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ORIGINAL ARTICLE

Gender-specific and menstrual cycle dependent differences in circulating microparticles†

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Abstract
In comparison to age-matched men, young women are at increased risk to suffer from venous thromboembolism (VTE). Some risk factors of inherited and acquired thrombophilia are known, but approximately 30% of the overall risk remains unexplained. Recently, a role for microparticles (MP) in coagulation has been suggested. We investigated, if gender- and menstrual cycle-specific differences in circulating MP exist. Platelet- and endothelial cell-derived microparticles (PMP, EMP) and subpopulations thereof were evaluated flow-cytometrically in healthy women ($n = 27$) in different phases of their menstrual cycles (follicular phase: $n = 14$, luteal phase: $n = 13$) and in healthy men ($n = 18$). Additionally, D-dimer levels were determined. Compared to men, women had elevated numbers of annexin V-binding MP ($p = 0.007$), PMP (CD61; $p = 0.013$), P-selectin-exposing PMP ($p = 0.002$) and E-selectin-exposing EMP ($p = 0.009$). During the luteal phase, women had strongly elevated concentrations of MP, PMP, P-selectin- and CD63-exposing PMP as well as E-selectin-exposing EMP ($p = 0.001$, $p < 0.001$, $p = 0.004$, $p = 0.003$, and $p < 0.001$, respectively), and the ratio of P-selectin-exposing PMP/platelet increased more than three-fold as compared to men ($p = 0.01$). When different phases of the menstrual cycle were analysed, MP (annexin V; $p = 0.025$), PMP (CD61: $p < 0.001$; CD63: $p = 0.015$) and E-Selectin-positive EMP ($p = 0.006$) were all increased in the luteal phase. Although D-dimer concentrations in women were increased compared to men ($p = 0 = 0.006$), no menstrual cycle-specific differences were observed. In summary, circulating MP and subpopulations thereof are increased in women when compared to men, and this increase seems to be modulated by the menstrual cycle. Therefore, circulating MP may be an additional risk factor contributing to the hitherto unexplained procoagulatory state of young women.

Keywords: *Circulating microparticles, gender, menstrual cycle, venous thromboembolism*

Introduction

Circulating blood cells and endothelial cells (EC) release microparticles (MP) during cell activation or apoptosis, partial or complete lysis (e.g. by complement), oxidative stress, or high shear [1, 2]. MP are therefore considered to be a general indicator of injury and stress [3]. They also promote thrombosis and inflammation [4].

Circulating numbers of MP and subpopulations thereof have been associated with various disease states. There is strong but still circumstantial evidence that altered concentrations of platelet-derived MP (PMP) are associated with e.g. diabetes, hypertension, myocardial infarction, and sepsis [5–8]. Endothelial cell-derived microparticles (EMP) are elevated in acute coronary syndrome, antiphospholipid syndrome, multiple sclerosis, preeclampsia, and thrombotic thrombocytopenic purpura [2, 9–12].

The pathophysiological relevance of changes in circulating numbers of MP is a topic of

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current research. The role of PMP is best known [13, 14]. They provide an anionic phospholipid surface that enables efficient formation of tenase- and prothrombinase coagulation complexes [13]. Detection of PMP-associated tissue factor (TF) emphasizes a possible role not only in supporting but also in initiating coagulation [15]. EMP are also capable of initiating coagulation via a TF- and factor VII-dependent pathway [16–18].

During platelet activation, intracellular granule membrane glycoproteins (GP) become exposed, including P-selectin (CD62P) and gp55 (CD63) [19, 20]. Van der Zee et al. [21] suggested that measuring P-selectin- or CD63-exposing PMP may be a feasible and reliable method to assess the in vivo platelet activation status. They showed that both P-selectin- as well as CD63-exposing PMP were increased in patients with peripheral arterial disease and myocardial infarction.

Women <45 years have a higher risk of developing venous thromboembolism (VTE) compared to age-matched men [22–25]. Possible risk factors are pregnancy, puerperium, oral contraceptives, and hormone replacement therapy. Nevertheless, about 30% of VTE remains unexplained [26, 27].

In this case-control study, we determined the concentrations of circulating MP as well as of PMP and EMP from activated platelets and endothelial cells, respectively, in healthy women in different phases of the menstrual cycle and age-matched healthy men.

Methods

Study population

Signed informed consent was obtained from all participants, allowing analysis of all clinical and laboratory data mentioned in this paper. The Human Investigation Review Board of the Ludwig-Maximilians-University Munich approved the study.

