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Inflammatory and Haemostatic Changes Following Pre-eclampsia: Potential Link with Development of Subsequent Cardiovascular Events?

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ABSTRACT

Pre-eclampsia (P-EC) is a major cause of maternal and neonatal mortality and morbidity. Despite intensive research, its aetiology remains poorly understood. However, underlying maternal cardiovascular risk factors are thought to be implicated. Changes in the maternal vasculature and coagulation profile may predispose women with P-EC to subsequent adverse cardiovascular consequences. Here we investigate the relationship between circulating levels of haemostatic factors and inflammatory cytokines in women with a previous history of P-EC.

The participents included 26 women who had had P-EC within the last three years and were more than 6 months postpartum and 14 age-matched healthy women with no past history of P-EC. Blood was collected and assayed for plasma IL-6, IL-8, TNF-α and IL-10, Tissue Factor (TF) and TF-Pathway Inhibitor (TFPI), using Enzyme-Linked Immunosorbent Assays.

Individually, plasma TF, IL-6, IL-8 and IL-10 levels increased in the P-EC group compared with their normal counterparts, whereas plasma TFPI and TNF-α level were reduced. Plasma TF/TFPI ratios and IL-10 values were significantly increased in the P-EC group compared with controls ($p<0.05$, $p<0.01$, respectively). There were positive and significant correlations between TFPI and IL-10 ($r= 0.5$; $p<0.01$) and TF/TFPI ratio and IL-10 ($r= 0.31$; $p<0.041$), and between IL-6 and TNF-α ($r=0.71$; $p<0.001$) and IL-6 and IL-10 ($r=0.42$; $p<0.01$).

In conclusion, our results suggest the presence of elevated inflammatory cytokines and an imbalance of the haemostatic system in women with a past-history of P-EC, which may contribute to the known increased risk of cardiovascular disease in these women later in life.

Keywords

Normal pregnancy, Pre-eclampsia, Post-delivery, Haemostasis, Inflammation, Cardiovascular disease.

Introduction

Pre-eclampsia (P-EC) is a pregnancy-specific syndrome. It is the second most common cause of maternal mortality and morbidity worldwide [1], affecting 5–7% of all pregnancies [2]. P-EC is believed to be of multifactorial origin. It is widely accepted that the placenta, has a major role in the development of P-EC [3]. The onset, severity and progression of P-EC are affected by the maternal response to factors and proteins derived from the placenta. Pre-eclampsia is generally defined by hypertension and proteinuria after 20 weeks' gestation in a previously normotensive woman [2].

During a normal pregnancy, the maternal spiral arteries are reconstructed to help the body cope with the increase in maternal

circulation linked to placental perfusion. Upon entry of the foetal syncytial trophoblasts, the vessels dilate, enlarge and become flaccid. These changes to the blood vessels do not occur in pre-eclamptic pregnancies and, as a result, the placenta is prevented from embedding into the maternal blood vessels. This can also cause Intrauterine Growth Retardation and have a range of other effects on foetal development. The maternal immune response to feto-placental factors is likely to be involved in orchestrating the platelet activation and vascular endothelial damage characteristic of the maternal disease [4]. T-cells may also play a role in the development of P-EC induced hypertension [5].

Inflammatory cytokines may play a role in the development of acute inflammation. The acute inflammatory phase i.e., the first response to damage can be characterised by an increase in blood flow and permeability of vessels, together with a build-up of fluid, leukocytes and cytokines. In contrast, in the chronic inflammatory process, humoral and cellular immune responses to the pathogens can be detected at the injury site. A number of soluble factors influence leukocyte recruitment through an increased expression of cellular adhesion molecules and chemo-attractants [6]. Such soluble factors also regulate the activation of resident cells (such as fibroblasts, endothelial cells, tissue macrophages and mast cells) and with the inflammatory cells create a systemic response. The cytokines that mediate acute inflammatory reactions include Interleukin-1 (IL-1), Interleukin-6 (IL-6), Interleukin-8 (IL-8) Interleukin-11 (IL-11), Tumour Necrosis Factor Alpha (TNF-α), and other Chemokines. Granulocyte Colony Stimulating Factor and Granulocyte Monocyte Colony Stimulating Factor are also involved.

