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Pre-eclampsia is associated with elevated CXCL12 levels in placental syncytiotrophoblasts and maternal blood

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ABSTRACT

Objectives: Placental derived vasculogenic/angiogenic substances in maternal blood are dysregulated in pre-eclampsia. We hypothesized that CXCL12, a chemokine with vasculogenic actions, is amongst such molecules.

Study design: CXCL12, CXCL16, CXCR4, and CXCR6 immunolocalization in placental tissue was analyzed in pre-eclampsia ($n = 8$) in comparison to controls ($n = 8$). CXCL12, measured by ELISA in blood, in women diagnosed with pre-eclampsia ($n = 14$) and prior to the development of pre-eclampsia (at 20 weeks' gestation, $n = 20$) was compared with CXCL12 concentrations in gestation-matched, healthy control subjects ($n = 34$).

Results: In placental tissue, syncytiotrophoblast staining for CXCL12 was increased in pre-eclampsia. Maternal serum CXCL12 was increased in pre-eclampsia [2000 (SD 402) vs 1484 (SD 261) pg/ml, $P = 0.01$] but not in plasma obtained at 20 weeks of gestation prior to the onset of pre-eclampsia [1183 (SD 336) vs 1036 (SD 144) pg/ml, $P = 0.09$].

Conclusion: Our data suggest that the syncytiotrophoblast contributes to a pre-eclampsia-associated increase in CXCL12 levels in maternal blood. These findings support the hypothesis that an imbalance of angiogenic factors contributes to the pathogenesis of pre-eclampsia.

1. Introduction

Pre-eclampsia affects about 5% of pregnancies and is a major contributor to maternal and perinatal morbidity and mortality [1]. It is diagnosed following the onset of maternal hypertension, proteinuria and variably, multi-organ complications and fetal growth restriction. Although the etiology is complex, abnormal placentation is a likely initiating factor [2–4]. The placenta forms the physical and functional interface between the mother and the embryo/fetus. Placental structure is established by differentiation of the organ's specialized epithelial cells, termed cytotrophoblasts (CTBs). In one differentiation pathway, CTBs that emanate from anchoring placental villi invade the uterine wall. In this location, they extensively remodel maternal spiral arterioles, replacing the endothelial lining of the vessels. This process establishes blood flow to the intervillous space at 10–12 weeks of gestation [5]. At a functional level, extravillous cytotrophoblasts, cells that mimic the phenotype of endothelial cells, channel maternal blood flow to the floating villi where nutrient, gas, and waste exchange takes place across the syncytiotrophoblast layer [6]. This process is defective in pregnancies complicated by the syndrome pre-eclampsia [1].

An imbalance of placenta-derived pro- and anti-angiogenic factors in maternal blood is thought to play a critical role in the development of pre-eclampsia [3,5,6]. Several groups have measured angiogenic/vasculogenic factors and their receptors in maternal blood during normal pregnancy and in pre-eclampsia. For example, in this syndrome, pro-angiogenic ligands (*e.g.* PlGF) are decreased and anti-angiogenic receptors (*e.g.* sFLT-1 and

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soluble endoglin) are increased [7]. These data suggest that placenta-derived molecules with potential maternal vascular effects may be dysregulated in pre-eclampsia.

The chemokine CXCL12 (also termed stromal-derived factor-1) fits this paradigm as interactions with its cell surface receptor, CXCR4, modulate haematopoiesis and angiogenesis in health and disease [8–10]. CXCL12 mobilises endothelial progenitor cells from the bone marrow and stimulates traffic of these cells from the circulation into the vessel wall where they modulate regeneration of endothelium following vascular damage [11–13]. Interestingly, CXCL12 also appears to have a role in key aspects of placental development including CTB migration [14], invasion, differentiation, and proliferation [15], as well as recruitment of immune cells to the decidua [16,17].

