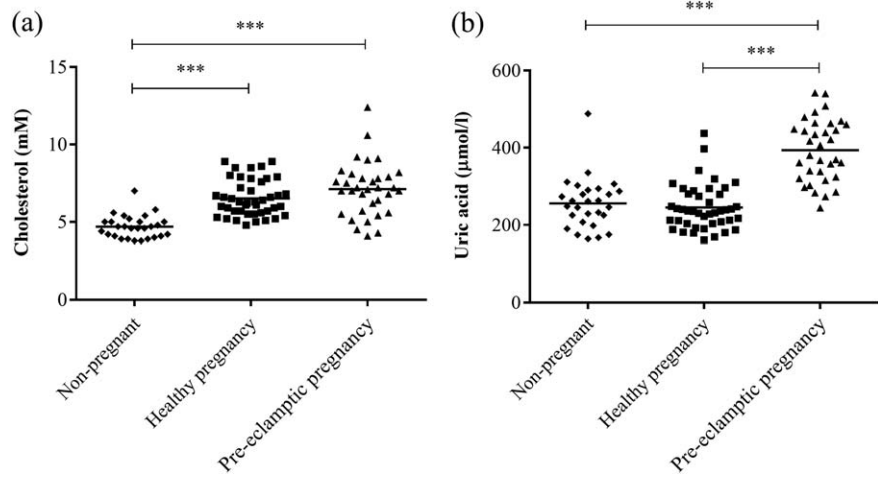


Fig. 1. Maternal serum levels of total cholesterol and uric acid. Circulating levels of (a) total cholesterol and (b) uric acid were measured in serum from non-pregnant ($n = 28$), healthy ($n = 43$) and pre-eclamptic ($n = 34$) pregnant women. Data were analysed using the Kruskal–Wallis test with Dunn’s multiple comparison *post-hoc* test. *** $P < 0.0001$.

with prior studies on the pre-eclampsia syndrome, the percentage of primiparae was higher and gestational age at delivery was lower in pre-eclamptic compared to healthy pregnant women (Supporting information, Table S1).

The clinical characteristics of the 105 subjects included for serum analyses are shown in Supporting information, Table S2. Among the pre-eclamptic pregnancies, 59% were additionally complicated by FGR and 79% had severe pre-eclampsia. The percentage of primiparae was higher and gestational age at delivery was lower in pre-eclamptic compared to healthy pregnant women (Supporting information, Table S2).

The average gestational age of the 10 first-trimester placentas was 10 (range = 7–12) weeks, and for the 10 healthy third-trimester placentas included for isolation of explants it was 39 (range = 38–40) weeks.

Maternal serum cholesterol and uric acid

Prior to delivery by caesarean section, maternal serum levels of total cholesterol were increased significantly in pre-eclamptic [7.1 mM (4.1–12.4), $P < 0.001$] and healthy pregnancies [6.5 mM (4.8–8.9), $P < 0.001$] compared to non-pregnant women [4.7 mM (3.8–7.0)] (Fig. 1a). Although the mean concentration was higher in pre-eclamptic than healthy pregnancies, the difference was not significant ($P = 0.0761$) (Fig. 1a).

Pre-eclamptic women had significantly higher uric acid levels [393.7 µmol/l (244.0–542.0)] compared to both healthy pregnant [245.2 µmol/l (161.0–437.0), $P < 0.001$] and non-pregnant women [255.5 µmol/l (164.0–488.0), $P < 0.001$] (Fig. 1b).

Both maternal serum levels of total cholesterol and uric acid correlated positively with maternal serum levels of sFlt-1 ($P < 0.0001$, Spearman’s $r = 0.58$; $P < 0.0001$, Spearman’s $r = 0.59$) and hsCRP ($P = 0.004$, Spearman’s $r = 0.28$; $P < 0.0001$, Spearman’s $r = 0.37$). For serum concentrations of sFlt-1 and hsCRP, see Supporting information, Table S2.

Placental expression of inflammasome pathway components and complement factors

Immunohistochemical staining of placental sections with CK7 identified the syncytiotrophoblast layer (the syncytium) and cytotrophoblasts (Fig. 2b). Within the placental villi, endothelial cells (Fig. 2c) and leucocytes (Fig. 2d) were identified.