Blood samples were obtained from 27 healthy women and from 18 healthy men (no history of thrombosis, no smoking, no medication). All participants were Caucasians. Body mass index was between 20 and 25. All women had regular menstrual periods and ovulatory cycles. None of them had taken oral contraceptives for at least 6 months. The menstrual cycle was subdivided into a follicular (progesterone ≤ 1 ng/ml; $n = 14$) and a luteal phase (progesterone ≥ 6 ng/ml; $n = 13$). Luteinizing hormone (LH) and 17ß-estradiol (E2) concentrations were also assessed.

On average, LH concentrations tended to be higher in the follicular phase (8.7 ± 12.6 mU/ml versus 4.5 ± 3.2 mU/ml), while E2 concentrations were comparable (104 ± 90 pg/ml versus 113 ± 44 pg/ml).

Blood sampling and measurements

Blood samples were taken by puncture of the antecubital vein without tourniquet through a 20-gauge needle. For MP analysis, platelet-poor plasma was prepared by centrifugation at $1550 \times g$ for 20 minutes within 15 minutes after collection. The plasma was then shock-frozen in liquid nitrogen for 15 minutes and stored at -80°C until assayed. Serum concentrations of progesterone, LH, and E2 were measured using an automated immunoassay system (Roche Elecsys 2010, Roche Diagnostics; Mannheim, Germany).

D-dimer concentrations were determined using a latex-amplified immunoassay system (STA-LIATEST-D-dimer, Diagnostica Stago; F-92600 Asnières, France). The test-specific detection limit was 0.25 µg/ml. D-dimer levels below detection limit were regarded as negative.

Reagents

Fluorescein isothiocyanate (FITC)-labelled annexin V, phycoerythrin (PE)-labelled annexin V, and IgG-PE were from Immuno Quality Products (Groningen, The Netherlands). Anti-CD61-PE and anti-E-selectin-PE were from BD Biosciences (Heidelberg, Germany), anti-P-selectin-PE, anti-CD63-PE, and IgG-FITC from Immunotech (Marseille, France), and anti-CD144-FITC from Acris (Hiddenhausen, Germany). All antibodies and annexin V were diluted with phosphate-buffered saline (PBS; 154 mmol/l NaCl, 1.4 mmol/l phosphate, pH 7.4). Final dilutions were: Annexin V-FITC 1:100 (v/v), annexin V-PE 1:200, anti-CD61-PE 1:100, anti-P-selectin-PE 1:100, anti-CD63-PE 1:20, anti-CD144-FITC 1:20, and anti-E-selectin-PE 1:20. The latter antibody specifically stains MP exposing E-selectin from TNF-α-stimulated human endothelial cells, as demonstrated previously [28].

Isolation and analysis of platelet- and endothelial cell-derived microparticles

MP isolation and analysis were performed as described by Nieuwland et al. [29]. A sample of 250 µl frozen plasma was thawed on melting ice for approximately one hour. After centrifugation of 250 µl plasma at $17570 \times g$ and 20°C for 30 minutes, 225 µl of MP-free supernatant was discarded. The remaining MP pellet was diluted with 225 µl of PBS containing 10.9 mmol/l trisodium citrate (PBS/citrate buffer). MP were resuspended and centrifuged again for 30 minutes at $17570 \times g$ and 20°C.

After removal of the supernatant (225 µl), 75 µl of PBS/citrate buffer was added, and the MP pellet was resuspended again. Five microlitres of the MP suspension was diluted in 35 µl $CaCl_2$

(2.5 mmol/L)-containing PBS. Then 5 µl APC-labeled annexin V was added plus 5 µl of a cell-specific monoclonal antibody or isotype-matched control antibodies. Samples were incubated in the dark for 15 minutes at room temperature. The reaction was stopped with 900 µl calcium buffer (2.5 mmol/l) except to the annexin V control, to which 900 µl citrate-containing PBS was added.

MP were analyzed in a FACScan flow cytometer (Becton Dickinson; Heidelberg, Germany) using the Cell Quest Software (Becton Dickinson; San Jose, CA, USA). Forward scatter (FSC) and side scatter (SSC) were set at a logarithmic gain. MP were identified on basis of their size and density and their capacity to bind a cell-specific monoclonal antibody and annexin V. Cell-specific labelling with monoclonal antibodies was corrected for identical concentrations of isotype-matched control antibodies and annexin V measurements were corrected for autofluorescence. The concentration of MP/L plasma was estimated according to Berckmans et al. [1].

Statistics

Parametrically distributed data were expressed as mean (±standard deviation (SD)). All other data were presented as the median ((Q1-Q3) = interquartile range). Independent variables were analysed by the Mann-Whitney-U test and Fisher's exact test. D-dimer data were analysed by using the Chi-Square test. P-values <0.05 were regarded as statistically significant. Data were examined with SPSS for Windows (release 14.0).