Furthermore the chemokine and receptor pair CXCL16 and CXCR6 is present in the human placenta. Cytotrophoblasts co-express CXCR6 and CXCL16 and are thought to induce their proliferation and invasion in an autocrine manner [18]. CXCR6 is the sole receptor of CXCL16 and is also expressed by T lymphocytes, natural killer cells, and monocytes, suggesting a role in leucocyte recruitment in the placenta [19].

To test the hypothesis that CXCL12, CXCL16, CXCR6, and CXCR4 are dysregulated in preeclampsia, expression of these molecules was examined in tissue sections at the fetal–maternal interface, including chorionic villi and basal plate, obtained from women diagnosed with pre-eclampsia, and compared with specimens from women who had an uncomplicated pregnancy. The staining showed a difference in CXCL12 expression which was further followed by investigating circulating CXCL12 levels in maternal blood after the diagnosis of pre-eclampsia and at 20 weeks of gestation prior to the onset of pre-eclampsia. CXCL12 levels were altered in pre-eclampsia, but not in the mid trimester prior to pre-eclampsia.

2. Materials and methods

2.1. Studies

Three separate studies were conducted. The first was a case–control study performed at the University of California San Francisco (UCSF), USA. CXCL12, CXCL16, CXCR6, and CXCR4 expression was investigated using immunohistochemistry of placental and basal plate biopsies obtained from women with pre-eclampsia and gestation-matched controls who had delivered following spontaneous preterm labour. In the second study, CXCL12 in serum from women with clinically manifest pre-eclampsia was compared with serum levels from gestation-matched, healthy controls who had an uncomplicated pregnancy. In the third study, CXCL12 was measured in plasma from women at week 20 of gestation who later developed pre-eclampsia and women who subsequently had uncomplicated pregnancies. The second and third prospective, clinical studies were performed in Auckland, New Zealand.

2.2. Study 1: immunolocalization of CXCL12, CXCL16, CXCR6 and CXCR4 in placental tissue

Placental tissue collection was performed with informed consent and approved by the UCSF Committee on Human Research. Placental specimens were obtained from women with pre-eclampsia (32.3, SD 4 weeks, $n = 8$) and gestational age matched control subjects (30.4, SD 4 weeks, $n = 8$) who delivered following spontaneous preterm labour without infection (absence of clinical criteria for infection and no evidence of chorioamnionitis on fetal membrane histology) or premature rupture of membranes. In the control group two cesarean sections were performed for fetal reasons (one breech position and one due to an abnormal fetal cardiotocograph). Placental basal plate was immediately processed after delivery, fixed and paraffin-embedded as described previously [20]. Immunohistochemistry was performed using a standard protocol. Initially tissue sections were deparaffinized, hydrated, quenched with 3% hydroxyperoxide and blocked for nonspecific reactivity for 20 min. Tissue sections were then incubated for 1 h at room temperature with either an anti-human CXCL12 mAb, CXCL16 mAb, CXCR6 mAb, CXCR4 mAb (R&D Systems, Minneapolis, MN) or an anti-human cytokeratin 7 mAb (Dako, Carpinteria, CA) to identify cytotrophoblasts. Primary antibody binding was then detected using VECTASTAIN ABC and DAB peroxidase substrate kits according to the manufacturer's instructions (Vector Laboratories, Burlingname, CA). Mouse IgG was used as a negative control instead of the primary antibody. Photomicrographs were taken with a Leica microscope (Bannockburn, IL) and a Nikon diaphot 300 (Amstelveen, Netherlands). The evaluation was done blindly by three independent investigators.

2.3. Study 2: serum CXCL12 after diagnosis preeclampsia

Serum CXCL12 concentrations were measured by ELISA in nulliparous women with clinically manifest pre-eclampsia ($n = 14$) and healthy nulliparous controls who delivered an appropriately grown, healthy baby at 37 or greater weeks' gestation ($n = 14$). Blood samples were obtained prior to delivery and controls were gestation-age matched at sampling (± 1 week) to cases. Twelve of the 14 cases were on anti-hypertensive medication (usually methyldopa) when the specimens were collected and blood pressure 'at sampling' was recorded. Ethical approval was obtained from the Auckland Ethics Committee (2000/157, AKX/02/00/364) and all participants provided written informed consent.