Tissue Factor (TF) is the main cellular initiator of blood coagulation. When the endothelial lining is damaged, TF binds to FVII in the presence of calcium (TF: FVII/FVIIa) and proteolytically activates downstream coagulation factors, eventually resulting in thrombin formation and fibrin generation [7]. Upon tissue injury or trauma, TF is expressed by monocytes, granulocytes, platelets and endothelial cells. It is regulated by bacterial lipopolysaccharide (LPS), complement C5a, hypoxia and pro-inflammatory cytokines (e.g., Interleukins, TNF-α, C-reactive protein and IFN) [8]. In vivo, TF is regulated by a specific inhibitor known as Tissue Factor Pathway Inhibitor (TFPI), which under normal condition, is synthesised primarily by the vascular endothelium.

Previously, we studied TF and TFPI levels in women who have had P-EC compared with normal counterparts [9]. In the present study we investigate the relationship between these and inflammatory cytokines (IL-6, IL8, IL10 and TNF-α) in women with a past medical history of P-EC.

Material and Methods

Subjects

Ethical approval was granted for the study by the Southampton and South West Hampshire Research Ethics Committee (REC reference number is 05/Q1702/131). Informed consent was obtained from all participants. The participants were asked to complete a general medical questionnaire to assess inclusion and exclusion criteria. A case-controlled study design was used to evaluate plasma TF and TFPI levels, as well as pro-inflammatory (IL-6, IL-8 and TNF-α) and an anti-inflammatory (IL-10) cytokines in 26 women who had a history of P-EC during previous pregnancies (spanning January 2008–October 2011) and 14 age-matched healthy women who have never had P-EC in previous pregnancies.

Inclusion criteria

Inclusion criteria for the study group was that participants had experienced P-EC between January 2008 and October 2011; for the control group, participants were women within the same age range but with no past history of P-EC.

Exclusion criteria

Exclusion criteria common for the two groups were: current pregnancy (including women who had given birth in the previous 6 months); chronic hypertension and obesity; the presence of cardiovascular, autoimmune and hepatic diseases; connective tissue disorders; diabetes; coagulation disturbances; and cancer. Women on anticoagulants or corticosteroid therapy were also excluded from the study.

Sample size

A sample size calculation was performed, based on a 0.6 correlation coefficient between the TF, TFPI and inflammatory cytokines levels and P-EC. The p value to assess this association was set to 5%, two-sided. The power was 0.95. Given these criteria, 30 subjects would need to be recruited in each arm, taking into account a dropout-rate of 25%.

Specimen collection

A 5 mL specimen of venous blood was collected using a 21-gauge needle, into vacutainer tubes containing 3.8% tri-sodium citrate. These then were centrifuged at 3000 rpm for 10 minutes at room temperature. Plasma samples then were immediately isolated and transferred into 250 µl aliquots, which then were stored at -86°C until used for batch-wise analysis. For each assay, a previously unthawed aliquot was used.

Assays

Commercially available enzyme-linked immuno-sorbent assay (ELISA) assays were used to measure IL-6, IL-8, IL-10 and TNF-α, according to the manufacturer's guidelines (R&D Systems, UK). The intra- and inter-assay coefficient of variations (CV) for TF and TFPI were 3.4 and 5.7%, and 3.6 and 5.9%. For cytokines these were 1.7 and 2.0%, 7.3 and 9.4%, 4.6 and 8.5%, 3.1 and 7.4%, respectively.

Statistical analysis

Data were analysed using the Statistical Package for the Social Sciences (SPSS), Version 23 for Windows (Statistical Analysis System, Chicago, Illinois, USA). The Shapiro-Wilk test was used to test normality. Data were not normally distributed, so results are expressed as a Box and Whisker plot, with outliers additionally identified, or as Median and Interquartile Range

(IQR). Comparisons between two groups were performed by Kruskal-Wallis and Mann-Whitney tests. P-values of <0.05 were considered statistically significant. Correlations between the pro-inflammatory cytokines were assessed by Spearman's correlation test. Assays results were either recorded as pg/ml of the original specimen for the inflammatory cytokines or ng/ml for TF and TFPI.

