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Increased Neutrophil Activation and Plasma DNA Levels in Patients with Pre-Eclampsia

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Abstract

Pre-eclampsia (PE) is a chronic inflammatory disease in pregnancy, which is associated with enhanced blood coagulation and high thrombotic risk. To date, the mechanisms underlying such an association are not fully understood. Previous studies reported high levels of plasma deoxyribonucleic acid (DNA) in PE women, but the cellular source of the circulation DNA remains unknown. In this study, we tested the hypothesis that activated neutrophils under-going cell death, also called NETosis, maybe responsible for the elevated plasma DNA levels in PE women. We analysed plasma samples from non-pregnant, normal pregnant and PE women and found high levels of double-stranded DNA, myeloperoxidase (an abundant neutrophil granular enzyme) and histones (the major nucleosome proteins) in PE-derived samples, indicating

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Conflict of Interest
None.

increased NETosis in the maternal circulation. The high plasma DNA levels positively correlated with enhanced blood coagulation in PEwomen. When isolated neutrophils from normal individuals were incubated with PE-derived plasma, an elevated NETosis-stimulating activity was detected. Further experiments showed that endothelial micro-particles, but not soluble proteins, in the plasma were primarily responsible for the NETosis-stimulating activity in PE women. These results indicate that circulating micro-particles from damaged maternal endothelium area potent stimulator for neutrophil activation and NETosis in PEwomen. Given the pro-coagulant and pro-thrombotic nature of granular and nuclear contents from neutrophils, enhanced systemic NETosis may represent an important mechanism underlying the hyper-coagulability and increased thrombotic risk in PE.

Keywords

hyper-coagulability; Hypertension; micro-particles; NETosis; Neutrophils; Pre-eclampsia

Introduction

Pre-eclampsia (PE), defined as newly onset hypertension and proteinuria after 20 weeks of gestation, is a leading cause of pregnancy-related morbidity and mortality.¹ The disease is associated with a hyper-coagulable state, as indicated by high levels of plasma pro-coagulant proteins, enhanced platelet activation and endothelial dysfunction.^{2–4} These protein profile and vascular changes increase the risk of thrombosis. It is well known that PE women are more likely to have microvascular clotting and tissue ischaemia, which could lead to organ failure.⁵

During pregnancy, uterine spiral artery remodelling is essential for adequate maternal–foetal blood flow.^{6,7} In PE, genetic and environmental factors that impair uterine spiral artery remodelling reduce the uteroplacental perfusion, causing placental hypoxia.^{6–11} Chronic placental hypoxia and oxidative stress, in turn, trigger local and systemic cellular and inflammatory responses that are pro-thrombotic. In a recent study,¹² for example, we found that high-mobility group box 1 (HMGB1), a pro-inflammatory protein,¹³ released from hypoxic trophoblasts promoted endothelial micro-particle (MP) production and blood coagulation in PE patients. We also showed that MPs from HMGB1-stimulated endothelial cells activated neutrophils in vitro.¹²

Neutrophils are major players in innate immunity. Upon challenge, neutrophils undergo a distinct process of cell death, called NETosis, during which chromatin fibres and granular contents are released to form neutrophil extracellular traps (NETs) that capture and kill invading pathogens.¹⁴ NETosis also occurs in non-infectious conditions such as autoimmune and vascular diseases, diabetes and cancer.^{15–19} As NET components, including long fragments of deoxyribonucleic acid (DNA), histones and neutrophil elastase, are procoagulant and pro-thrombotic, excessive NETosis can lead to arterial and venous thrombosis.^{20–24}

To date, increased neutrophil activation in the maternal circulation has been reported in PE.^{25–28} There are also reports of local neutrophil activation in the placenta in response to

inflammation and oxidative stress in PE women.^{19,29} Moreover, NET formation has been detected in the intervillous space in PE women,^{30–32} consistent with local neutrophil activation in the hypoxic placenta. Given the pro-coagulant nature, NET components are expected to promote thrombus formation in the microvasculature, exacerbating placental ischaemia in PE.

To understand the role of neutrophil activation and NETosis in PE-associated hyper-coagulability, we analysed plasma levels of NET components, including double-stranded DNA, myeloperoxidase (MPO) and nucleosome proteins, in non-pregnant, normal pregnant and PE women. We also examined potential mechanisms underlying neutrophil activation and NETosis in PE women. Our results show that levels of plasma DNA, MPO and histones are elevated and associated with enhanced blood coagulation in PE women. Moreover, our data indicate that endothelial cell-derived MPs, but not soluble plasma factors, are primary stimulators for NETosis in the maternal circulation in PE women.