In third-trimester placentas NLRP3, caspase-1 and IL-1 β were expressed in the syncytiotrophoblast layer, cytotrophoblasts, fetal endothelium and stromal leucocytes (Fig. 3), with similar staining patterns in healthy and pre-eclamptic placentas. A more distinct staining of NLRP3 was observed towards the apical side of the syncytiotrophoblast layer (Fig. 3a,b).

Complement factors TCC and C5a were observed in the syncytiotrophoblast layer and leucocytes of both healthy and pre-eclamptic placentas (Fig. 4). The C5a staining was apparent in distinct syncytium regions and appeared stronger in placentas from pre-eclamptic pregnancies (Fig. 4c,d).

In first-trimester placentas, NLRP3 and IL-1 β were expressed predominantly in the syncytiotrophoblast layer and cytotrophoblasts, but also in stromal cells of various sizes (Supporting information, Fig. S2).

Quantified NLRP3, IL-1 β and TCC expression levels in normal and pre-eclamptic placentas

No differences were detected in NLRP3 expression levels in the syncytiotrophoblast layer (Fig. 5a) or cytotrophoblasts (data not shown) when comparing healthy pregnancies with pregnancies complicated with pre-eclampsia alone or combined with FGR.

Quantification of IL-1 β staining revealed that the syncytium IL-1 β expression was significantly higher in pregnancies complicated with pre-eclampsia and FGR compared to healthy pregnancies [76 arbitrary units (AU) ± 3 versus 88 AU ± 2 , $P = 0.0174$] (Fig. 5b). Statistical significance was not reached when comparing pre-eclamptic pregnancies without FGR to healthy pregnancies (86 AU ± 3 versus

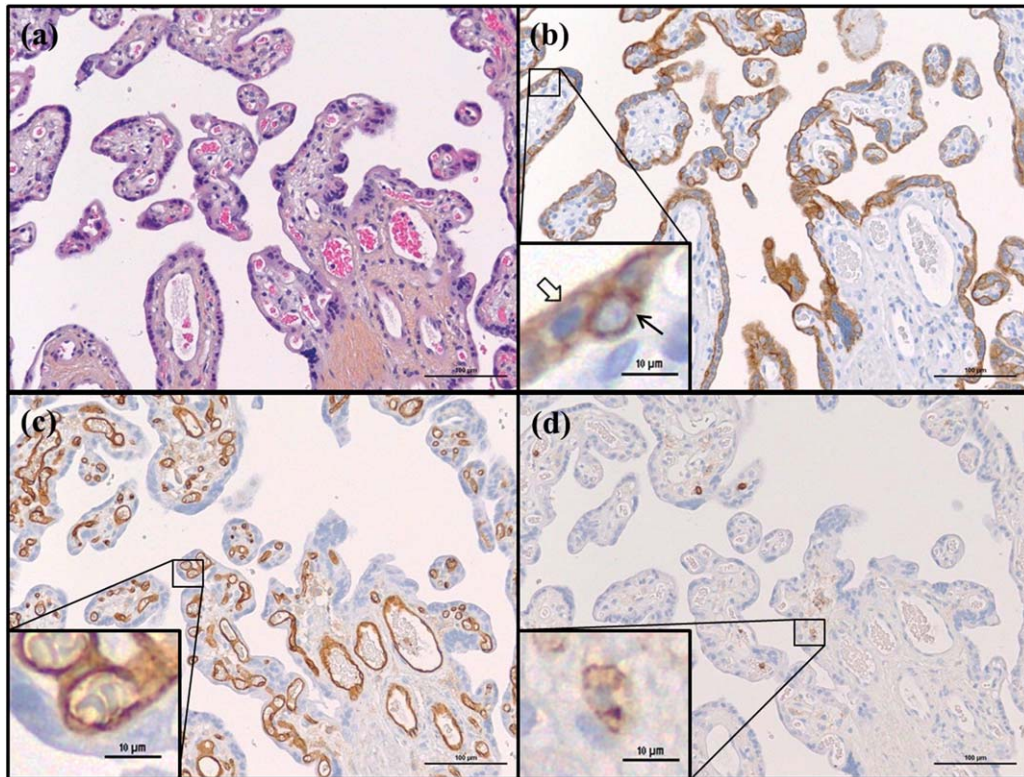


Fig. 2. Haematoxylin erythrosine saffron (HES) staining and cell type markers in third trimester placenta. Representative images of healthy placental tissue at gestational age 38 weeks are shown stained by (a) HES and immunohistochemical staining of (b) the trophoblast marker cytokeratin 7 (CK7), (c) the endothelium marker CD31 and (d) the leucocyte marker CD45. Black arrow indicates cytotrophoblast, transparent arrow indicates syncytiotrophoblast.

