

Circulating Cytokines and Alarmins Associated with Placental Inflammation in High-Risk Pregnancies

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Introduction

Inflammation occurring during pregnancy is an important clinical problem that can have devastating consequences for the developing fetus.¹ Exposure to inflammation increases the incidence of stillbirth and surviving fetuses are at higher risk of fetal growth restriction (FGR) and preterm birth.^{2,3} These serious complications affect approximately 10–15%

Problem

Inflammation during pregnancy has devastating consequences for the placenta and fetus. These events are incompletely understood, thereby hampering screening and treatment.

Method of study

The inflammatory profile of villous tissue was studied in pregnancies at high-risk of placental dysfunction and compared to uncomplicated pregnancies. The systemic inflammatory profile was assessed in matched maternal serum samples in cases of reduced fetal movements (RFM).

Results

Placentas from RFM pregnancies had a unique inflammatory profile characterized by increased interleukin (IL)-1 receptor antagonist and decreased IL-10 expression, concomitant with increased numbers of placental macrophages. This aberrant cytokine profile was evident in maternal serum in RFM, as were increased levels of alarmins (uric acid, HMGB1, cell-free fetal DNA).

Conclusion

This distinct inflammatory profile at the maternal-fetal interface, mirrored in maternal serum, could represent biomarkers of placental inflammation and could offer novel therapeutic options to protect the placenta and fetus from an adverse maternal environment.

of all pregnancies in the UK and USA.⁴ Although there is strong evidence that placental dysfunction underlies FGR and stillbirth,⁵ the association between inflammation and placental dysfunction in humans remains unclear. Infections are an important cause of inflammation at the maternal-fetal interface and are found in up to 30% of cases of preterm labor and some instances of FGR and stillbirth. However, a large proportion of cases lack evidence

of an infectious cause, which is borne out by the minimal effects of antibiotic therapy.⁶ Importantly, there are no treatment options available to prevent these inflammatory pathologies, as the causes are still speculative. Multiple non-pathogenic initiators of inflammation such as damage-associated molecular patterns (DAMPs), also known as alarmins, have recently been shown to be elevated in high-risk pregnancies, mainly in cases of preterm birth⁷ and preeclampsia^{8,9} and could be potential inducers of maternal-fetal inflammation in cases without infection.

We hypothesized that inflammation is a key player in pregnancy pathologies with placental dysfunction. In the current study, we sought to determine the inflammatory profile in the placenta and maternal circulation in human high-risk pregnancies known to have placental dysfunction, namely pregnancies associated with reduced fetal movements (RFM), and to determine levels of alarmins as potential non-pathogenic inducers of inflammation.

Materials and methods

Ethics

Approval was obtained from North West Research Ethics Committee (Ref: 08/H1010/28) for placenta from uncomplicated term ($n = 37$), FGR ($n = 16$) and PE ($n = 16$) pregnancies. Approval for placentas ($n = 53$) and maternal serum from pregnancies associated with RFM was obtained from Oldham Research Ethics Committee (Ref: 08/H1011/83). Approval was obtained from Greater Manchester South Research Ethics Committee (Ref: 12/NW/0015) and from North West Research Ethics Committee (Ref: 08/H1010/28) for maternal serum from uncomplicated pregnancies. All participants provided written informed consent.

Patient Demographics

Fetal growth restriction was defined as an individualized birthweight centile (IBC) below the 5th,¹⁰ and PE as hypertension ($>140/90$ mmHg) in previously normotensive women and proteinuria (>300 mg/L) as previously described.¹¹ RFM was defined as a subjective maternal perception of reduced movements for at least 12 hr after 28-weeks gestation (fetal anomaly and multiple pregnancies excluded).¹² The RFM population is heterogeneous and two

subgroups, either with normal or poor pregnancy outcome (PPO) was defined. Poor pregnancy outcome was defined by birth before 37 weeks of gestation, small for gestational age infant (IBC below 10th) or admission to the neonatal intensive care unit. In some cases, RFM is thought to be a compensatory response to reduced fetal oxygen/nutrient supply and is associated with a significantly increased risk of FGR and stillbirth by placental dysfunction.¹³ Importantly, placental dysfunction is not restricted to those with presenting with a poor outcome.¹³ Placentas were collected if delivery occurred within 7 days of an episode of RFM to allow the association between RFM and placental findings.¹³ Placenta villous tissue samples were collected within 30 min of birth, and the same sampling strategy used across groups. Biopsies (approximately 1–3 cm³) were obtained from three regions of the placenta (i.e. close to the center of the placental mass, edge of the placenta, and in the middle).

Maternal serum collection and analysis

Maternal venous blood was collected at presentation with their first episode of RFM ($n = 34$) or at 34–36 weeks of gestation in uncomplicated pregnancies ($n = 26$ for cytokine analysis and $n = 19$ for alarmins analysis). Blood was centrifuged at $3000 \times g$ at 4°C for 10 min. Supernatant was collected and further centrifuged at $4000 \times g$ for 10 min and aliquoted and stored at -80°C until analysis.

Alarmins Levels in Maternal Serum

Levels of uric acid were determined using an assay, following manufacturer's instructions (Abcam, Cambridge, UK), and HMGB1 levels determined by ELISA (IBL International, Toronto, ON, Canada). Cell-free DNA (cfDNA) was isolated from 200 μL of maternal serum using the QIAamp DNA Blood Mini Kit (Qiagen, UK), following manufacturer instructions. qPCR was performed (as described below). Cell-free fetal DNA (cffDNA) was detected through amplification of the Y chromosome specific *DYS1* gene (forward = TCCTGCTTATCCAAATTCACCAT, reverse = ACTTCCCTCTGACATTACCTGATAATTG) and GAPDH (forward = CCTAGTCCCAGGGCTTTGATT, reverse = CCCCACACACATGCACTTACC) to assess the presence and quality of the isolated DNA performed blindly with DNA isolated from pregnancies with either male or female fetuses. No amplification for *DYS1* was detected in pregnancies with

female fetuses. An arbitrary value of 6.6 pg DNA per genome equivalent (GE) was used for analysis as previously reported.¹⁴

Protein Analysis

Villous tissue was homogenized in lysis buffer containing 1% Triton X-100 (Sigma-Aldrich, Dorset, UK) and protease inhibitor cocktail (Calbiochem, Nottingham, UK) and centrifuged at $11,000 \times g$ for 10 min at 4°C. Supernatants were taken and kept at -20°C until analysis. Bicinchoninic acid (BCA) assay was performed to determine protein concentration (Thermo Scientific, Loughborough, UK). Levels of cytokines (interleukin (IL)-1 α , IL-1 β , IL-6, IL-1 receptor antagonist (IL-1Ra), IL-10, tumor necrosis factor (TNF)- α) were determined by DuoSet ELISAs (R&D Systems, UK).