2.4. Study 3: plasma CXCL12 levels in early pregnancy (week 20) by ELISA

CXCL12 concentrations were measured in plasma obtained at 20 ± 1 weeks from women who subsequently developed pre-eclampsia ($n = 20$) and controls who had an uncomplicated pregnancy outcome ($n = 20$). The participants were healthy nulliparous women with singleton pregnancies recruited to the SCOPE (Screening for Pregnancy Endpoints) study in Auckland, New Zealand [21]. SCOPE is a prospective, multi-centre cohort study with the main aim of developing screening tests to predict pre-eclampsia, small for gestational age (SGA) infants and spontaneous preterm birth. Participants were interviewed at 15 ± 1 and 20 ± 1 weeks and a blood sample was obtained at each visit. The women were then followed prospectively, with pregnancy outcome data collected by research midwives, usually within 72 h of birth. Cases were randomly selected from the first 700 recruits in Auckland, of whom 37 (5.2%) developed pre-eclampsia. Controls were randomly selected from women who had an uncomplicated pregnancy in the same cohort. The study was approved by the Auckland Ethics Committee (New Zealand AKX/02/00/364) and all women provided written informed consent.

2.5. Specimen collection and processing

Blood samples were collected by venepuncture into plain tubes for serum (after diagnosis of pre-eclampsia) or EDTA tubes for plasma (prior to pre-eclampsia), placed on ice, and centrifuged at $2400 \times g$ for 10 min at 4 °C. Serum was transferred to a new tube and centrifuged again at $3200 \times g$ for 15 min at 4 °C to obtain

platelet-poor serum. Serum and plasma samples were stored in 250 μL aliquots at −80 °C within 4 h of collection. Specimens from matched cases and controls were stored under the same conditions over a similar time period.

2.6. CXCL12 ELISA

CXCL12 was measured in these samples with a quantitative sandwich ELISA (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. Plate to plate variability was controlled by comparison with an independent standard curve that was evaluated on each plate. Samples from cases and their respective controls were run in duplicate on the same ELISA plate.

2.7. Definitions

When gestational age was calculated from a certain last menstrual period (LMP) date it was only adjusted if either (1) a scan performed at <16 weeks' gestation found a difference of ≥7 days between the scan gestation and that calculated from the LMP or (2) a 20-week scan showed a difference of ≥10 days between the scan gestation and that calculated from the LMP. If the LMP date was uncertain, then scan dates were used to calculate gestational age.

Blood pressure at sampling was measured once using a standardised protocol with the women in the same position (sitting), rested and using the correct sized cuff for arm circumference. Korotkoff sounds 1 and 5 were used to determine the systolic and diastolic blood pressure, respectively. Maximum blood pressure was the highest recorded blood pressure at the end of pregnancy before the onset of labour, or in the postpartum period.

Pre-eclampsia was gestational hypertension (systolic BP ≥ 140 mmHg and/or diastolic BP ≥ 90 mmHg on at least 2 occasions 4 h apart after 20 weeks' gestation, but before the onset of labour, or postpartum systolic BP ≥140 mmHg and/or diastolic BP ≥ 90 mmHg on at least 2 occasions 4 h apart) with proteinuria (24 h urinary protein 300 mg or spot urine protein:creatinine ratio ≥30 mg/mmol creatinine or urine dipstick protein ≥2+) or any multi-system complication of pre-eclampsia [20]. Multi-system complications included thrombocytopenia (platelets $<100 \times 10^9$/L), disseminated intravascular coagulation, micro-angiopathic hemolysis, liver dysfunction with aspartate transaminase and/or alanine transaminase >45 IU/L, imminent eclampsia (severe headache with hyperreflexia and persistent visual disturbance), eclampsia, cerebral haemorrhage, acute renal failure and pulmonary oedema. SGA was defined as a birth weight less than the 10th percentile adjusted for infant gender and maternal parity, ethnicity, height and weight, as determined by the customized centile calculator at http://www.gestation.net. Uncomplicated pregnancy was a pregnancy with no antenatal obstetric or medical complications resulting in delivery of an appropriately grown, healthy baby at 37 or greater weeks' gestation.