88 AU \pm 2, $P = 0.52$) (Fig. 5b), or when comparing severe to non-severe pre-eclamptic pregnancies (not shown).

The scattered C5a expression pattern was not quantified, but the syncytium TCC staining was significantly higher in pre-eclamptic compared to healthy pregnancies (94 AU \pm 3 versus 107 AU \pm 3, $P = 0.0286$) (Fig. 5c).

Cholesterol crystal-mediated IL-1 β response in placental explants

Incubation with cholesterol crystals (Fig. 6) and uric acid crystals (data not shown) significantly increased the release of IL-1 β from LPS-primed cultured chorionic villi explants. Inhibition of the NLRP3 inflammasome by MCC950 reduced the cholesterol crystal-induced IL-1 β response in placental tissue more than twofold, demonstrating NLRP3 inflammasome dependence (Fig. 6).

C5a and TNF- α priming of cholesterol crystal-mediated NLRP3 activation in placental explants and trophoblasts

When priming cultured chorionic villi explants with C5a and TNF- α , the cells responded to cholesterol crystals and ATP by significantly increased IL-1 β production (Fig. 7).

When priming SGHPL-5 trophoblasts with C5a and TNF- α , the cells responded to cholesterol crystals by releasing significantly higher levels of IL-1 β (Fig. 8). The IL-1 β response in trophoblasts was confirmed to be NLRP3-dependent by a threefold reduction in IL-1 β production when inhibiting NLRP3 activity (Fig. 8).

Lactate dehydrogenase viability analyses confirmed that the stimuli had no toxic effect on placental explants or trophoblast cells (Supporting information, Figs S3 and S4).

Discussion

In this study, we have identified that components of the NLRP3 inflammasome and the complement system are co-localized with IL-1 β in the syncytiotrophoblast layer. We demonstrate elevated syncytium expression of IL-1 β and TCC and raised maternal serum cholesterol and uric acid in pre-eclampsia. When primed with C5a/TNF- α or LPS, placental explants and trophoblasts became responsive to cholesterol crystal-mediated activation of the NLRP3 inflammasome, resulting in release of mature IL-1 β . Together, these data suggest that crystal-mediated NLRP3 inflammasome activation leads to IL-1 β production in the syncytium and that this potent inflammatory mechanism