Materials and Methods

Participants

Normal and hypertensive individuals and pregnant women with or without PE were recruited at the Second Affiliated Hospital of Soochow University. The study was approved by the ethics committee of Soochow University and conducted in accordance with the Declaration of Helsinki. All participants gave written informed consent. Participant characteristics are shown in ►Supplementary Tables S1 and S2 (available in the online version). PE is diagnosed based on the published guidelines,¹ that is, newly onset hypertension (140/90 mm Hg) and proteinuria (300 mg/24 hour) at 20 weeks of gestation. For PE women, blood samples were taken after the disease was diagnosed and before treatments started. In controls, blood samples were taken from age-matched non-pregnant women and age- and gestation-matched normal pregnant women. In a sub-set study, blood samples were taken from normal pregnant and PE women before (30–34 weeks of gestation) and after (1 week) child delivery. Blood cell counts and coagulation assays were done at the hospital laboratory.

Plasma Sample Preparation

Venous blood samples were collected into coagulation tubes with 3.2% sodium citrate and centrifuged at $2,000 \times g$, at 4°C for 10 minutes. Plasma was taken, aliquoted and snap-frozen in liquid nitrogen, and stored at –80°C till use.

Quantification of Plasma DNA

Plasma DNA levels were measured by a fluorescent method for double-stranded DNAs (PicoGreen dsDNA quantitation assay, Invitrogen, P7589). Briefly, plasma (50 µL) was diluted (1:10) with phosphate-buffered saline (PBS) in assay plates (Corning, 3925). SytoxGreen reagent (2 µM in final concentration) was added to the plasma-containing plates that were monitored in a plate reader (SpectraMax M5, Molecular Devices) at 485 nm (excitation) and 538 nm (emission) wavelength, respectively, at room temperature. Fluorescent signals were recorded and DNA concentrations were calculated based on standard curves.

Plasma MPO

Plasma MPO levels were measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, DMYE00B). Plasma was diluted with a sample solution (1:10) and added to a micro-titer plate (50 μ L/well). MPO was detected by the antibodies included in the kit. The optical absorbance was monitored at 450 nm for 30 minutes in a plate reader (SpectraMax M5, Molecular Devices). MPO concentrations were calculated based on standard curves.

Plasma Nucleosome Proteins

Plasma nucleosome proteins were quantified by ELISA (Cell Death Detection kit, Roche, 11774425001) that included monoclonal antibodies recognizing histones H1, H2A, H2B, H3 and H4 in nucleosome DNA-protein complexes. Plasma histone H3 protein levels were measured by a colorimetric assay (EpiGentek, P-3097–96). All procedures were performed according to the manufacturers' instructions. One unit of nucleosome proteins is referred to be the average nucleosome protein level in pooled plasma from non-pregnant controls.

Isolation of Human Neutrophils

Peripheral blood samples from normal individuals were collected into coagulation tubes with 3.2% sodium citrate. DextranT-500 (Pharmacia, 17–0320–01, 1% final concentration) was added to PBS-diluted blood (1:1). After 1 hour at room temperature, neutrophils in the upper layer were collected and centrifuged over Percoll II (GE Healthcare, 17–0891–09) discontinuous gradients (75 and 60%). Neutrophils in the middle layer were collected and washed twice with PBS. The purity of the isolated neutrophils was > 95%, as assessed by Wright–Giemsa's staining and microscopic examination (► Supplementary Fig. S1, available in the online version).

Analysis of Plasma MPs

Plasma MPs were isolated from platelet-poor plasma by sequential centrifugations, as described previously.¹² MP-free plasma was collected and pelleted MPs were washed and re-suspended in RPMI 1640 (Hyclone). MP surface markers were analysed by flow cytometry, using antibodies against annexin V (BD Biosciences, 556547, for total MPs), CD31 (BD Biosciences, 555448, for endothelial MPs), CD41a (BD Biosciences, 555467, for platelet MPs), CD235a (BD Biosciences, 340947, for red blood cell MPs) and CD45 (BD Biosciences, 347464, for leukocyte MPs), as described previously.¹²

Quantification of Neutrophil-Released DNA

Freshly isolated neutrophils (1×10^5) in RPMI 1640 medium with calcium nitrate (0.42 mM) and magnesium sulphate (0.41 mM) were added to 96-well plates (100 μ L per well) and stimulated with phorbol 12-myristate 13-acetate (PMA) (50 nM, Sigma), plasma (1:1 ratio), MP-free plasma (1:1 ratio) or isolated plasma MPs ($0.5\text{--}2 \times 10^6$ /mL) from non-pregnant, normal pregnant and PE women. After 4 hours at 37°C, DNA levels in the conditioned medium were measured by the Quant-iT PicoGreen dsDNA assay, as described above.