2.8. Statistical analysis

Data are expressed as means ± SD. Clinical data were analyzed using Student's *t*-test assuming equal variance and categorical variables were analyzed using either Chi-square test with Yates correction or Fisher's Exact test. For correlation analysis the Pearson's correlation coefficient '*r*' was reported. CXCL12 values measured by ELISA were compared using Student's *t*-test (two-tailed). Statistical significance was assumed at $P < 0.05$. Calculations were performed using SPSS (version 18. SPSS Inc., Chicago, IL).

Table 1
Clinical characteristics of women in study of CXCL12 immunolocalization in placenta.

Characteristics	Controls ($n=8$)	Preeclampsia ($n=8$)	*P*-value
Age (years)	29.6 (7.1)	32 (9.1)	0.88
Parity	1.6 (0.9)	1.3 (0.9)	0.79
Gestation at delivery (wks)	30.4 (4.6)	32.3 (3.3)	0.51
Mode of delivery			0.59
Vaginal	6	5	
Cesarean section	2	3	

Mean (SD) or *n*.

3. Results

3.1. CXCL12 protein expression is up-regulated in the placental syncytiotrophoblast in pre-eclampsia

There was no difference in maternal age, parity, gestational age at delivery or mode of delivery between the women with pre-eclampsia and controls in the placental histology study (Table 1). Adjacent tissue sections of chorionic villi and basal plate were obtained from subjects with pre-eclampsia or healthy control subjects and examined by immunolocalization of CXCL12, CXCL16, CXCR4, and CXCR6 (Fig. 1). In control third trimester placental tissue (Fig. 1A, C, E, G, I and K), CXCL12 is localized mainly to the extravillous cytotrophoblasts (EVT). In pre-eclampsia (Fig. 1B, D, F, H, J and L), tissue sections of the placenta showed much stronger staining of the syncytiotrophoblast layer (STB) (Fig. 1B) and the EVT stained as well. These results were confirmed in eight different control and pre-eclampsia placentas. The expression of CXCR4, CXCR6 and CXCL16 in the STB, EVT or cells in the decidua was not different in pre-eclampsia (Fig. 1F, H and J) compared to healthy control tissue sections (Fig. 1E, G and I).

3.2. Serum CXCL12 levels after diagnosis of preeclampsia

Maternal and infant characteristics are summarized in Table 2. All women diagnosed with pre-eclampsia had proteinuria and one had abnormal liver function. Serum CXCL12 levels in pre-eclampsia were significantly increased compared to gestation matched, healthy controls [2000 SD 402 pg/ml and 1484 SD 261 pg/ml, respectively, $P = 0.01$] (Fig. 2). Amongst the women with pre-eclampsia, serum CXCL12 did not correlate with either gestational age at the onset of disease ($R = 0.33$) or gestation at delivery ($R = 0.08$).

3.3. Plasma CXCL12 levels at week 20 of gestation prior to the onset of pre-eclampsia

Table 2 shows the maternal characteristics and pregnancy outcome of participants. Nineteen out of the 20 women had significant proteinuria with hypertension, and one woman met the definition of pre-eclampsia as she had HELLP syndrome. Amongst the women with pre-eclampsia, three developed abnormal liver function, two had acute renal insufficiency and one woman had a placental abruption. Plasma CXCL12 levels at 20 weeks' gestation were not significantly different in women who later developed pre-eclampsia compared to women with a healthy pregnancy outcome [1183 (SD 336) vs 1036 (SD 144) pg/ml; $P = 0.09$] (Fig. 2). Given the trend to slightly elevated CXCL12 prior to pre-eclampsia, we explored whether CXCL12 plasma levels at week 20 correlated with gestation at the onset of pre-eclampsia or delivery. Amongst the women with pre-eclampsia, plasma CXCL12 at 20 weeks did not correlate with either gestational age at the onset of disease ($R = 0.095$) or gestation at delivery ($R = 0.07$).