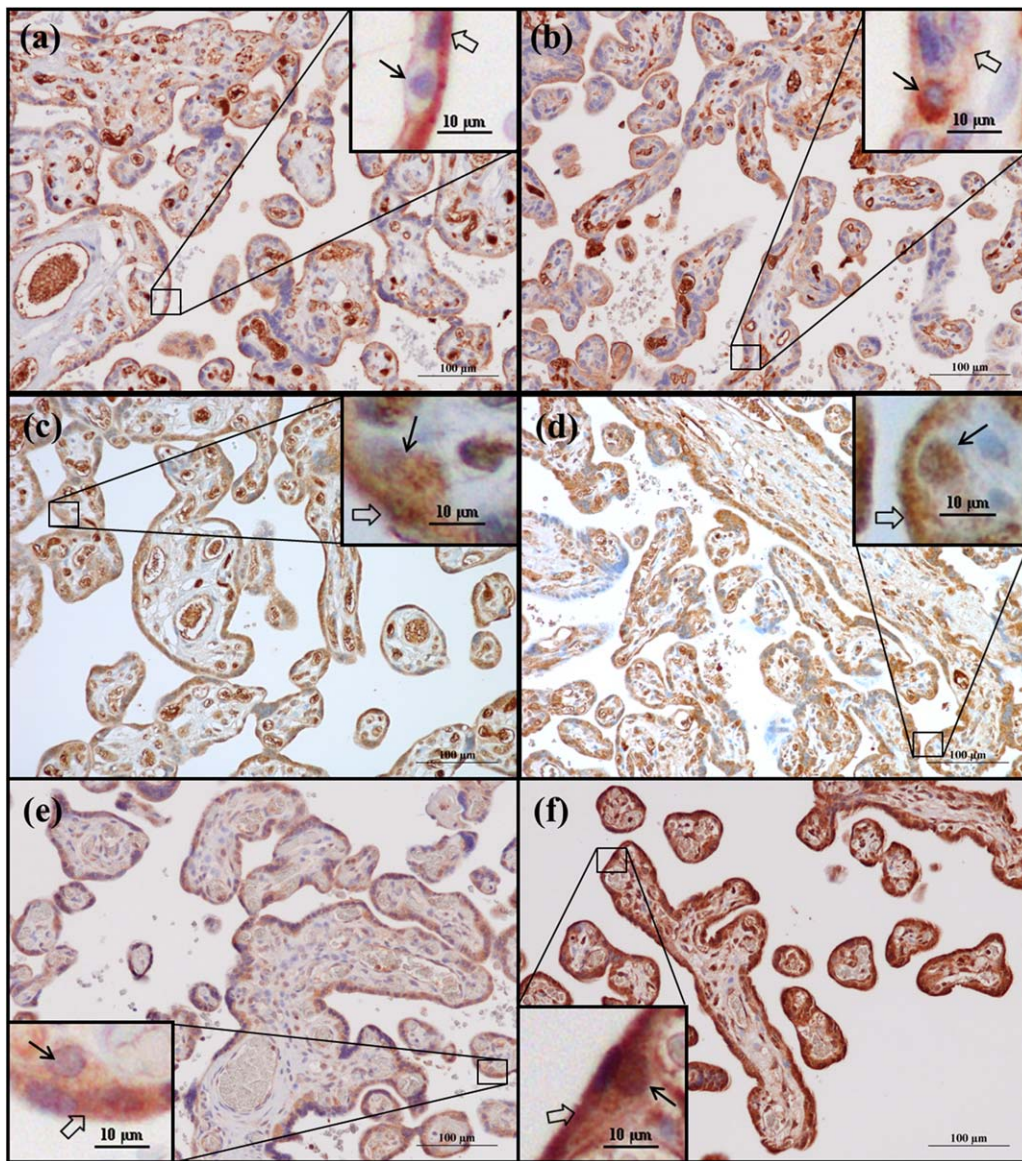


Fig. 3. Nod-like receptor protein 3 (NLRP3), caspase-1 and interleukin (IL)-1 β protein expression in third-trimester placenta. Immunohistochemical staining of (a,b) Nod-like receptor protein 3 (NLRP3), (c,d) caspase-1 and (e,f) interleukin (IL)-1 β . Representative images of healthy placentas at gestational age (a,c) 40 + 0 and (e) 39 + 0 weeks, and pre-eclamptic placentas at gestational age (b,d) 33 + 6 and (f) 33 + 5 weeks are shown. Black arrows indicate cytotrophoblasts, transparent arrows indicate syncytiotrophoblast.

plays a role in the harmful placental inflammation underlying pre-eclampsia.

The cell-specific co-localization of components essential for NLRP3 activity strongly supports a link between complement priming, NLRP3 inflammasome activation and IL-1 β production in the syncytiotrophoblast. Our findings are sustained in a recent report showing NLRP3, caspase-1 and IL-1 β protein expression in the syncytiotrophoblast layer [32] and by NLRP3 expression in placental lysates and primary trophoblasts [31,33,34,45,46]. Syncytium IL-1 β production in first- and third-trimester placentas is in accordance with previous findings [47–49]. NLRP3

expression in the syncytiotrophoblast layer in the first trimester of pregnancy has not been described previously, and also suggests a role for the NLRP3 inflammasome in the initial phase of placental development. Studies of the NLRP3 inflammasome pathway in pre-eclampsia are limited. Recently, Weel *et al.* [32] showed elevated NLRP3 and caspase-1 in placental tissue from pre-eclamptic pregnancies; however, our study is the first to quantify cell-specific NLRP3 protein expression in the syncytiotrophoblast layer. Our identification of elevated placental IL-1 β expression in pre-eclampsia is in accordance with previous findings [31,32,50]. Maternal serum IL-1 β in pre-eclampsia has