Results

Demographic data

The clinical and demographic characteristics of the study groups are shown in table 1. No significant differences were found in the participants' age, body mass index, smoking status and alcohol consumption; however, six of the participants had a family history of P-EC whilst eight had a previous history of hypertension. The control group also was sampled at least a year after last delivery.

Characteristics	Preeclampsia	Controls
Number of participants	26	14
Mean age of participants	33.6 years	30.5 years
Minimum to maximum age	24-47 years	22-43years
Mean Body mass index of participants	27.1	24.9
Family history of P-EC	6	None
Family history of hypertension	8	4
Family history of type II diabetes	4	7
Family history of myocardial infarction	5	5
Family history of deep vein thrombosis	1	None
Ethnic group	24 British; 1 European; 1 Black African	10 British;1 European; 1 Mexican; 1 Black African; 1 Indian
Current smokers	4	2
Regular exercise	5	3
Alcohol consumers	22 (Average Units)	10 (Average Units)
Unit consumed	3.3 (Per Week)	3.2 (Per Week)
Personal history of Anaemia; during or after pregnancy	3	None
Use of contraception	6	9

Table 1: Subjects' demographic and clinical data. Data shown are the results of the general medical questionnaire completed by the study population.

Inflammatory cytokines

Plasma IL-10 levels were significantly raised in women with a history of P-EC post-pregnancy compared with controls (P<0.01; Figure 1). Plasma IL-6 levels appeared elevated in the study group compared with controls, but this rise was not significant (Figure 2). On the other hand, plasma TNF-α levels tended to be reduced in the study group, compared to controls, but not reaching statistical significance (Figure 3). For the other inflammatory cytokines measured, we observed a trend towards raise levels in the P-EC group compared with controls. There were positive and significant correlation between IL-6 with TNF-α and IL-10; r=0.71; p<0.001; r=0.42; p<0.01), respectively. No statistically significant correlations were observed between IL-8 and IL-10 (r=0.103; P<0.27), nor between TNF- and IL-10 (r=0.1; p<0.32).

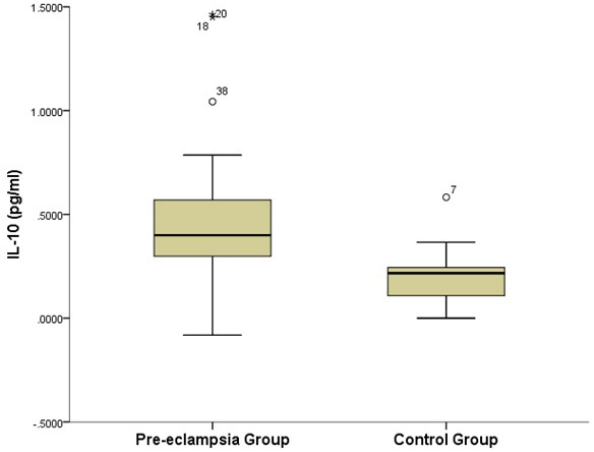

Figure 1: Plasma IL-10 in women with P-EC and controls. Results are shown as Box and Whisker plot. The bottom and top of the 'box' represent the 25th and 75th centile, respectively, while the line within the box represents the median value. The 'whiskers' represent the range.

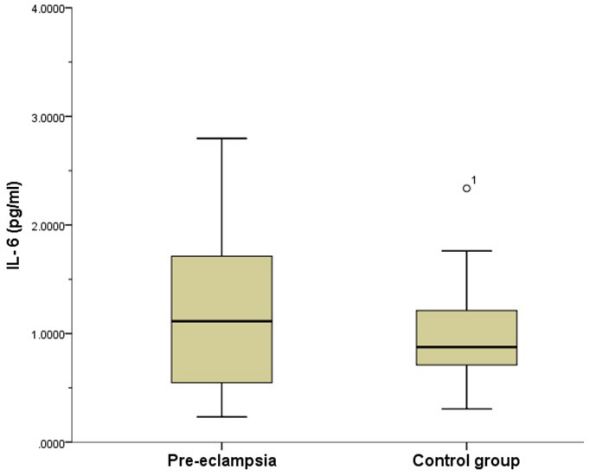

Figure 2: Plasma IL-6 in women with P-EC and controls. Results are shown as Box and Whisker plot.