RNA Analysis

Total RNA was extracted from placental villous tissue using the RNeasy mini kit (Qiagen). RNA purity and concentration was determined using a Nanodrop Spectrophotometer (Thermo Scientific). RNA (250 ng) was reverse transcribed as previously described.¹⁵ Primers for the following were used: IL-1 β , IL-1 α , IL-6, TNF α , IL-18, IL-12 β , IFN γ , IL-1Ra, IL-10, IL-4, TGF β 1, IL-1R1, IL-1R2, Casp1, and NLRP3 (Qiagen, UK). Real time PCR system (7900 HT; Applied Biosystem, Warrington, UK) was used, mRNA expression quantified using SYBR green I with a dissociation curve analysis performed to ensure amplification specificity. Expression in villous tissue samples was normalized to the house-keeping gene YWHAZ (forward= CCTGCATGA AGTCTGTAAGTCTGAG, reverse= TTGAGACGACCCTC CA-AGATG, Invitrogen, UK), and the data are presented as relative expression.

Histological Analysis of Placental Villous Tissue

Villous tissue biopsies were fixed in 10% neutral buffered formalin for 24 hr and paraffin embedded. Five micrometer thick sections were processed as previously described.¹³ The following antibodies were used: CD45 (0.5 μ g/mL; Dako, Cambridgeshire, UK), CD163 (10 μ g/mL; Serotec, Oxford, UK), IL-1Ra (4 μ g/mL; Santa Cruz Biotechnology, Dallas, TX, USA), HMGB1 (10 μ g/mL; Abcam) and negative controls: mouse IgG (Invitrogen, Paisley, UK) or rab-

bit IgG (R&D System, Abingdon, UK). Matched secondary antibody HRP-conjugated (antirabbit-HRP, Serotec, UK or antimouse-HRP, Invitrogen, UK) were used, staining revealed using 3, 3'-diaminobenzidine (DAB; Sigma-Aldrich) and slides counterstained with hematoxylin. Images were obtained with a Leitz light microscope, QIcam Fast 1394 camera (QImaging, Surrey, BC, Canada) and Image Pro Plus 6.0 software (Media Cybernetics Inc, Rockville, MD, USA). For every section, 10 images of terminal villi were taken randomly (total of 30 images per placenta). Image analysis was performed using Histoquest software (Tissue Gnostics, Vienna, Austria). For immunofluorescence, sections were processed as above with autofluorescence being quenched by 20 min incubation in 1% sodium borohydride (Sigma-Aldrich) and fluo-conjugated secondary antibody were used (antirabbit Alexa 594 and antimouse Alexa 488 (Life Sciences, Paisley, UK). Coverslips were applied with Dapi-containing medium (Life Sciences). Images were obtained using an Olympus upright BX51, CoolSnap ES camera and MetaVue software.

Statistical Analysis

Data are presented as median with range or number with percentage as specified. Statistical comparisons between multiple groups were assessed using Kruskal-Wallis with Dunn's post-test or Mann-Whitney when only two groups were compared. Linear regression analysis was used to correlate cytokines levels with maternal or fetal characteristics (i.e. birthweight, gestational age, and BMI). Categorical data were analyzed using Fishers' exact test. Statistical analysis was performed with GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA).

Results

Patient Population Characteristics and Demographic Information

Patient clinical and demographic information are presented in Table I. By design, median birthweight, gestational age at delivery, and the proportion of infants with IBC <10th, were lower in all groups with pregnancy complications, except RFM with normal pregnancy outcome (NPO). Maternal body mass index (BMI) was higher in pregnancies complicated by PE and RFM than those with normal pregnancies.

Table 1 Demographics Information of the Patient Population used for Placental Villous Tissue Analysis

	RFM				
	Normal term (n = 34)	NPO (n = 39)	PPO (n = 14)	All (n = 53)	PE (n = 16)
Maternal age (years) ^a	31 (25–42)	28 (17–46)	25.5 (24–41)	27 (17–46)	27 (19–39)
Gravidity ^a	2 (1–18)	2 (1–9)	2 (1–8)	2 (1–9)	1 (1–3)
BMI ^a	22.9 (18.9–27.1)	25.5 (17.4–45.6)***	24 (18.1–34.3)	25.2 (17.4–45.6)**	27.3 (18.2–46)***
GA (weeks) ^a	40 (37–42)	40.1 (37.4–42)	38 (30–42)**	40 (30–42)	37 (28–40)***
Birthweight (g) ^a	3525 (2850–4800)	3400 (2750–4720)	2355 (850–3220)***	3330 (850–4720)*	2720 (880–4340)***
IBC ^a	60.5 (10–100)	41.6 (10–100)	1 (0–38)***	26 (0–100)*	25 (0–98)
Gender (% of male)	13 (38%)	21 (54%)	7 (50%)	28 (53%)	0 (0%)
Cigarette zsmoking ^b	2 (5.9%)	6 (15.4%)	4 (28.6%)	10 (18.9%)	6 (37.5%)
Delivery by CS ^b	19 (55.9%)	16 (41%)	7 (50%)	23 (43.4%)	8 (50%)***
Preterm birth (<37 weeks) ^b	0 (0%)	0 (0%)	6 (42.9%)***	6 (11.3%)	5 (31.3%)**
IBC below 10th centile ^b	0 (0%)	0 (0%)	13 (92.9%)***	13 (24.5%)**	5 (31.3%)**

BMI, body mass index; GA, gestational age; IBC, individualized birthweight centile; N/A, Not applicable; NPO, normal pregnancy outcomes; PPO, poor pregnancy outcomes; RFM, Reduced fetal movements. ^aOne-way anova, data presented as median (range); ^bFisher's exact test; data presented as number (percentage%). **P* < 0.05 vs normal term; ***P* < 0.01 vs normal term; ****P* < 0.001 vs normal term.

Levels of Cytokines in Placenta from High-Risk Pregnancies Associated with RFM

We measured the concentration of cytokines known to be associated with pregnancy pathologies and altered fetal development, in placental villous tissue from high-risk pregnancies associated with placental dysfunction, compared with uncomplicated term pregnancies. Protein levels of IL-1β were significantly elevated in placental villous tissue from high-risk pregnancies associated with RFM compared with normal term tissue (Fig. 1a), but this was not the case in other pathological pregnancies (namely FGR and PE). IL-1Ra was also significantly elevated in placentas from RFM pregnancies (Fig. 1b). Even though IL-1Ra is primarily an anti-inflammatory mediator, it is induced as a result of prior activation of the IL-1 system and therefore used as a marker of a prior pro-inflammatory activation of the IL-1 pathway. Furthermore, concentrations of anti-inflammatory IL-10 were decreased selectively in placentas from RFM pregnancies compared with term normal tissue (Fig. 1c). Protein levels of pro-inflammatory IL-1α, IL-6, and TNF-α were unchanged (Figure S1).