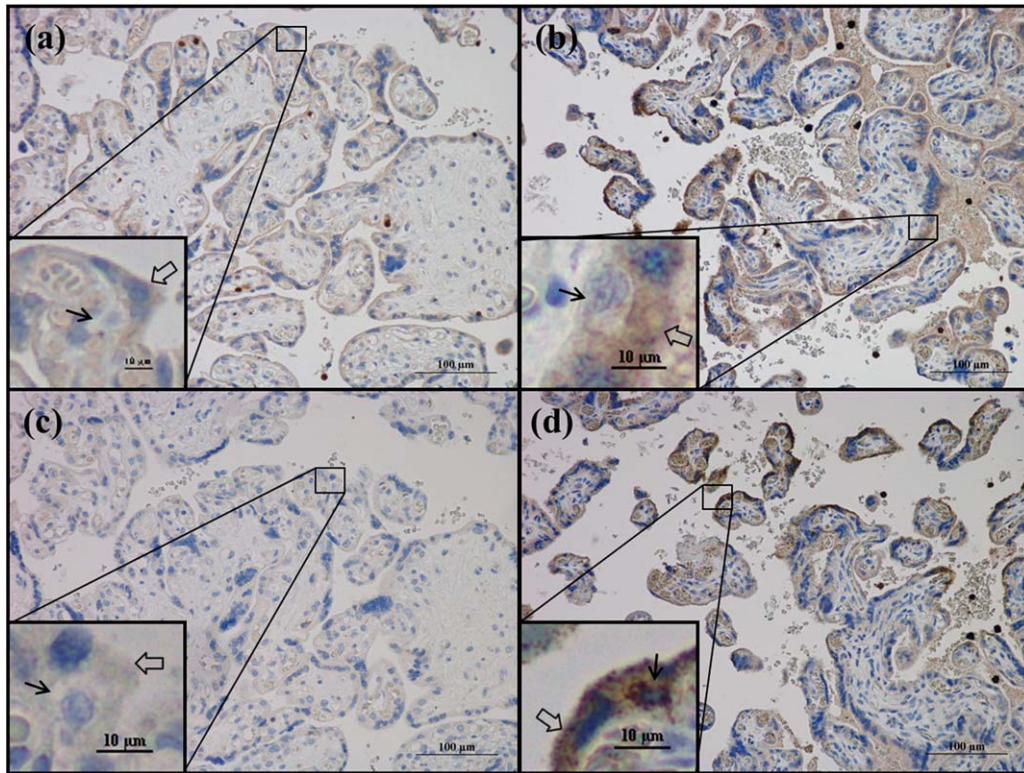


Fig. 4. Terminal complement complex (TCC) and complement component 5a (C5a) protein expression in third-trimester placenta. Immunohistochemical staining of (a,b) TCC and (c,d) C5a. Representative images of (a,c) a healthy placenta at gestational age 40 + 0 and (b,d) a pre-eclamptic placenta at gestational age 29 + 6 weeks are shown. Black arrows indicate cytotrophoblasts, transparent arrows indicate syncytiotrophoblast.

been suggested to originate from an inflamed placenta [50–52], and our findings point to NLRP3 activation at the syncytium as a probable source. To our knowledge, this is an original discovery and suggests a prominent role for inflammation located at the syncytiotrophoblast layer in the pre-eclampsia pathogenesis.

Our *in-vitro* studies in placental explants and trophoblasts confirmed functional activity of the NLRP3 inflammasome pathway. This is the first study demonstrating cholesterol crystal-mediated NLRP3 activation in trophoblasts, as inflammasome responses have only been shown previously for uric acid, ATP and nigericin in these cells [30,31,34,45,53]. NLRP3 inflammasome activation requires an already exaggerated inflammatory setting, as a priming signal is needed to achieve sufficient intracellular levels of NLRP3 and pro-IL-1 β [15,54]. The choice of priming signal in the placenta was based on reports of elevated C5a and TNF- α in pre-eclampsia [28,32,35–38,55–57], and supported by our discovery of C5a and TCC in the syncytium of pre-eclamptic placentas. The functional response confirms that C5a and TNF- α may serve as endogenous priming factors potentiating placental NLRP3 inflammasome activation. The low-grade inflammation in normal pregnancy may be sufficient for priming trophoblasts for

NLRP3 activation, leaving pregnancy particularly vulnerable for activation of this potent inflammatory mechanism.