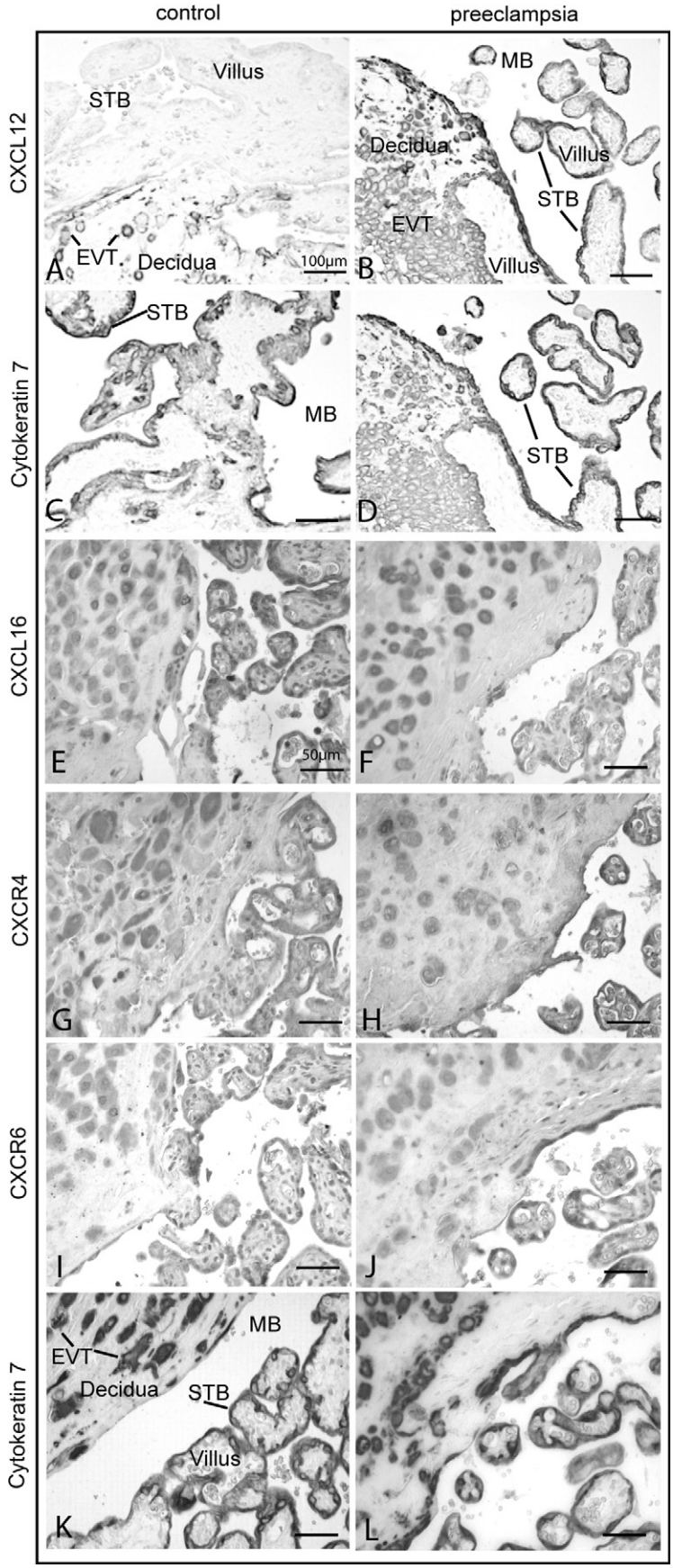

Fig. 1. CXCL12, CXCL16, CXCR6 and CXCR4 in placental tissue. CXCL12 expression by syncytiotrophoblasts is increased in pre-eclampsia. CXCL12 (A), CXCL16 (E), CXCR4 (G), CXCR6 (I) protein expression in placental tissue samples from control subjects ($n = 8$) in comparison to subjects with pre-eclampsia (B, F, H, J; $n = 8$). Anti-cytokeratin 7 was used for the detection of cytotrophoblast cells in placentae from control (C and K), and pre-eclamptic tissues (D and L). Maternal blood (MB), Syncytiotrophoblast (STB), extravillous trophoblast (EVT). Scale bar: 100 μm (A–D) and 50 μm (E–L).