The increase in IL-1Ra levels detected in RFM pregnancies was not due to the presence of infection (one patient presented with group B streptococcus and was excluded from analysis). Furthermore, labor induction, maternal BMI, sex of the fetus IBC and mode of delivery (cesarean section vs. vaginal delivery) were not associated with any of the differences observed in either IL-1Ra or IL-10 expression (data not shown). There was a weak but significant negative correlation observed between IL-1Ra concentrations and both birthweight ($R^2 = -0.2699$, $P < 0.001$) (Fig. 2a) and gestational age at birth ($R^2 = -0.2618$, $P < 0.001$) (Fig. 2b). Levels of IL-1Ra were higher in RFM pregnancies delivering prematurely (<37 weeks), but were still significantly elevated in tissue from RFM pregnancies delivering >37 weeks compared with uncomplicated term births (Fig. 2c). These associations were observed only in the RFM population. Levels of IL-1Ra were significantly elevated in both subgroups of RFM (NPO and PPO) as compared to normal term, although higher in those with PPO (Fig. 2d). This placental cytokine profile in high-risk pregnancies associated with RFM revealed a pro-inflammatory imbalance at the maternofetal interface. Based on these findings, we further focused on the population with RFM for the remainder of this study.

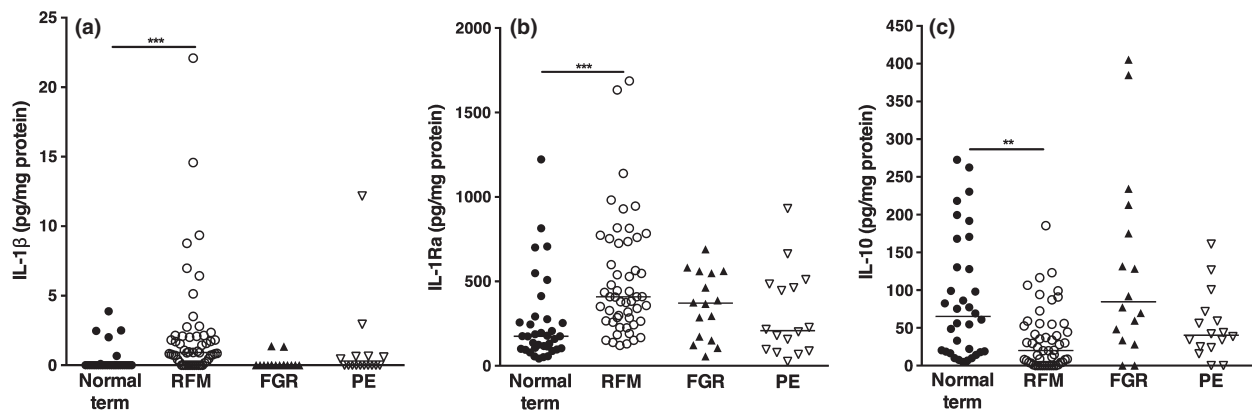


Fig. 1 Inflammatory profile in placentas from high-risk pregnancies. (a) Protein concentrations of pro-inflammatory IL-1 β were significantly elevated in high-risk pregnancies associated with reduced fetal movements (RFM, $n = 53$) ($P < 0.001$) as compared to placentas from normal term ($n = 34$) and other high-risk pregnancies ($n = 16$). (b) Levels of IL-1Ra were also significantly elevated ($P < 0.001$) specifically in RFM pregnancies. (c) Levels of anti-inflammatory IL-10 were significantly decreased ($P < 0.001$) in pregnancies with RFM. Results presented as median. Statistical analysis by Kruskal–Wallis with Dunn's multiple comparison. ** $P < 0.01$, *** $P < 0.001$.

Pro-Inflammatory Imbalance in Cytokine Gene Expression in Placentas from High-Risk Pregnancies with RFM

mRNA levels of inflammatory mediators were determined in villous tissue from normal term or RFM pregnancies. We extended the range of mediators studied from those determined at the protein level. A similar pattern of expression was determined as for the protein. This was characterized by increased expression of pro-inflammatory members of the IL-1 system, namely IL-1 α , IL-1 β and IL-18, compared with normal term placenta (Fig. 3a–c). mRNA for IL-1Ra was induced, while anti-inflammatory cytokines IL-10 and IL-4 were significantly decreased in RFM placentas (Fig. 3d–f). Levels of IL-1 receptor 1 (IL-1R1), the receptor responsible for signal transduction, were elevated (Fig. 3g), but levels of IL-1R2, a decoy receptor, were unchanged, as were caspase-1 and NLRP3 which are both necessary for IL-1 β and IL-18 processing (Figure S2). Other pro-inflammatory cytokines TNF- α and IL-6 were also significantly elevated (Fig. 3h,i), but mRNA levels of pro-inflammatory mediators typically associated with infections (namely IL-12 and IFN γ) were low and unchanged between RFM and normal term tissues (data not shown). There was no significant difference between the NPO and PPO subgroups of the RFM population in any of the inflammatory mediators studied at the mRNA level. These gene expression data further emphasize the pro-inflammatory imbalance within the placental villi, and the potential central role of

the IL-1 system in high-risk human pregnancies associated with RFM.

IL-1Ra is Localized at the Interface Between Maternal and Fetal Circulations and in Placental Immune Cells

Placentas stained for IL-1Ra revealed expression within the syncytiotrophoblast layer in the terminal villi, the fetal cell layer in direct contact with maternal blood, as well as in cytotrophoblast in both normal term and RFM tissue (Fig. 4a, black arrowheads). IL-1Ra positive cells were also detected within the placental parenchyma (Fig. 4a, white arrowheads) and were morphologically identified as immune cells. The intensity of IL-1Ra staining in the syncytiotrophoblast layer appeared markedly reduced in placentas from RFM pregnancies, possibly reflecting release into the maternal circulation, while elevated numbers of IL-1Ra positive immune cells were detected (Fig. 4a). Detailed analysis of immune cells within the placenta, using a broad immune cell marker CD45, revealed an increased number in RFM placenta as compared to normal term tissue (Fig. 4b). These immune cells were primarily CD163 + macrophages, as showed by analysis of adjacent sections (Fig. 3c). There were few neutrophils present. Similar to CD45+ cells, CD163+ cells were also significantly increased in RFM tissue (Fig. 4c). IL-1Ra staining colocalised with the macrophage marker CD163 (Fig. 4d), as well as with the trophoblast marker CK7 (Fig. 4e) providing further evidence of expression by both cell types.