NLRP3 activation in macrophages induces a potent inflammation culminating in cell death by pyroptosis [54]. Loss of surface integrity at the syncytium is part of the pathophysiological processes in pre-eclampsia development, with distorted microvilli and shedding of cell components to the maternal circulation evoking harmful maternal responses [58]. NLRP3 inflammasome activation may be responsible for parts of the destructive process observed at the syncytium in pre-eclampsia. Interestingly, the marked NLRP3 expression on the syncytium surface facing maternal blood may imply a contingent interaction between the inflamed syncytium and maternal blood. This localization of the inflammasome emphasizes that danger signals present in maternal blood, such as cholesterol and uric acid, have a strong likelihood to disrupt the homeostasis at this crucial fetal–maternal interaction site by activating NLRP3. Similar to earlier studies [22,26], maternal serum levels of total cholesterol and uric acid were shown elevated in pre-eclamptic women, and correlated with hsCRP and sFlt-1. Although cholesterol has been shown to accumulate in the placenta and decidua in pre-eclampsia and trophoblasts are involved in cholesterol uptake [29,59],

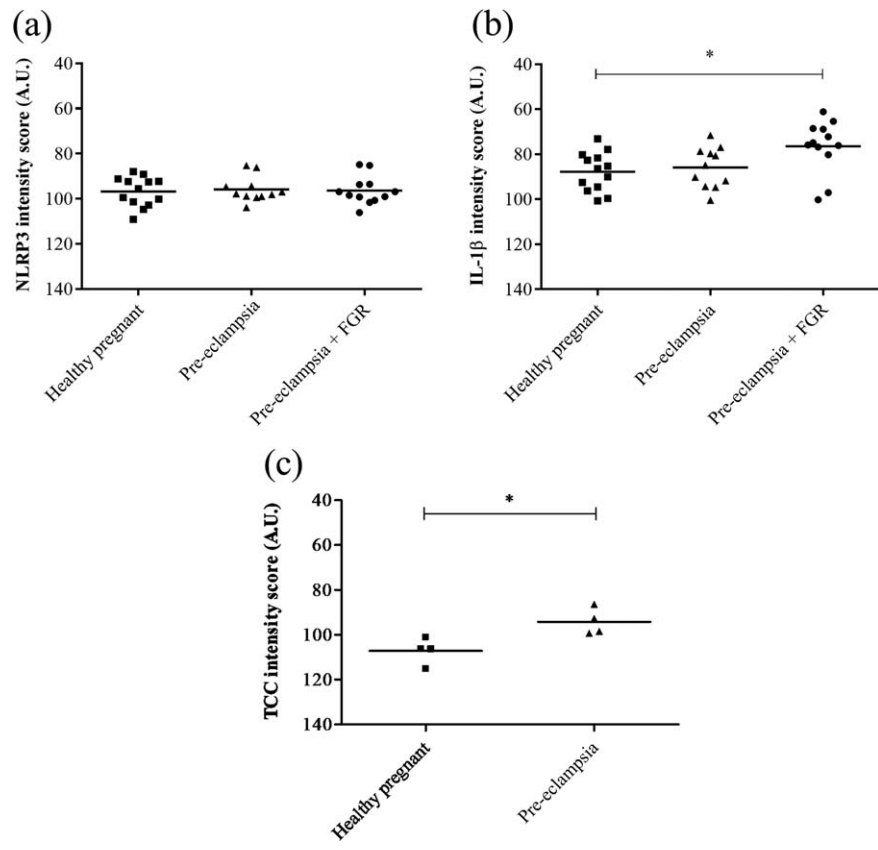


Fig. 5. Protein expression levels of Nod-like receptor protein 3 (NLRP3), interleukin (IL)-1 β and terminal complement complex (TCC) in third-trimester placenta. (a,b) For NLRP3 and IL-1 β the syncytiotrophoblast cytoplasmic staining was quantified in healthy ($n = 13$) and pre-eclamptic placentas without ($n = 11$) and with ($n = 12$) fetal growth restriction (FGR), and data were analysed using one-way analysis of variance (ANOVA) with Tukey's multiple comparison *post-hoc* test. (c) TCC expression level was quantified in the syncytiotrophoblast cytoplasm of healthy ($n = 4$) and pre-eclamptic placentas ($n = 4$), and data were analysed using the two-tailed Mann-Whitney test. * $P < 0.05$. AU = arbitrary units.