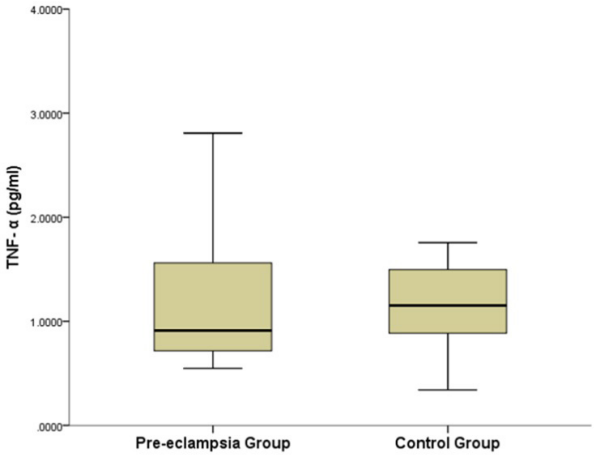

Figure 3: Plasma TNF-α in women with P-EC and controls. Results are

shown as Box and Whisker plot.

Tissue factor and tissue factor pathway inhibitor

There was a slight increase, albeit statistically insignificant, in plasma TF levels when P-EC group was compared to the control group. On the other hand, plasma TFPI levels were slightly reduced in the P-EC group; however, this also was not significant. The TF/TFPI ratio was significantly raised in women P-EC (Median=0.01; IQR=0.007-0.014) compared to controls (Median=0.006; IQR=0.005-0.009; $p<0.05$).

The relationship between inflammatory cytokines and tissue factor, tissue factor pathway inhibitor. There were positive and significant correlations between IL-10 and TFPI ($r= 0.5$; $p<0.01$), as well as IL-10 and TF/TFPI ($r= 0.31$; $p<0.041$).

Discussion

Changes to the immune system, haemostasis and endothelial status may have a profound effect on maternal health. However, it is not yet fully understood how such changes relate to the aetiology and pathogenesis of P-EC [10]. In this study, we show that mothers who have experienced P-EC show altered levels of both inflammatory and coagulation markers postpartum. Our results have several implications for clinical practice. First, several studies have suggested that a past history of P-EC is associated with an increased risk of cardiovascular disease later in life [11]. Knowledge of the factors underlying this association is lacking. The findings of our study may therefore be relevant in broadening this understanding especially with respect to the monitoring of subsequent risk progression. Second, alterations in immune and haemostatic factors, in women with previous P-EC, during a non-pregnant state, may be of aetiological significance in explaining maternal disease susceptibility.

The immune system pertaining in pregnant women is key to the maintenance of a normal healthy pregnancy. The pro-inflammatory Th1 cells which produce interleukin IL-2, interferon γ and TNF-α are involved in cell-mediated responses and delayed-type hypersensitivity reactions, whereas the anti-inflammatory Th2 cells produce IL-4, IL-5, IL-10 and IL- 13 are involved in evoking humoral immunity. In order for a pregnancy to remain successful, the maternal immune response must shift from Th1 to Th2 phenotypes [12]. Pregnant women with P-EC have been shown to have increased concentrations of serum IL-6, IL-8 and soluble IL-4 receptors [13]. Th1 and Th2 cytokines reciprocally regulate one another's functions [14]. In P-EC, pro-inflammatory cytokines are predominantly sourced in the maternal circulation; however, both monocytes and macrophages are also known to express certain cytokines when activated by non-specific immune reactions [15]. Considering the immunological impact on P-EC progression, we investigated the relationship between plasma cytokines in women with a past history of P-EC and age-matched healthy women with no previous history of P-EC. All plasma cytokines measurements were made at least six months postpartum (Table 1).

It has been reported that IL-6 plays a vital role in regulating the body's immune response, and has a significant effect on B lymphocyte differentiation and the production of acute phase proteins, such as CRP [8]. Similarity, in women with P-EC, a significant increase in plasma IL-6 levels was observed between the first- and third-trimester; the same was not seen in the control group [16]. Recently our group demonstrated that women with a history of previous P-EC show altered levels of circulating inflammatory markers and an increased acute-phase response to influenza vaccination postpartum [17]. In the present study we observed raised levels of IL-6 in postpartum P-EC women compared with the control group, although this was not statistically significant.