Table 2
Clinical characteristics of participants in studies to evaluate CXCL12 levels in blood.

Maternal characteristics	After diagnosis of preeclampsia study			Prior to preeclampsia study		
	Controls ($n=14$)	Preeclampsia ($n=14$)	P value	Controls ($n=20$)	Preeclampsia ($n=20$)	P value
Age (years)	32.4 (3.7)	29.2 (5.4)	0.08	31.3 (4.7)	30.0 (4.7)	0.40
Ethnicity						
Caucasian	9 (64%)	6 (43%)	0.45	18 (90%)	14 (70%)	0.24
Other	5 (36%)	8 (57%)		2 (10%)	6 (30%)	
Body mass index (kg/m^2)	22.5 (4.0)	24.4 (4.4)	0.23	23.7 (3.1)	26.5 (3.9)	0.01
Smokers, n (%)	3 (21%)	2 (14%)	1.0	1 (5%)	0 (0%)	0.33
Gestation at sampling (weeks)	35.6 (3.3)	35.9 (2.8)	0.83	19.6 (0.6)	19.9 (0.7)	0.19
Blood pressure (mmHg)						
<20 w systolic[a]	114 (14)	113 (14)	0.85	106 (10)	117 (12)	<0.01
<20 w diastolic[a]	63 (7)	70 (11)	0.05	63 (9)	73 (10)	0.001
Systolic at sampling	113 (9)	135 (16)	<0.001	104 (9)	117 (10)	<0.001
Diastolic at sampling	71 (8)	91 (11)	<0.0001	63 (7)	70 (9)	<0.01
End of pregnancy						
Systolic blood pressure	122 (7)	168 (18)	<0.0001	121 (12)	160 (15)	<0.0001
Diastolic blood pressure	75 (8)	107 (6)	<0.0001	74 (8)	102 (8)	<0.0001
Infant characteristics						
Gestation at delivery (weeks)	39.7 (1.6)	36.9 (1.6)	<0.001	39.9 (1.0)	36.1 (3.2)	<0.001
Birthweight (g)	3535 (340)	2647 (605)	<0.0001	3494 (457)	2643 (787)	<0.001
SGA < 10th customised centile	0	7 (50%)	<0.01	0	8 (40%)	<0.01

Mean (SD) or n (%).
[a] Blood pressure was measured at the first antenatal visit in after diagnosis of preeclampsia study and at 15 ± 1 weeks in prior to preeclampsia study.

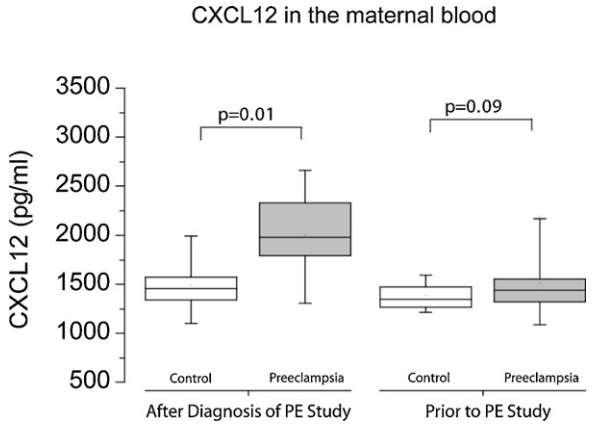

Fig. 2. CXCL12 levels are increased in blood of pre-eclamptic patients. Total CXCL12 concentration (pg/ml) was assayed in serum collected after the diagnosis of pre-eclampsia (PE) in comparison with gestation matched controls (After diagnosis of PE study) and in 20 ± 1 weeks plasma prior to the onset of pre-eclampsia and controls with an uncomplicated pregnancy outcome (Prior to PE study). Box plots with median and ranges are shown.