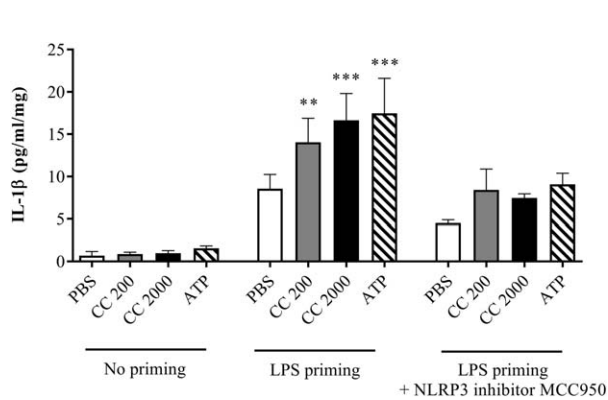


Fig. 6. Interleukin (IL)-1 β response following cholesterol crystal stimulation of lipopolysaccharide (LPS)-primed placental explants, and the effect of Nod-like receptor protein 3 (NLRP3) inhibition. Third-trimester chorionic villous explants from six healthy pregnancies were first incubated with or without LPS, before adding cholesterol crystals (CC, 200 or 2000 $\mu\text{g}/\text{ml}$) or adenosine triphosphate (ATP). Three biological replicates were also treated with the NLRP3 inflammasome inhibitor MCC950. Six technical replicates were included for each experimental condition. Release of IL-1 β was measured by enzyme-linked immunosorbent assay (ELISA), and is presented as mean \pm standard error of the mean (s.e.m.) relative to explant weight. Data were analysed using the Kruskal-Wallis test with Dunn's multiple comparison *post-hoc* test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Lactate dehydrogenase viability analysis showed no toxic effect on the placental cells (Supporting information, Fig. S3a). PBS = phosphate-buffered saline.

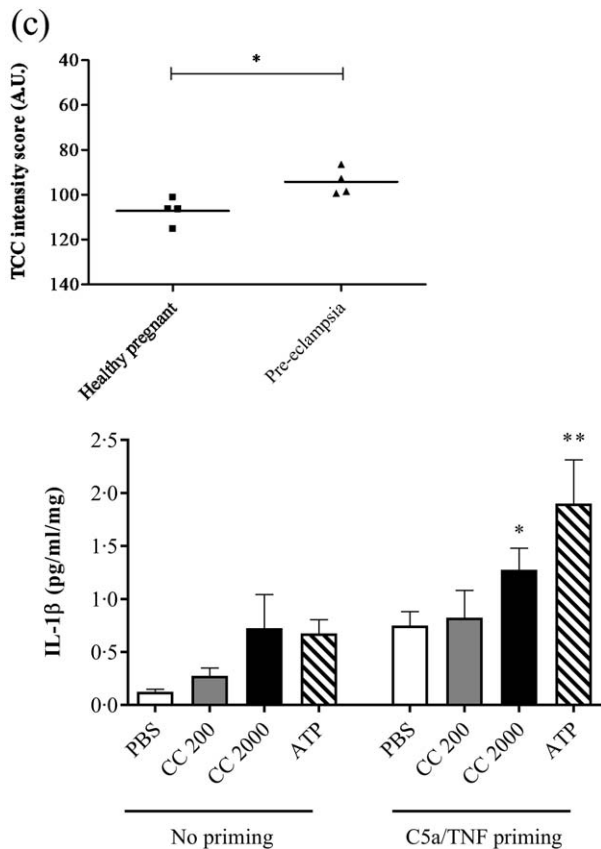


Fig. 7. Interleukin (IL)-1 β response following C5a/tumour necrosis factor (TNF)- α primed cholesterol crystal stimulation of placental explants. Third-trimester chorionic villous explants from four healthy pregnancies were incubated with or without complement component 5a (C5a) and TNF- α before stimulation with cholesterol crystals (CC, 200 or 2000 $\mu\text{g}/\text{ml}$) or adenosine triphosphate (ATP). Six technical replicates were included for each experimental condition. The level of IL-1 β was measured in supernatant using enzyme-linked immunosorbent assay (ELISA) and presented as mean \pm standard error of the mean (s.e.m.) relative to explant weight. Data were analysed using the Kruskal-Wallis test with Dunn's multiple comparison *post-hoc* test. * $P < 0.05$; ** $P < 0.01$. Lactate dehydrogenase viability analysis showed no toxic effect on the placental cells (Supporting information, Fig. S3b). PBS = phosphate-buffered saline.