Two samples from women in the follicular phase and in the luteal phase were excluded from the analysis of CD62E and CD63 positivity, since the numbers were outside the normal range (mean $\pm 2 \times$ SD) after log transformation.

Results

Study population

Mean age of women and men did not differ significantly. This was also the case with regard to women in the follicular and in the luteal phase of the menstrual cycle. Platelet counts tended to be higher in women than in men and showed a significant difference in the menstrual cycle, being highest in the luteal phase ($p = 0.009$). Hemoglobin concentrations were significantly higher in men ($p < 0.001$). Twelve women had D-dimer levels above the test-specific limit of 0.25 µg/ml compared to one aged-matched man ($p = 0.006$), but no menstrual cycle-specific differences were detected. See Table I.

Microparticle analysis

Compared to age-matched men, annexin V-binding MP were significantly increased in women ($p = 0.007$). This difference was even more pronounced, when men were compared to women in the luteal phase ($p = 0.001$), as annexin V-binding MP were elevated in the luteal phase compared to the follicular phase ($p = 0.025$) of the menstrual cycle (Figure 1).

Platelet-derived microparticles (PMP)

CD61-exposing PMP showed gender-specific as well as menstrual cycle-dependent differences (Figure 2).

Concentrations of PMP were increased in women as compared to men ($p = 0.013$), and this effect was solely due to women in the luteal phase of the menstrual cycle ($p < 0.001$). When menstrual phases were compared, significantly higher concentrations were observed in the luteal phase compared to the follicular phase ($p < 0.001$) (Figure 2).

Subpopulations of PMP exposing P-selectin were higher in women than in men ($p = 0.002$) (Figure 3). On average, the fraction of P-selectin-exposing PMP was two-fold increased in women versus men (1.9% versus 0.8% of total PMP; $p = 0.025$), and the ratio of P-selectin-exposing PMP per platelet was more than two-fold increased (0.20 $(0.1–0.5) \times 10^{-3}$ versus 0.09 $(0.04–0.2) \times 10^{-3}$; $p = 0.004$). Differences were most pronounced in the luteal phase of the menstrual cycle: Circulating P-selectin-positive PMP were five-fold higher than in men ($p = 0.004$; Figure 3).

Regarding the exposure of CD63 on PMP, differences were present between women in the luteal phase and both men ($p = 0.003$) and women in the follicular phase ($p = 0.015$) (Figure 4). The ratio of CD63-exposing PMP per platelet was higher in women in the luteal phase compared to men (0.3 $(0.5–0.7) \times 10^{-3}$ versus 0.2 $(0.09–0.5) \times 10^{-3}$; $p = 0.04$).

Endothelial microparticles (EMP)

In contrast to PMP, no gender-specific or menstrual cycle-dependent differences were present in circulating CD144-exposing EMP (Figure 5). In contrast, E-selectin-exposing EMP, i.e. EMP from activated endothelial cells, were elevated in women compared to men ($p = 0.009$), particularly in the luteal phase ($p < 0.001$; Figure 6).

Discussion

Our data demonstrate considerable gender-specific and menstrual cycle-dependent differences in circulating (sub-) populations of MP. In light of these results, we suggest that circulating MP or

Table I. Study population.

Gender	Women		*p*-value	Women	Men	*p*-value
Menstrual cycle (phase)	follicular $n=14$	luteal $n=13$	follicular vs luteal	(total) $n=27$	(total) $n=18$	women vs men
Age (years)	29 ± 8.4	35 ± 7.9	$p=0.07$	32 ± 8.5	29 ± 3.2	$p=0.5$
Hemoglobin (g/dl)	13.7 ± 0.9	13.7 ± 0.9	$p=0.98$	13.7 ± 0.9	15.7 ± 0.9	$p<0.001$*
Leukocytes (g/l)	6.6 ± 1.3	7.0 ± 1.5	$p=0.3$	6.8 ± 1.4	6.6 ± 1.0	$p=0.8$
Platelets (g/l)	247 ± 50	310 ± 63	$p=0.009$*	277 ± 64	251 ± 47	$p=0.26$

Data are presented as mean $\pm$ SD ($p<0.05$ = significant). Hemoglobin levels were higher in men compared to women. Platelet counts differed significantly during menstrual cycle. *represents $p<0.05$.