Immunofluorescent Staining of NET-Forming Cells

Isolated human peripheral neutrophils (1×10^6 cells) on coverslips pre-treated with 0.001% polylysine were stimulated with PMA (50 nM), plasma, MP-free plasma or isolated MPs from non-pregnant, normal pregnant and PE women. After 4 hours at 4°C the cells were fixed in 4% paraformaldehyde, treated with (permeable) or without (non-permeable) 0.5% Triton X-100, and blocked with 5% bovine serum albumin for 1 hour. The cells were then incubated with an anti-elastase antibody (1:200; Abcam, ab68672) at 4°C overnight. An Alexa-594-conjugated secondary antibody (1:200; Invitrogen, A11012) was used for detection, as described previously.³³ Coverslips were mounted on Fluoromount-G medium containing 4',6-diamidino-2-phenylindole (DAPI) (Southern Biotech) to stain DNA. Percentages of NET-forming neutrophils were examined under a confocal microscope (FV1000MPE, Olympus) at $\times 400$ magnification. A NET-forming cell was defined as a cell with fluorescence-stained DNA filaments covering an area larger than a neutrophil size.³⁴

Thrombin Generation Assay

A fluorimetric assay (Thrombinoscope BV, TS50.00) was used to measure thrombin generation in plasma from non-pregnant, normal pregnant and PE women, as described previously.¹² Fluorescent absorbance was monitored with an automated coagulation analyser (Ceveron α , TGA, Technoclone) to calculate thrombin generation.

Statistical Analysis

Data were analysed and graphed using the SPSS 17.0 and Prism 7 software. Equal variance and normality of the data were verified by Levene's test and Kolmogorov–Smirnov test, respectively. If the data passed the normality and equal variance tests, Student's *t*-test was used to compare two groups or one-way analysis of variance for comparisons among three or more groups. If the data did not pass the normality or equal variance test, Mann–Whitney test was used for two independent sample comparisons, and Kruskal–Wallis test and Mann–Whitney test with Bonferroni correction were used for multiple comparisons. Correlations were analysed by computing Pearson's correlation coefficient. Results were expressed as means $\pm$ standard deviation. *p*-Values of < 0.05 were considered statistically significant.

Results

Plasma DNA Levels in Pregnant Women

Compared with that in non-pregnant women (2.32 ± 0.44 $\mu\text{g/mL}$), plasma DNA levels were higher in normal pregnant women (2.96 ± 0.43 $\mu\text{g/mL}$, $p < 0.001$). The levels were even higher in PE women (3.87 ± 1.39 $\mu\text{g/mL}$, $p < 0.001$ vs. non-pregnant and normal pregnant women) (►Fig. 1A). In PE women, there were no correlations between plasma DNA levels and systolic (►Fig. 1B) or diastolic (►Fig. 1C) blood pressure. In normotensive and hypertensive non-pregnant women, plasma DNA levels were comparable (2.32 ± 0.44 vs. 2.50 ± 0.47 $\mu\text{g/mL}$, $p = 0.54$) (►Fig. 1D). The levels were also similar between normotensive and hypertensive males (2.40 ± 0.49 vs. 2.56 ± 0.46 $\mu\text{g/mL}$, $p = 0.56$) (►Fig. 1E). These results indicate that the elevated plasma DNA levels in PE women are likely

related to pregnancies but not hypertension per se. Consistently, plasma DNA levels decreased in normal pregnant and PE women after child delivery (►Fig. 1F).