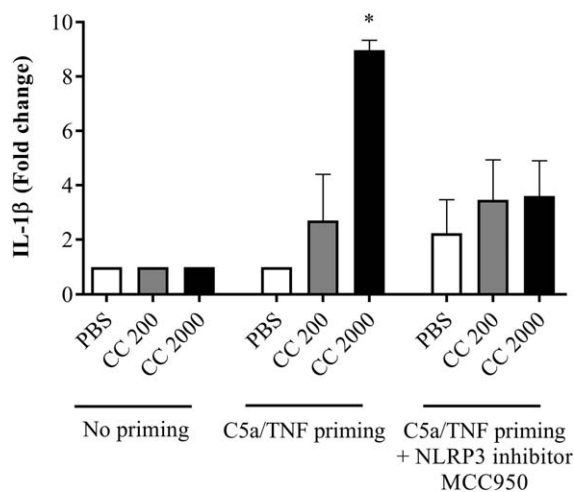


Fig. 8. Interleukin (IL)-1 β response following complement component 5a (C5a)/tumour necrosis factor (TNF)-primed cholesterol crystal stimulation of trophoblasts. The trophoblast cell line SGHPL-5 was incubated with or without C5a and TNF- α before stimulation with cholesterol crystals (CC, 200 or 2000 μ g/ml). Treatment with the Nod-like receptor protein 3 (NLRP3) inflammasome inhibitor MCC950 was included. IL-1 β release was measured by enzyme-linked immunosorbent assay (ELISA) and presented as fold change of triplicates from three independent experiments. Data were analysed using the Kruskal–Wallis test with Dunn's multiple comparison *post-hoc* test. * $P < 0.05$. Lactate dehydrogenase viability analysis showed no toxic effect on the cells (Supporting information, Fig. S4). PBS = phosphate-buffered saline.

crystallization of cholesterol in the placenta has not yet been confirmed, and needs to be investigated further. Taken together, these findings suggest that components at the syncytium surface and in maternal blood directly influence each other and may induce escalated inflammatory responses if disturbed or dysregulated. Consequently, placental dysfunction and maternal response in pre-eclampsia must be addressed as an interdependent interactive process.

Underlying placental pathology is partly shared in pre-eclampsia with restricted fetal growth, pre-eclampsia with normal fetal growth and normotensive FGR [57,60,61]. The maternal systemic inflammatory state and immune cell activation, however, is known to be more vigorous in isolated pre-eclampsia compared to normotensive FGR [57,62,63]. The data presented here suggest that further elucidation of placental NLRP3 activation in subgroups of pre-eclampsia and FGR will provide valuable knowledge about shared and distinct placental pathophysiological processes in these disorders.

To summarize, syncytial expression of the NLRP3 inflammasome pathway components and the *in-vitro* support of pathway functionality shown here strongly support an important role for the NLRP3 inflammasome in

placental inflammation and in the pathogenesis of pre-eclampsia. Initial activation by complement factors is likely to facilitate NLRP3 inflammasome responsiveness to crystalline cholesterol and uric acid and induce IL-1 β processing at the inflamed syncytium in pre-eclampsia. This suggests a novel inflammatory role for cholesterol at the maternal-fetal interface.

Author contributions

U. E. D., C. S., L. C. V. T. and L. B. collected the clinical material and information. G. S. S. and A. C. I. conceived and designed the experiments. G. S. S., G. B. S., L. H. T., L. M. G., I. N. and M. H. A. performed the experiments and analysed the data, and along with A. C. I. interpreted the data. G. S. S., G. B. S., L. B. and A. C. I. drafted the paper. All authors critically revised the article and approved the final version.

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Disclosure

The authors have no financial or commercial conflicts of interest.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Isotype controls for immunohistochemical staining protocols. Third-trimester placentas were stained with isotype controls matching the specific antibodies used for (a) interleukin (IL)-1 β , (b) Nod-like receptor protein (NLRP)3, (c) caspase-1, (d) complement component 5a (C5a) and (e) terminal complement complex (TCC).

Fig. S2. Immunohistochemical staining of (a) Nod-like receptor protein (NLRP)3 and (b) interleukin (IL)-1 β in first-trimester placental tissue ($n = 10$).

Fig. S3. Lactate dehydrogenase (LDH) release from placental explants [$n = 6$ in (a), $n = 4$ in (b)]. Kruskal–Wallis test with Dunn's multiple comparison *post-hoc* test. Mean with standard error of the mean (s.e.m.).

Fig. S4. Lactate dehydrogenase (LDH) release from trophoblast cells ($n = 3$). Kruskal–Wallis test with Dunn's multiple comparison *post-hoc* test. Mean with standard error of the mean (s.e.m.).

Table S1. Clinical characteristics for subjects included in third-trimester placental analyses ($n = 36$).

Table S2. Clinical characteristics and markers for subjects included in serum analyses ($n = 105$).