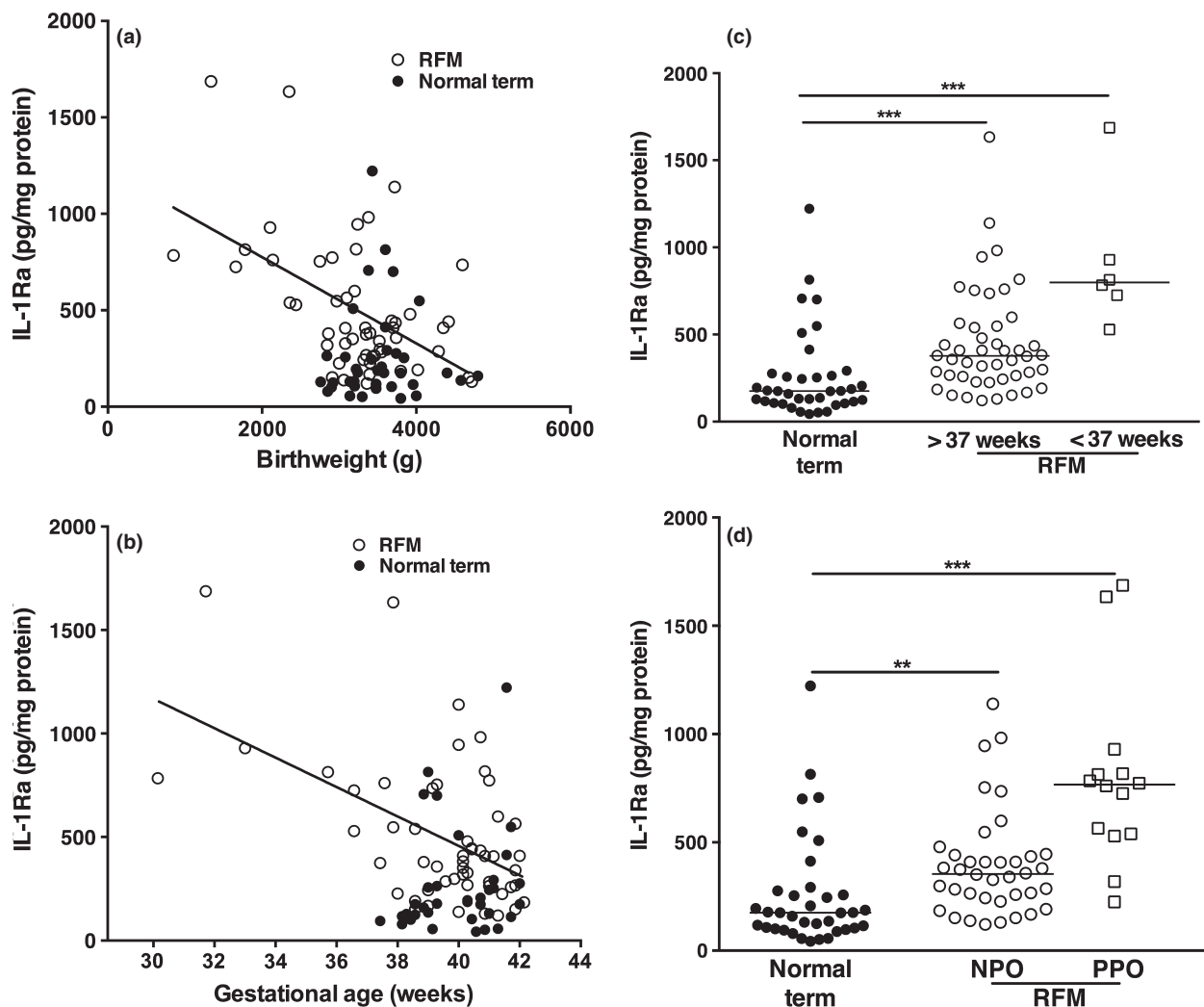


Fig. 2 Correlation between placental IL-1Ra levels and pregnancy characteristics. Placental levels of IL-1Ra were negatively correlated with birthweight (a) ($R^2 = -0.2699$, $P < 0.001$) and gestational age at birth (b) ($R^2 = -0.2618$, $P < 0.001$). Although levels of IL-1Ra were higher in premature deliveries (<37 weeks of gestation) levels were still significantly elevated in those with term delivery (c). Levels of IL-1Ra were higher in the subgroup of RFM presenting with PPO, but still significantly elevated in those with NPO (d). Results presented as median. Statistical analysis by Kruskal–Wallis with Dunn’s multiple comparison or linear regression for correlation analysis. $N = 34$ normal term and 53 placenta from RFM pregnancies. $**P < 0.01$, $***P < 0.001$.

Circulating Levels of IL-1Ra and IL-10 in the Maternal Serum: Identification of Potential New Biomarkers of Placental Inflammation in Pregnancies With RFM

Based on the altered placental cytokine profile, we next assessed if this pattern was also observed within the maternal circulation and could therefore be used as potential biomarker of compromised maternal-fetal environment in patients with RFM. Cytokine

concentrations in maternal serum collected at the time of presentation with RFM were compared with serum from normal pregnancies collected at 36 weeks of gestation (see Table II for demographic information). Our results revealed that, similarly to the placenta, serum levels of IL-1Ra were significantly higher and IL-10 significantly lower, in women presenting with RFM compared with those with normal pregnancies (Fig. 5a,b). The elevation in IL-1Ra was only apparent in women who went