Plasma MPO and Histone Levels in Pregnant Women

To understand the cellular origin of the detected plasma DNA, we analysed correlations between plasma DNA levels and blood cell counts. Significant correlations were found between the plasma DNA levels and white blood cell counts (►Fig. 2A) or neutrophil counts (►Fig. 2B) in PE women. In contrast, no such correlation was found with monocyte or lymphocyte counts (►Supplementary Fig. S2, available in the online version). To verify if the detected plasma DNAs were from activated neutrophils, we measured plasma levels of MPO (a neutrophil granular protein) and histones (nucleosome proteins) that are released during NETosis. Levels of plasma MPO (►Fig. 3A) and histones (►Fig. 3B), including histone H3 (►Fig. 3C), were higher in PE women, compared with those in non-pregnant and normal pregnant women. Moreover, levels of plasma MPO (►Fig. 3D) and histones (►Fig. 3E) positively correlated with plasma DNA levels. These data indicate enhanced neutrophil activation and NETosis in PE women.

DNA Release and NET Formation in Plasma-Treated Neutrophils

To understand the mechanisms underlying the enhanced NETosis in PE, we incubated normal peripheral neutrophils with plasma from non-pregnant and pregnant women, and measured DNA levels in the conditioned medium collected over time. Compared with those in the non-pregnant group, DNA levels were higher in the normal pregnant group (►Fig. 4A, $p < 0.001$). The levels were even higher in the PE group ($p < 0.001$ vs. non-pregnant and normal pregnant). To verify these results, we examined elastase- and DAPI-staining in the plasma-treated neutrophils. Under both permeable and non-permeable conditions, higher percentages of NET-forming cells were found in the neutrophils treated with PE-derived plasma (►Fig. 4B, $p < 0.001$ vs. non-pregnant and normal pregnant). These results indicate that plasma from PE women had an elevated NETosis-stimulating activity.

DNA Release and NETosis in MP-Stimulated Neutrophils

To test if plasma-soluble proteins or cellular components such as MPs were responsible for the observed NETosis-stimulating activity in PE-derived plasma, we incubated neutrophils with MP-free plasma or isolated MPs from non-pregnant, normal pregnant and PE women and measured DNA levels in the conditioned medium. DNA levels in the conditioned medium from neutrophils treated with PE-derived MP-free plasma were higher than that in the nonpregnant group ($p = 0.002$) but similar to that in the normal pregnant group ($p = 0.247$) (►Fig. 5A). In contrast, the levels were much higher in the medium from neutrophils treated with MPs from normal pregnant and PE women, with the highest values being in the PE group (►Fig. 5B). High levels of MPO were also found in the conditioned medium from the PE group (►Fig. 5C). As another control, endothelial cell-derived MPs stimulated MPO release from normal peripheral neutrophils (►Fig. 5D). In immunostaining and confocal microscopy, higher percentages of NET-forming cells were found when the neutrophils were treated with MPs from PE women (data not shown). These results indicate that MPs are mostly responsible for the NETosis-stimulating activity in the plasma from PE women.

Correlations between Plasma DNA Levels and Endothelial MPs

Recently, we reported high levels of MPs in PE women, most of which were of endothelial cell origin.¹² Consistently, we found positive correlations between DNA levels and numbers of total MPs (►Fig. 5E) or endothelial (CD31⁺/CD41⁻) MPs (►Fig. 5F) in plasma samples from the pregnant women. In contrast, no correlation was found between DNA levels and numbers of platelet (CD41a⁺)-, red blood cell (DC235a⁺)- or leukocyte (CD45⁺)-derived MPs in plasma from these women (►Supplementary Fig. S3, available in the online version).

Correlations between Plasma DNA Levels and Blood Clotting Parameters

NET components are pro-coagulant. We measured thrombin generation in plasma from non-pregnant, normal pregnant and PE women. PE-derived plasma was found to have the shortest thrombin generation time (►Fig. 6A) and the highest peak of thrombin generation (►Fig. 6B). Consistently, there were significant correlations between plasma DNA levels and clotting times (activated partial thromboplastin time and pro-thrombin time) (►Fig. 6C and D) and levels of anti-thrombin III (►Fig. 6E) and fibrinogen D-dimer (►Fig. 6F) in the pregnant women. These results support the idea that enhanced neutrophil activation and NETosis promoted blood coagulation in PE women.