There are many potential sources of IL-8 spanning a number of cell types (all nucleated cells). The main sources of IL-8 are monocytes and macrophages, as the role of IL-8 is to recruit monocytes and neutrophils, the main cells at work during an acute inflammatory response. We found a slight increase in plasma IL-8 level of post-pregnancy pre-eclamptic women compared to their counterparts; this in agreement with other reports, where IL-8 has been shown to increase in pre-eclamptic subjects [18].

It is noted that TNF-α promotes apoptosis and further encourages leakage in endothelial vessels, resulting in a systemic endothelial activation response and some of the symptoms of P-EC. The low TNF-α level in postpartum pre-eclamptic women reported in our studies are in accordance with results from another study [19], which reported a reduced TNF-α in its patient group over controls. The reduction in TNF-α levels seen in the two studies could be attributed to its short half-life.

IL-10 acts as an immuno-regulatory cytokine in balancing any increases of pro-inflammatory cytokines through its anti-inflammatory action. Khalid et al. [20] reported that IL-10 plays a key role in regulating inflammatory responses in the placenta and is thought to be essential for a healthy pregnancy, and that women with P-EC had raised levels of IL-10 in comparison to normotensive women. Similarly Benian et al. [21] found increased plasma levels of IL-10 in pre-eclamptic subjects and attributed this to the pathophysiological processes occurring in P-EC. In our study, plasma IL-10 was significantly increased in P-EC compared to controls.

Inflammatory cytokines activate coagulation through TF and protein C expression, and inhibition of fibrinolysis. Inflammation is modulated by the components of thrombin/fibrin pathway [22]; endothelium and monocytes/macrophages become activated and IL-1 and IL-8 secretion increases [23]. Similarly, immunoglobulins could potentially exert many prothrombotic and antifibrinolytic activities, especially through interaction with mast cells [24].

Intravascular coagulation activation may play a part in the pathogenesis of P-EC [25]. It has been suggested that coagulation abnormalities may be more relevant to fetal outcome than blood pressure [26] and coagulation indices may be of value in monitoring the P-EC progress [27]. Indices of a prothrombotic

state correspond with those of inflammation and may be related to the underlying vascular disease and co-morbidities [28]. Vascular changes are prominent features of P-EC [25]. Indeed, both cardiovascular disease and P-EC share many risk factors [9].

Previously we investigated the relationship between TF and TFPI in women who had P-EC compared to their normal counterpart and reported a significant increase in TF/TFPI ratio in women with P-EC [9]. Our result suggested an imbalance between TF/TFPI levels in women with past history of P-EC post-pregnancy and we proposed that such imbalance may contribute to the development of a maternal hypercoagulable states and may predispose women with a history of P-EC to cardiovascular risks later in life [9].

In the present work we examined the relationship between TF, TFPI, TF/TFPI ratio and both pro- and anti-inflammatory cytokines levels in the same cohort of subjects. We observed a positive and significant correlations between IL-10 and TFPI ($r= 0.5$; $p<0.01$) and between IL-10 and TF/TFPI ratio ($r= 0.31$; $p<0.041$). This is in agreement with previous report, where both IL-10 and TFPI levels were reported to be higher in severe pre-eclamptic women compared to the controls post-delivery [29]. Taken together, these results lend further support to the link between inflammation and haemostasis in disease conditions [30]. It also suggests that both anti-inflammatory cytokines and anti-TF-dependent coagulation pathway are activated in women with P-EC, arguably due to endothelial dysfunction and vascular damage, both of which are known to be associated with P-EC. Indeed, vascular endothelial changes are recognised as being a central process in pregnancy-induced hypertension [31,32].

In conclusion, we acknowledge that this work should be seen as a pilot study, which might add beneficial information to more focused studies in the future. Our results suggest the presence of elevated inflammatory cytokines and an imbalance of the haemostatic system in women with a past-history of P-EC. This may contribute to the known increased risk of cardiovascular disease in these women later in life.

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