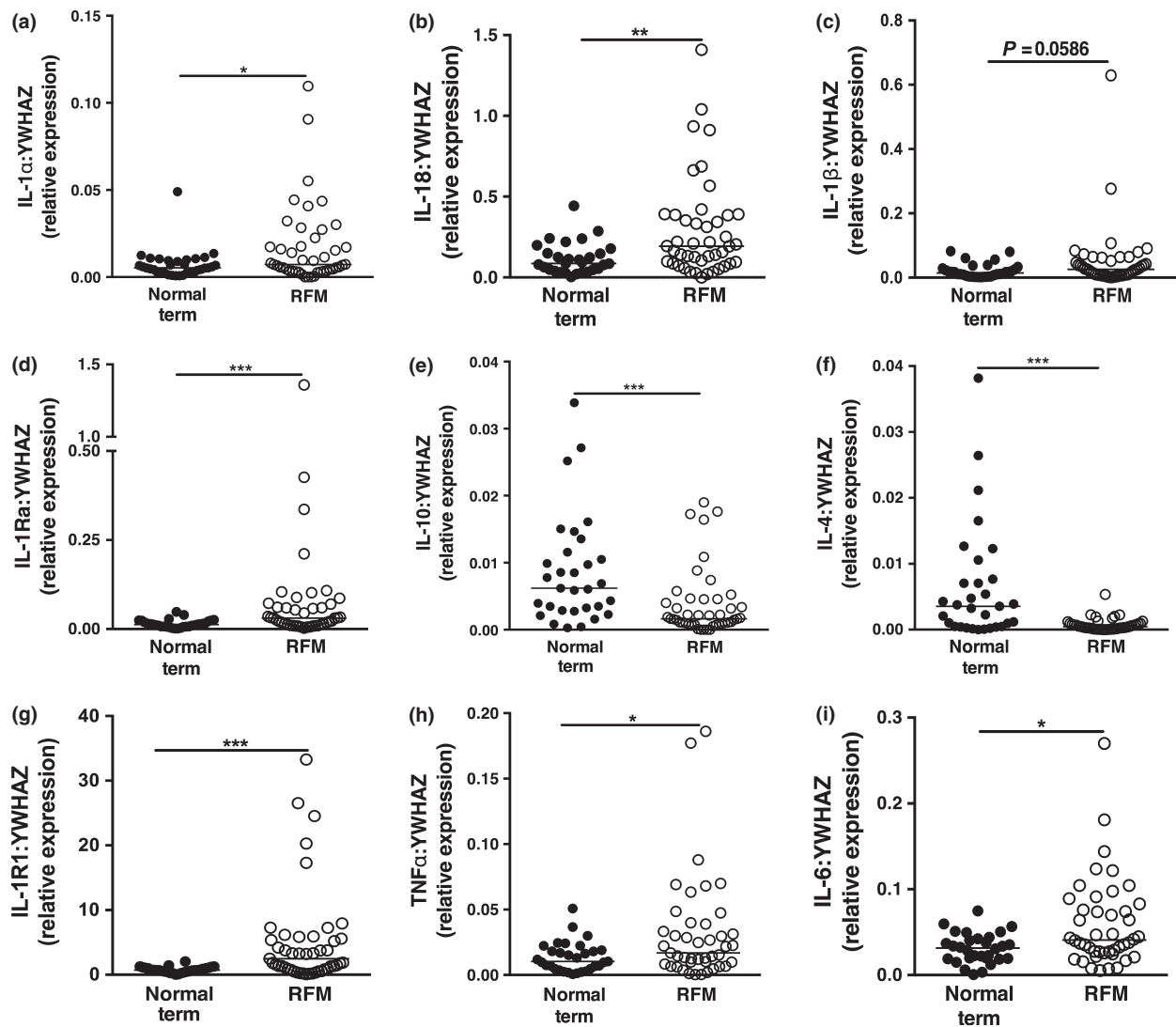


Fig. 3 Inflammatory mediators RNA expression in high-risk pregnancies with RFM as compared to normal term pregnancies. Relative mRNA expression of pro-inflammatory members of the IL-1 system namely: IL-1 α (a), IL-18 (b), IL-1 β (c) as well as IL-1Ra (d) were all elevated in placentas from RFM ($n = 53$) compared to normal term pregnancies ($n = 34$). On the other hand, anti-inflammatory cytokines IL-10 (e) and IL-4 (f) expression were significantly decreased. IL-1R1 was significantly elevated (g), as were pro-inflammatory TNF- α (h) and IL-6 (i). Results presented as median. Statistical analysis by Mann-Whitney *t*-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

on to deliver within 7 days of the episode of RFM, indicating an acute (<7 days) relationship with RFM. There were no differences in IL-1Ra between women with normal or poor outcomes in the RFM population. Conversely, levels of IL-10 in maternal serum were significantly decreased regardless of the interval between presentation with RFM and delivery (Fig. 5d), suggesting a more sustained relationship between circulating IL-10 levels and placental inflammation.

Elevated Circulating Levels of Alarmins in Maternal Serum from Pregnancies Associated with RFM

We next addressed potential upstream causes of inflammation. As no infection was detected in these cases, and the inflammatory profile within the placenta did not suggest an infectious stimulus, we focused on non-pathogenic inducers of inflammation. Due to the availability of maternal serum, the