Discussion

High levels of circulating foetal and maternal DNAs have been reported in PE.^{35–39} The circulating foetal DNA, which accounts for approximately 1% of total plasma DNA, may come from foetal nucleated red blood cells or damaged trophoblasts that entered the maternal circulation.^{36–39} It remains unknown if the high plasma DNA levels are caused directly by hypertension in PE women and what the cellular sources are for the maternal circulating DNA. In this study, we detected high levels of cell-free double-stranded DNA in plasma from PE women, which was associated with pregnancy but not high blood pressure. Moreover, we found that activated neutrophils that undergo NETosis are likely the primary source of the maternal circulating DNA, as indicated by high levels of MPO, an abundant neutrophil granular enzyme, and histones, the major nucleosome proteins, in plasma from PE women, which correlated with the plasma DNA levels. It should be pointed out that the PE plasma samples in our study were taken after the disease was diagnosed. Further studies are needed to examine if enhanced neutrophil activation occurs before the clinical symptoms of PE manifest.

PE is a chronic inflammatory disease.^{40–42} High levels of pro-inflammatory factors have been detected in PE women, which are expected to promote neutrophil activation, locally or systemically.^{12,31,43–48} Unexpectedly, we found that the NETosis-stimulating activity in PE-derived plasma was mainly from MPs but not soluble plasma proteins. Recently, we reported that most circulating MPs in PE women were from damaged maternal endothelial cells.¹² Consistently, we found a strong correlation between plasma DNA levels and circulating endothelial (CD31⁺/CD41⁻) MPs in PE women, supporting a role of endothelial MPs in inducing NETosis. In a previous study, NETosis was observed when isolated human neutrophils were incubated with MPs from cultured placental villous explants.³¹ It appears

that neutrophils in pregnant women are more susceptible to MP-mediated stimulation to undergo NETosis. Alternatively, levels of soluble pro-inflammatory proteins in PE-derived plasma were not high enough to directly trigger NETosis under our experimental conditions.

Innate immunity and blood coagulation are closely connected.⁴⁹ In primitive invertebrates such as horseshoe crabs that lack adaptive immunity, haemolymph coagulation acts as a primary anti-microbe defence mechanism.⁵⁰ When stimulated by bacterial lipopolysaccharides, horseshoe crab haemocytes release serine proteases to trigger coagulin protein aggregates that trap invading microbes. In mammals, NET components, including long DNA filaments and associated histones, enhance blood coagulation and platelet activation.^{20–22,24,51} Neutrophil-derived elastase also promotes factor XII-dependent coagulation and inactivates tissue factor pathway inhibitor.²³ Thus, the coupling of neutrophil activation and blood coagulation enhances innate immunity by restraining pathogens in localized blood clots. If neutrophil activation is uncontrolled, however, excessive NETosis can cause vascular damage and thrombosis. In PE woman, fibrin deposition and micro-infarctions in the placenta are common.^{5,52} In our study, we found strong correlations between plasma DNA levels and shortened blood clotting times, decreased anti-thrombin III levels and increased fibrinogen D-dimer levels in PE women, suggesting that high levels of circulating NET components enhance blood coagulation in PE women.

In summary, we detected enhanced systemic NETosis in PE women. Moreover, we identified endothelial MPs, but not soluble plasma proteins, as primary stimulators of NETosis in PE women. Previously, we and others have reported that pro-inflammatory proteins from the hypoxic placenta are responsible for maternal endothelial damage and MP production.^{12,53} These data suggest a mechanism, in which pro-inflammatory proteins from the hypoxic placenta stimulate maternal endothelial cells to generate MPs that, in turn, induce NETosis in the maternal circulation. High levels of NET components are expected to promote blood coagulation, contributing to the hyper-coagulable state common in PE. Additional studies are needed to directly verify NETosis in neutrophils isolated from PE women. These studies should help to determine if targeting neutrophil activation could be a therapeutic strategy to prevent or treat thrombophilia in PE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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What is known about this topic?

- Enhanced neutrophil activation and NETosis promote blood coagulation and increase thrombotic risk.
- PE is associated with thrombophilia, but the underlying mechanisms are not fully understood.

What does this paper add?

- This study indicates increased systemic NETosis in PE women, which correlates with enhanced blood coagulation.
- Endothelial cell-derived MPs, but not soluble plasma proteins, are primarily responsible for stimulating NETosis in the maternal circulation, thereby contributing to thrombophilia in PE women.

Fig. 1.