4. Comment

Taken together, these results show that the syncytiotrophoblast layer of the placenta from subjects with pre-eclampsia has up-regulated CXCL12 protein expression. Furthermore we found that compared to gestation matched healthy controls, CXCL12 levels in the maternal blood are elevated in women with diagnosed pre-eclampsia, indicating a possible involvement of the chemokine in the manifestation of the disease. Prior to the onset of pre-eclampsia, plasma CXCL12 was not significantly elevated at 20 weeks' gestation. Whilst CXCL12 levels at 20 weeks did not correlate with the gestational age at the onset of pre-eclampsia, our study was underpowered to investigate the subgroup with early onset disease. To shed further light on the role of CXCL12 in pre-eclampsia, it would be of interest to investigate plasma CXCL12 levels prior to the development of early onset disease or pre-eclampsia with a small for gestational age infant.

Our laboratory and other groups have studied the expression and function of chemokines and their receptors in the placenta of healthy subjects [14–16,22]. In this context, the CXCL12/CXCR4-axis appears to be important for placental development. Throughout pregnancy, CXCL12 is expressed by extravillous cytotrophoblasts in the uterine wall and in remodelled blood vessels. CXCL12 secretion by extravillous cytotrophoblasts has chemotactic as well as chemokinetic functions and attracts natural killer cells, which comprise 70% of all lymphocytes in the decidua, to the maternal–fetal interface [17].

CXCL12 is rarely detected in the syncytiotrophoblast layer especially in healthy second and third-trimester pregnancies [16]. In the present study, we show that in addition to the characteristic localization of CXCL12 to extravillous cytotrophoblasts, the syncytiotrophoblast layer also expresses CXCL12 in pre-eclampsia. In contrast, the expression of the receptor CXCR4 and another chemokine receptor pair, CXCL16 and CXCR6 was not different between pre-eclampsia and control subjects. These results suggest that the up-regulation of CXCL12 in the syncytium is a distinct process in pre-eclampsia that is not part of a global perturbation of chemokine or receptor expression. The mechanisms that regulate CXCL12 expression during cytotrophoblast differentiation from progenitor cells to the syncytiotrophoblasts are unknown, as are CXCL12's biological actions in this location.

Our findings suggest that the syncitium of placental villi may be the source of the elevated levels of CXCL12 seen in maternal blood in pre-eclampsia. Alternative origins for the elevated circulating CXCL12 associated with pre-eclampsia include platelet activation or endothelial damage, processes known to result in the release of CXCL12 [23,24].

At the tissue level, CXCL12 serves as a chemotactic factor enhancing angiogenic signals and the recruitment of tissue-specific progenitors and immune cells [24,25]. Currently, its role in pre-eclampsia is uncertain but increased CXCL12 may be involved in immune cell recruitment or activation at the maternal–fetal interface or possibly, the repair of damaged endothelium in maternal or placental vessels. Circulating endothelial progenitor cells can be mobilised by CXCL12 and play an important role in angiogenesis associated with the repair of tissues after an ischemic insult [11,12,25] and the regeneration of endothelium [13]. We speculate that the elevated circulating levels of CXCL12 observed in pre-eclampsia may function as a rescue mechanism in the repair of maternal endothelium by facilitating the recruitment and homing of endothelial progenitor cells.

In conclusion, our data suggest that over-expression of CXCL12 in placental tissues at the maternal–fetal interface contributes to a pre-eclampsia-associated increase of circulating CXCL12 which is measurable in maternal blood. These findings support the hypothesis that an imbalance of angiogenic factors may play a role in the development of pre-eclampsia. In the future, it will be interesting to further examine this process and follow potential patho-physiologic consequences, particularly immunologic events such as natural killer cell attraction or stimulation, and the recruitment of endothelial progenitor cells to the site of placental growth.

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