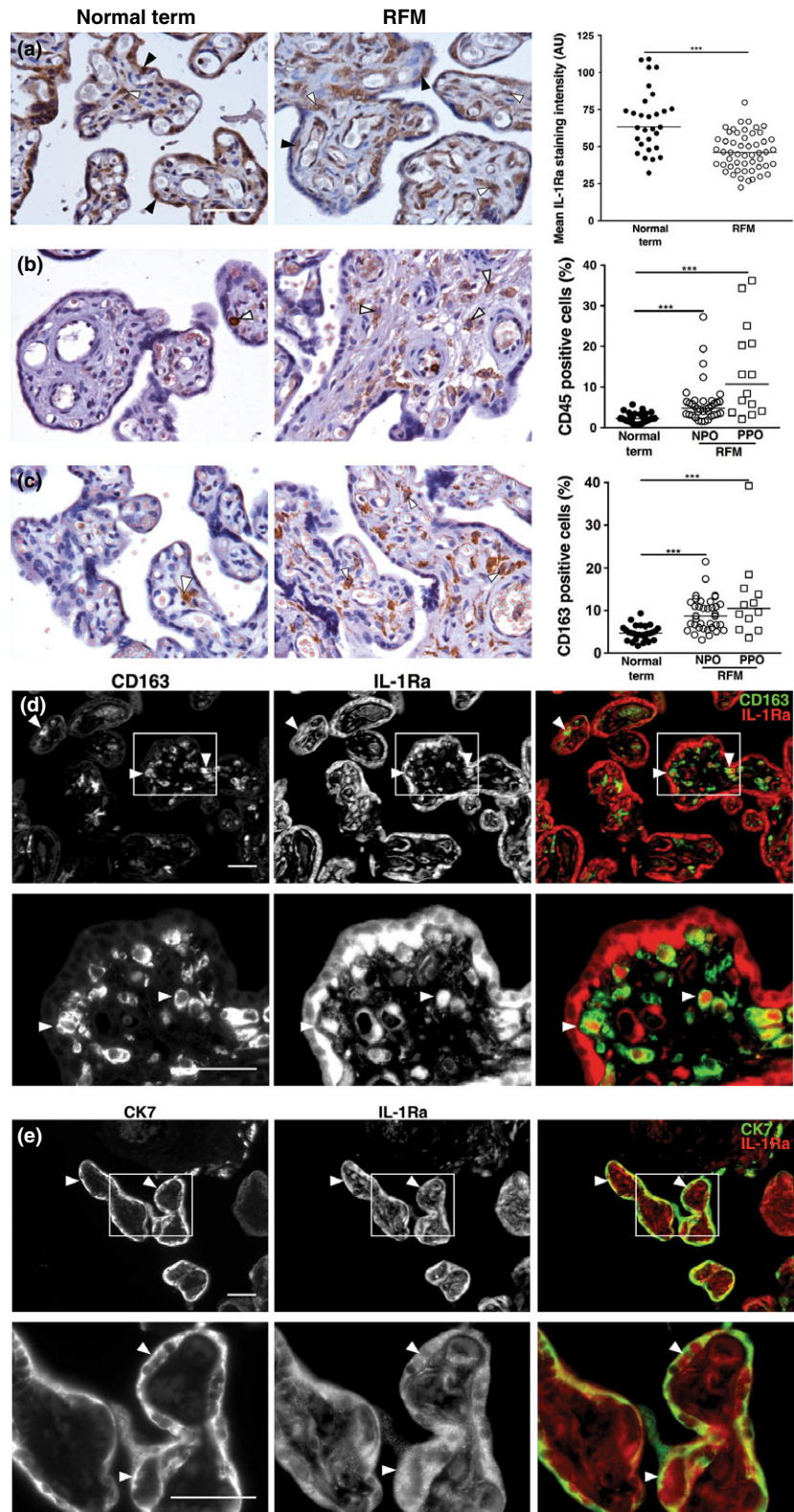


Fig. 4 IL-1Ra localization and immune cells quantification within normal term and RFM placenta. (a) IL-1Ra is pre-dominantly localized in syncytiotrophoblast (black arrowheads) and immune cells, the latter mainly in RFM pregnancies (white arrowheads), with decreased intensity in RFM placenta as compared to normal term. (b) IHC for CD45 revealed an increased number of immune cells within RFM placenta (both NPO and PPO) (white arrowheads). (c) The increased in immune cells in RFM tissue is due to higher number of CD163+ macrophages (white arrowheads). IL-1Ra expression colocalized with CD163+ macrophages (d) as well as with CK7+ trophoblast (e). Scale bars: 50 μm (a, b, c) and 25 μm (d, e). Statistical analysis by Mann–Whitney t-test (a) or Kruskal–Wallis with Dunn’s post-test (b, c). ***P < 0.001.

Table II Demographics Information for the Patient Population used for Serum Analysis

	Normal term (n = 26)	RFM		
		NPO (n = 31)	PPO (n = 8)	All (n = 39)
Maternal age (years)	35 (21–38)	28 (17–46)	25 (24–41)	27 (17–46)
Gravidity	2 (1–7)	2 (1–7)	2 (1–4)	2 (1–7)
BMI	23 (19.9–33.6)	25.5 (20.4–45.6)*	23.7 (18.1–29.7)	25.2 (18.1–45.6)
GA at presentation (weeks)	N/A	39.6 (32.4–41.9)	35.1 (29.6–41.3)	39.4 (29.6–41.9)
GA at delivery (weeks)	39.9 (37.4–42.3)	40.1 (37.4–42.1)	37.1 (30.1–41.9)***	40 (30.1–42.1)
Birthweight (g)	3378 (2680–4338)	3400 (2750–4720)	2245 (850–3000)***	3350 (850–4720)
IBC	42.0 (10–87)	51.3 (10.4–99.9)	1.2 (0–37.9)**	28.0 (0–99.9)
Gender (% of male)	14 (54%)	17 (55%)	5 (63%)	28.0 (56%)

BMI, body mass index; GA, gestational age; IBC, individualized birthweight centile; N/A, Not applicable; NPO, normal pregnancy outcomes; PPO, poor pregnancy outcomes; RFM, Reduced fetal movements. Serum in the normal term group was collected at 36 weeks of gestation. Data are presented as median (range). * $P < 0.05$ vs normal term; ** $P < 0.01$ vs normal term; *** $P < 0.001$ vs normal term.

control group used for this part of the study was different from the cytokine analyzes performed (see Table III for demographic information). Levels of uric acid were elevated in maternal serum in pregnancies associated with RFM as compared to normal pregnancies (Fig 6a). This increase was pre-dominant in the subgroup of pregnancies with PPO ($P < 0.001$ vs. normal) but still significantly elevated in those with NPO ($P < 0.05$ vs normal) (Fig 6a). Elevated circulating levels of cffDNA were also detected in pregnancies associated with RFM ($P < 0.01$, Fig 6b), due to lack of serum availability, it was not assessed in those with PPO. Circulating levels of HMGB1 were also elevated in RFM pregnancies in both subgroups with NPO ($P < 0.001$) or PPO ($P < 0.001$) to a similar extent (Fig 6c).

Within the placenta, HMGB1 staining intensity was increased in villous tissue from RFM pregnancies (Fig 6d) and was mainly localized to the syncytiotrophoblast layer (black arrowheads, Fig 6e), but could also be observed in immune cells within the villous core (white arrowheads, Fig 6e) in both normal and RFM pregnancies. A redistribution of the HMGB1 staining was observed in RFM pregnancies with PPO as determined by the increased stained area ($P < 0.01$, Fig 6f) and the localization to the cytoplasm (Fig 6g) as compared to a predominant nuclear localization in normal term tissues (Fig 6e).

Discussion

A distinct inflammatory profile was detected within the placentas of high-risk human pregnancies

associated with RFM, characterized by increased IL-1Ra and decreased IL-10 expression. Importantly, the same pattern was also detected in the maternal circulation several days prior to birth. Concomitantly, levels of non-pathogenic inducers of inflammation, the alarmins uric acid, HMGB1, and cffDNA, were elevated in the maternal circulation, suggesting they are associated with placental inflammation and thus, may play a role in its induction.

In the current study, we focused on high-risk pregnancies with RFM as these were already shown to have altered placental structure and function (even in cases with apparently NPO) without infection,¹³ and this is the first study to present evidence of inflammation in both the placenta and maternal circulation. Studies to date have focused mainly on infection-induced inflammation, using animal models to provide evidence of a causal link between pathogenic inflammation and fetal compromise.^{16,17} However, in human pregnancies, inflammation is commonly detected without infection and seen in various pregnancy pathologies associated with FGR and pregnancy loss. The inflammatory profile we report in the current study differs from that reported in a previous study on infection and chorioamnionitis in which IL-6 and TNF α involvement was reported.¹⁸ Moreover, no involvement of neutrophils was seen in the current study, contrary to the often reported infiltration of neutrophil in chorioamnionitis of infectious origin. In contrast, the hallmark of placental inflammation in RFM pregnancies was altered expression of anti-inflammatory cytokines, namely IL-1Ra and IL-10. The elevated IL-1Ra