Analysis of plasma deoxyribonucleic acid (DNA) levels. (A) Plasma DNA levels in non-pregnant (Non-P), normal pregnant (NP) and preeclampsia (PE) women were measured by a fluorescent method. Correlations between plasma DNA levels and systolic blood pressure (SBP) (B) or diastolic blood pressure (DBP) (C) in PE women were analysed by computing Pearson's correlation coefficient. (D and E) Plasma DNA levels in normotensive (Normal) and hypertensive (Hypertension) non-pregnant women (D) and male individuals (E). (F) Plasma DNA levels in non-pregnant women (Non-P), and normal pregnant (NP) and PE women before (pre-) and after (post-) child delivery. Sample numbers (n) in each group are indicated. Data in (A) and (F) were analysed by Kruskal–Wallis and Mann–Whitney tests with Bonferroni correction for multiple comparisons. Data in (D) and (E) were analysed by Student's t -test.

Fig.2. Correlations between plasma deoxyribonucleic acid (DNA) levels and peripheral white blood cell (WBC) (A) or neutrophil (B) counts in preeclampsia (PE) women. Analysis was done by computing Pearson's correlation coefficient.

Fig. 3.

Plasma myeloperoxidase (MPO) and histone levels. MPO (A), histone (B) and histone H3 (C) levels were measured by enzyme-linked immunosorbent assay (ELISA) in plasma from non-pregnant (Non-P), normal pregnant (NP) and pre-eclampsia (PE) women. Sample numbers (n) in each group are indicated. Data were analysed by Kruskal–Wallis and Mann–Whitney tests with Bonferroni correction for multiple comparisons. ns, not statistically significant. Correlations between plasma deoxyribonucleic acid (DNA) levels and MPO (D) or histone (E) levels in normal pregnant and PE women were analysed by computing Pearson's correlation coefficient.

Fig. 4.

Deoxyribonucleic acid (DNA) release and neutrophil extracellular trap (NET) formation in plasma-treated neutrophils. **(A)** Neutrophils isolated from normal individuals were incubated with plasma from non-pregnant (Non-P), normal pregnant (NP) and pre-eclampsia (PE) women for 1 (left), 2 (middle) or 4 (right) hours (h). DNA levels in the conditioned media were measured by a fluorescent method. $n = 9$ for each group. **(B)** Neutrophils treated with plasma from non-pregnant (Non-P), normal pregnant (NP) and PE women were stained for elastase (red) and DNA (blue) under permeable (top row) or non-permeable (bottom row) conditions to identify NET-forming cells, as indicated by extracellular DNA filaments. Percentages of NET-forming cells in neutrophils treated with plasma from Non-P, NP and PE women were examined in 6 randomly selected fields under a high magnification ($\times 400$). $n = 3$ in each group. Quantitative data are presented in bar graphs. Data in **(A)** and **(B)** were analysed by one-way analysis of variance (ANOVA).

Fig. 5.

Deoxyribonucleic acid (DNA) and myeloperoxidase (MPO) release in micro-particle (MP)-treated neutrophils. Neutrophils from normal individuals were incubated with MP-free plasma (A) or MPs (B and C) isolated from 1.5 mL plasma from non-pregnant (Non-P), normal pregnant (NP) and pre-eclampsia (PE) women. DNA (A and B) and MPO (C) levels in the conditioned media were measured. Numbers (n) per groups are indicated. Data were analysed by one-way analysis of variance (ANOVA). ns, not statistically significant. (D) Isolated normal neutrophils were incubated with control buffer or endothelial MPs (EMPs) from tumor necrosis factor (TNF)- α -treated human umbilical vein endothelial cells (HUVECs). MPO levels in the conditioned media were measured. Data were analysed by Student's t -test. (E and F) Correlations between plasma DNA levels and plasma total MPs (E) or endothelial (CD31⁺/CD41⁻) MPs (F) in PE women, as analysed by computing Pearson's correlation coefficient.

Fig. 6.

Thrombin generation in plasma and correlations between plasma deoxyribonucleic acid (DNA) levels and blood clotting parameters in pre-eclampsia (PE) women. (**A** and **B**) Thrombin generation was assayed in plasma from non-pregnant (Non-P), normal pregnant (NP) and PE women. Data for total thrombin generation (**A**) and peak thrombin generation (**B**) are shown. Data in (**B**) were analysed by one-way analysis of variance (ANOVA). Correlations between plasma DNA levels and activated partial thromboplastin time (aPTT) in seconds (s) (**C**), pro-thrombin time (PT) in seconds (s) (**D**), plasma anti-thrombin III (ATIII) (**E**) or fibrinogen D-dimer (**F**) levels were analysed by computing Pearson's correlation coefficient.