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

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\textbf{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 participants 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.

\textbf{Keywords}

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

\textbf{Introduction}

Pre-eclampsia (P-EC) is a pregnancy-specific syndrome. It is the second most common cause of maternal mortality and morbidity worldwide \cite{1}, affecting 5–7% of all pregnancies \cite{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 \cite{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 \cite{2}.

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

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 unwahed 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 analysised 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.
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.

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

| 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  | 22-43 years |
| 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        |

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).
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]. Similarly, 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 state 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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