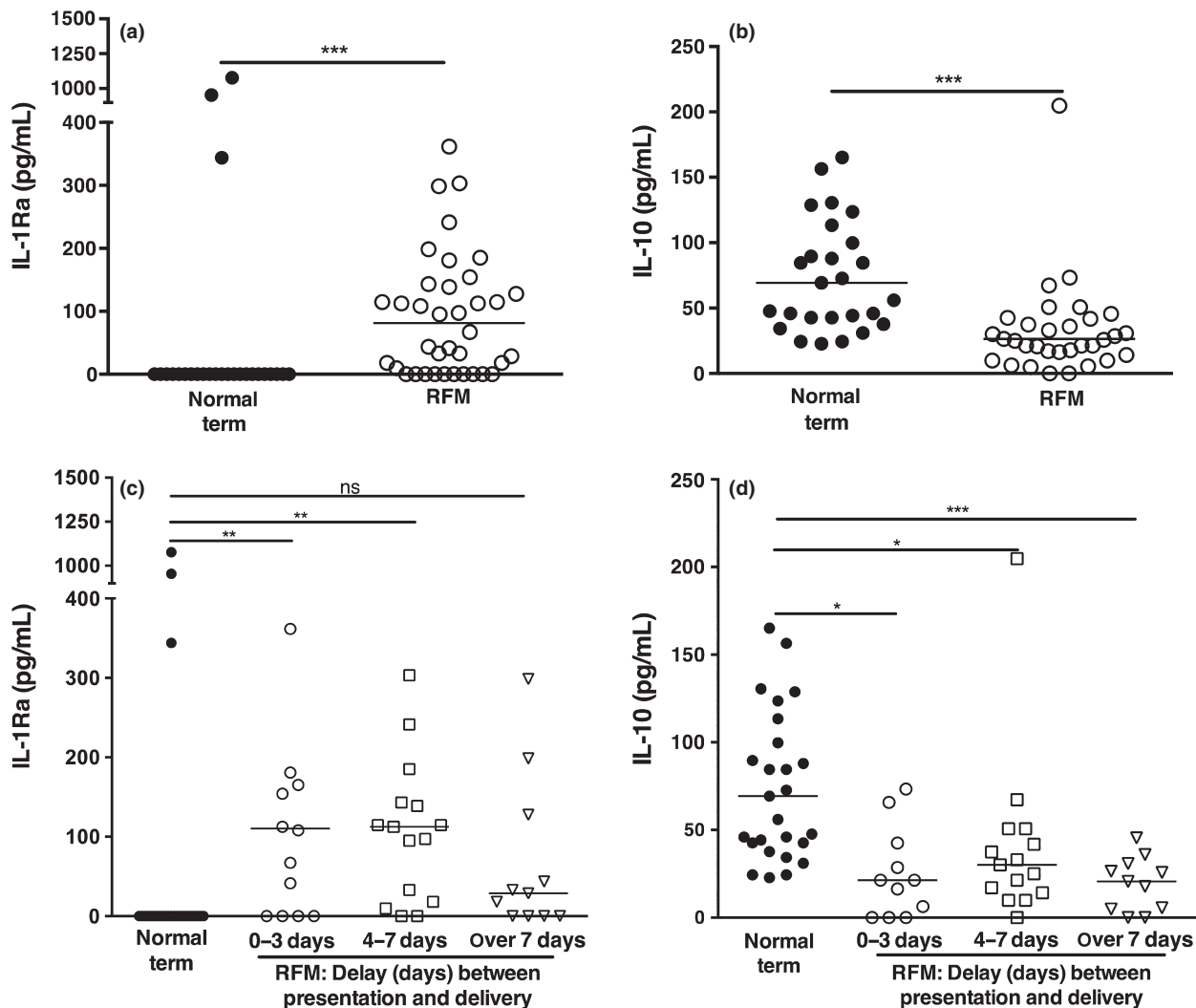


Fig. 5 Detection of cytokine levels in maternal serum as potential biomarkers of placental inflammation. Levels of IL-1Ra were significantly elevated (a) and IL-10 significantly decreased (b) in maternal serum from RFM ($n = 39$) as compared to normal pregnancies ($n = 26$) following the same pattern as determined within the placenta. (c) The increased in IL-1Ra was only apparent when the interval between presentation with RFM (time of serum collection) and delivery (placenta collection) was between 0 and 7 days. (d) The decreased in IL-10 was observed regardless of the interval between RFM and delivery. Results presented as median. Statistical analysis by Mann–Whitney t-test (a, b) or Kruskal–Wallis with Dunn's post-test (c, d). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

provides confirmation of prior-inflammatory IL-1 system activation¹⁹ while, combined with reduced IL-10, suggests a pro-inflammatory bias. Our data provide evidence that these two cytokines could be used as potential biomarkers of placental inflammation, as altered levels could be detected in maternal serum several days prior to delivery. This is in agreement with a recent study which reported that maternal serum IL-1Ra/IL-10 ratio in the second trimester was the best predictor of preterm delivery.²⁰ The fact that we did not observe changes in the

placental inflammatory profile in FGR and PE may be explained by the temporal association between placental dysfunction and cytokine expression, which is more likely to arise in the first trimester in PE and FGR, as compared to the shorter temporal association between RFM (occurring in the third trimester) and delivery. Furthermore, PE has been shown to have a systemic inflammatory component, and evidence is currently lacking to suggest a local placental inflammatory imbalance: this requires further investigation.

Table III Demographics Information for the Patient Population used for Serum Analysis of Alarmins

	Normal term (n = 19)	RFM (cffDNA) NPO (n = 16)	RFM (uric acid and HMGB1)		
			NPO (n = 20)	PPO (n = 20)	All (n = 40)
Maternal age (years)	29 (20–42)	27.5 (19–37)	28 (17–46)	26 (20–41)	27 (17–46)
Gravidity	2 (1–4)	1.5 (1–6)	1 (1–7)	2 (1–8)	2 (1–8)
BMI	29.6 (21.5–35)	26.0 (20.4–45.6)	26.0 (20.4–45.6)	23.7 (18–34)*	25.3 (18.1–45.6)
GA at presentation (weeks)	34.9 (27.9–41.7)	40.2 (33.6–41.9)	39.7 (33.6–41.9)***	38.6 (28.7–41.3)*	39.6 (28.7–41.9)***
GA at delivery (weeks)	40.0 (37.7–43)	40.8 (37.9–42.1)	40.4 (38.9–42.1)	39.1 (30–41.1) #	40.1 (30.1–42.1)
Birthweight (g)	3450 (2595–4100)	3710 (2860–4720)	3580 (2860–4720)	2732 (850–3220)***,###	3100 (850–4720)
IBC	46 (8–89)	43.3 (11–99.8)	51.44 (11–99.8)	5.9 (0–58.6)**,##	11.6 (0–99.8)
Gender (% of male)	12 (63%)	10 (50%)	10 (50%)	13 (65%)	23 (57.5%)
Smoking	0 (0%)	3 (18%)	3 (18%)	8 (40%)**	11 (27.5%)*
Delivery by CS	5 (26%)	6 (35%)	6 (35%)	3 (15%)	9 (22.5%)

BMI, body mass index; GA, gestational age; IBC, individualized birthweight centile; N/A, Not applicable; NPO, normal pregnancy outcomes; PPO, poor pregnancy outcomes; RFM, Reduced fetal movements. Serum in the normal term group was collected at 36 weeks of gestation. Data are presented as median (range). * $P < 0.05$ vs normal term; ** $P < 0.01$ vs normal term; *** $P < 0.001$ vs normal term, # $P < 0.05$ vs RFM-NPO; ## $P < 0.01$ vs RFM-NPO; ### $P < 0.001$ vs RFM-NPO.

The lack of difference between both subgroups of RFM (namely NPO and PPO) is an indication that not only conventional poor outcomes (which are based on the relatively crude endpoint of birthweight) are of importance. RFM has previously been associated with long-term neonatal neurological impairments²¹ and placentas from pregnancies associated with RFM, regardless of their outcomes, present evidence of infarction and dysfunction.¹³ However, the lack of long-term follow-up in the present study is a weakness and post-natal evaluation should be included in future studies.