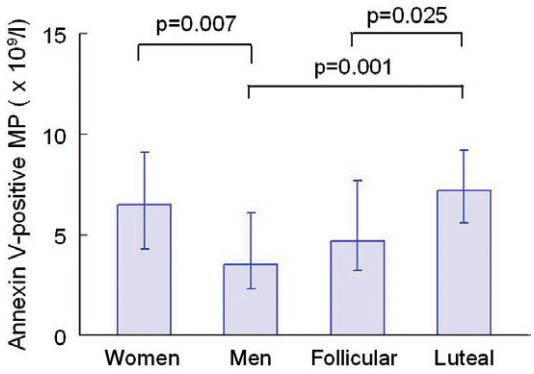

Figure 1. Annexin V-positive MP ($\times10^9$/L; data presented as median (interquartile range)).

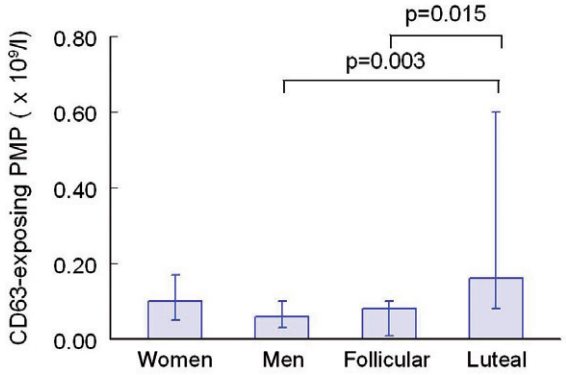

Figure 4. CD63-positive PMP ($\times10^9$/L; data presented as median (interquartile range)).

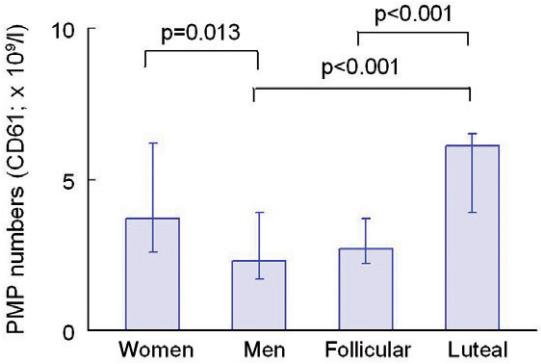

Figure 2. CD61-positive PMP ($\times10^9$/L; data presented as median (interquartile range)).

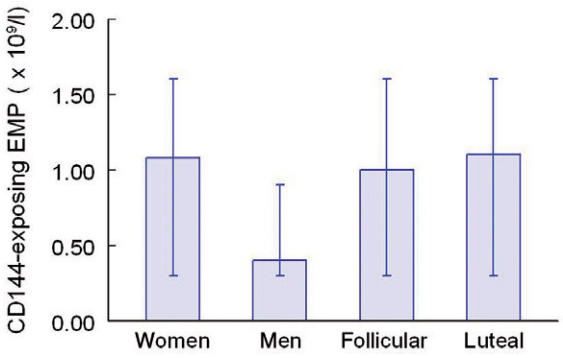

Figure 5. CD144-positive EMP ($\times10^9$/L; data presented as median (interquartile range)).

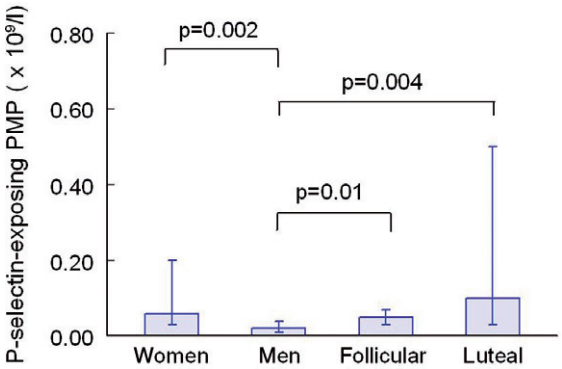

Figure 3. CD62P-positive PMP ($\times10^9$/L; data presented as median (interquartile range)).

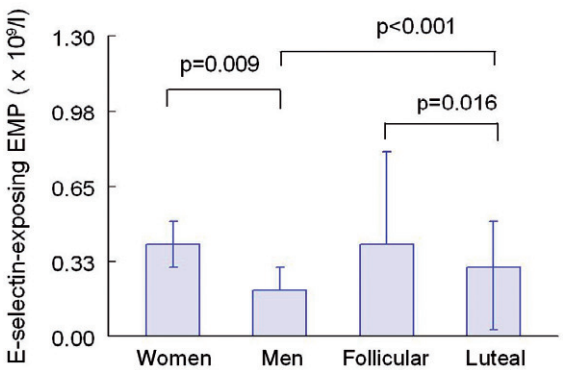

Figure 6. CD62E-positive EMP ($\times10^9$/L; data presented as median (interquartile range)).

subpopulations thereof may contribute to a procoagulant state in young women and increase their VTE risk.

So far, there are no data on gender- and menstrual cycle-specific differences in MP release. Our finding, that concentrations of PMP subpopulations exposing activation markers such as P-selectin or gp55 are elevated in the luteal phase, may reflect enhanced activation of platelets in young women. The observed increases in numbers of circulating platelet-derived MP, however, may also reflect higher platelet counts in the luteal phase.

Focusing the literature, there are conflicting data about platelet counts and platelet activation during menstrual cycle [30–33]. Therefore, a longitudinal study on individual women with emphasis on the effects of sexual steroids on circulating levels of PMP and platelet activity is part of our current research.

Our finding, that women of our study population had higher D-dimer levels compared to age-matched men, confirms earlier data of Rudnicka et al. [34]. These data indicate that coagulation activation may indeed have occurred. The lack of difference in D-dimer concentrations during the menstrual cycle may be due to the relatively small size of our study population.

Focusing on menstrual cycle-specific changes in the expression of PMP from activated platelets, we found that P-selectin-exposing PMP were increased in the luteal phase. P-selectin is involved in coagulation activation in various ways. First, P-selectin binds to monocyte-exposed P-selectin GP ligand-1 (PSGL-1), thus triggering the expression and production of coagulant TF, which subsequently may be released on MP [35, 36]. Such TF-exposing monocyte-derived MP have been shown to occur both in vitro and in vivo [8, 37]. Second, once platelets are activated after adhering to an adhesive surface, e.g. a wound, P-selectin becomes exposed and is involved in capturing "blood-borne" TF from the circulation [38]. Third, TF-exposing monocytic MP have recently been shown to fuse with P-selectin-exposing platelets via interaction with PSGL-1, thereby delivering procoagulant TF to the platelet surface [38]. Thus, the increase in P-selectin-exposing PMP may be a risk factor for VTE in young women, particularly in the luteal phase of the menstrual cycle.

There is evidence from other studies for an increased platelet activation and fibrinolytic activity in the luteal phase [30, 32, 39–43]. Feuring et al. as well as Roell et al. [39, 44] investigated alterations in platelet function during the menstrual cycle by using a platelet function analyzer (PFA-100^{TM}). They found platelets to be more responsive in the luteal phase as compared to the follicular phase. Moreover, other authors have also reported on higher concentrations of fibrinogen and fibrinogen degradation products in the luteal phase than in the follicular phase, indicating a higher fibrinolytic activity in the luteal phase [32, 40–43].

Other investigators studied gender-specific differences in platelet activation and aggregation and reported enhanced platelet reactivity in women compared to men [30, 31, 45–47]. Faraday et al. examined the ability of in vitro activated platelets from healthy male and female subjects to bind fibrinogen through activated integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa), which is the receptor responsible for platelet aggregation [30]. They observed that, upon standardized activation of the platelets in vitro, fibrinogen binding to platelets was significantly increased in women compared to men. Furthermore, fibrinogen binding depended on the menstrual cycle and was significantly increased in the luteal phase as compared to the follicular phase. Since binding of fibrinogen to activated GPIIb-IIIa has been suggested by some investigators to be a prerequisite for the formation and release of PMP, it is tempting to speculate that activation of this integrin is somehow affected by the menstrual cycle, which may lead to the observed increase in circulating PMP in women, particularly in the luteal phase.

The endothelium plays an important role in maintenance of vascular integrity and haemostasis, with endothelial cell dysfunction having been implicated in the pathogenesis of both atherosclerosis and plaque instability [48]. Although several studies suggested gender-dependent differences in endothelium-dependent vasodilatation [49–51], no studies on menstrual cycle- and gender-specific differences in EMP release exist.

In accordance with previous findings, Chirinos et al. [18] demonstrated elevated numbers of EMP in patients with VTE, which suggested that the release of EMP and their binding to monocytes are key events in thrombogenesis [15, 52]. Koga et al. [48] verified that CD144-positive EMP occurs in human plasma and that CD144-exposing EMP in plasma can be a clinically relevant and quantitative marker of endothelial cell dysfunction and injury. We observed menstrual cycle- and gender-specific differences in the occurrence of CD62E-positive EMP, which, similarly to PMP and their subpopulations, may reflect ongoing cellular activation.

Conclusion

Our data suggest that circulating MP may be gender- as well as menstrual cycle-dependent. The increase in PMP and subpopulations thereof may be associated with an increased VTE risk in young women. A prospective follow-up of individual women in different phases of the menstrual cycle is now being performed to confirm and extend these observations.

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