Non-pathogenic inducers of inflammation, known as alarmins, are increasingly associated with high-risk pregnancies^{7–9,22,23} and are a possible cause of inflammation at the maternal-fetal interface. Increased levels of uric acid have previously been reported in PE, believed to be due to the renal impact of raised blood pressure.⁸ In the present study, all patients were normotensive and therefore this cannot account for the raised uric acid levels in maternal serum. A recent *in vitro* study reported the release of uric acid by human first trimester trophoblast after stimulation with antiphospholipid antibodies²⁴, and our results suggest that this might also be possible in the third trimester associated with placental dysfunction. Elevated HMGB1 has previously been associated with PE as well as with preterm birth^{7,9,22} and cffDNA was reported to be elevated in PE²⁵ and cases of FGR.²⁶ These reports are in accordance with our

current study. There are no previous reported studies of alarmin levels in pregnancies complicated by RFM and no previous studies of pregnancy pathologies have examined all three alarmins together; the fact that in the current study they were elevated concomitantly prior to birth suggest that they have a common source, possibly the placenta. Due to serum availability, cffDNA analysis was performed only in the RFM subpopulation with NPO. The fact that we detected an elevation in cffDNA in these mildly affected cases is a strong indicator that it would also be elevated in the PPO subpopulation, but further studies are needed to ascertain these observations. Similarly the causal link between alarmins, cytokines, and placental dysfunction remains to be confirmed.

In summary, this study provides evidence of a unique inflammatory profile in high-risk human pregnancies with central involvement of the IL-1 system, identifies new potential biomarkers of a compromised maternal-fetal environment, easily detectable in maternal serum, and gives insight into possible upstream inducers of inflammation in the absence of infection.

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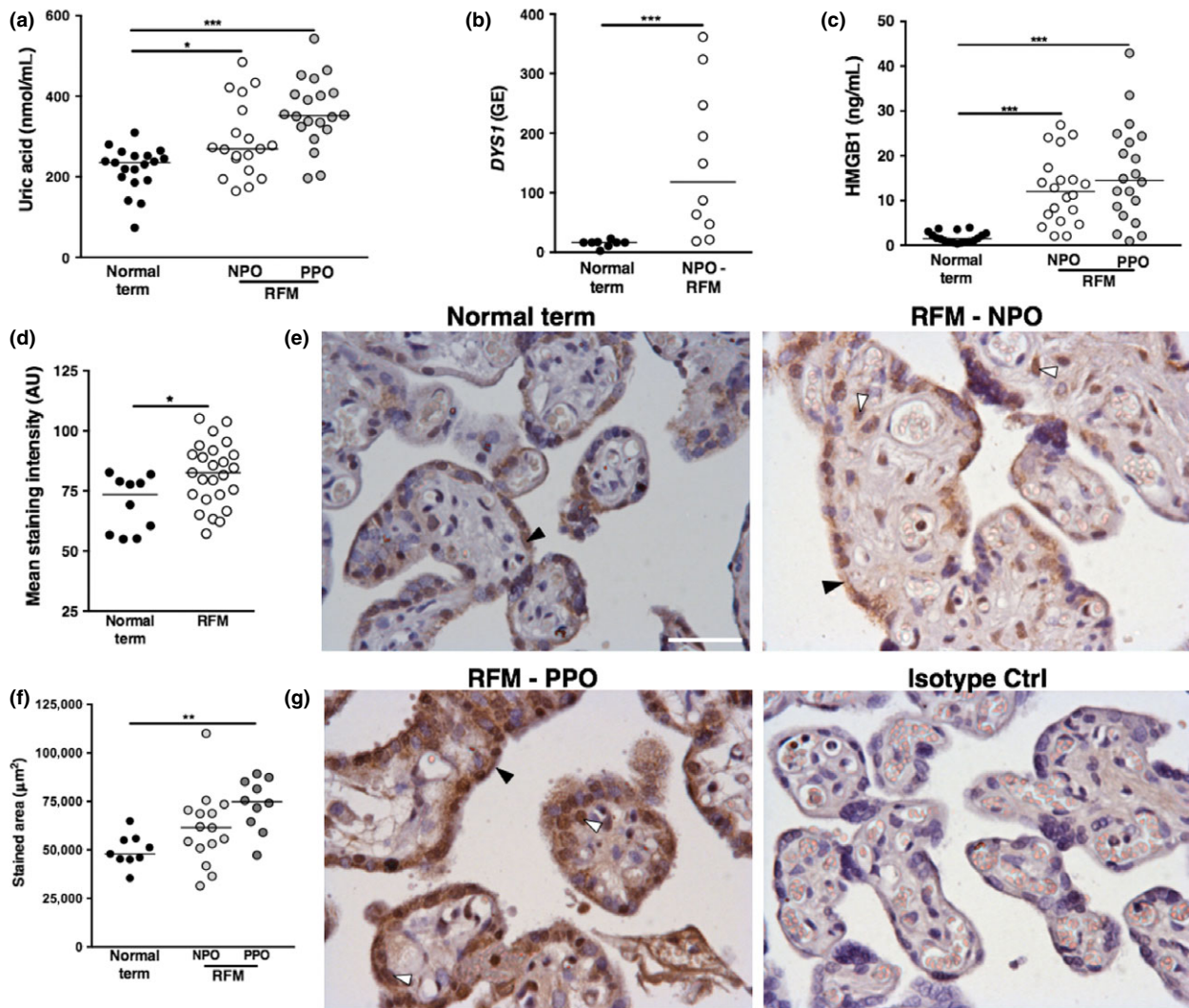


Fig. 6 Circulating levels of alarmins in high-risk pregnancies associated with RFM. Levels of uric acid (a), cffDNA (DYS1 gene) (b) and HMGB1 (c) were elevated in maternal serum from RFM pregnancies. HMGB1 staining intensity in the placenta was increased in RFM pregnancies (d) and mainly localized to the syncytiotrophoblast (black arrowhead, (e) and immune cells (white arrowhead, (e). HMGB1 staining was mainly localized to the cytoplasm in RFM with PPO as seen by the increased stained area (f, g). Results presented as median. Statistical analysis by Kruskal–Wallis with Dunn’s post-test (a, c, f) or Mann–Whitney (b, d). Scale bar: 50 µm * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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Conflict of interest

The authors have no conflict of interest to disclose.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Levels of IL-1 α , TNF- α and in high-risk pregnancies. Protein levels of IL-1 α (A), TNF- α (B), and IL-6 (C), were unchanged in placentas from high-risk pregnancies associated with RFM, FGR or PE as compared with normal term control placenta. Results presented as median.

Figure S2. mRNA levels of inflammatory mediators in placentas from pregnancies with RFM. mRNA levels of IL-1R2 (A), NLRP3 (B), and caspase-1 (C) were unchanged in placenta from high-risk pregnancies associated with RFM as compared to normal term tissue. Results presented as median.