

## Original Paper

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# Climacteric Lowers Plasma Levels of Platelet-Derived Microparticles: A Pilot Study in Pre- versus Postmenopausal Women

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Erhard Hiller<sup>e</sup> Bettina Toth<sup>c</sup><sup>a</sup>Department of Clinical Chemistry, Academic Medical Center, Amsterdam, The Netherlands;<sup>b</sup>2. Medizinische Klinik, Klinikum Augsburg, Augsburg, <sup>c</sup>Department of Gynecological Endocrinology and Fertility Disorders, Ruprecht-Karls University, Heidelberg, <sup>d</sup>Department of Radiotherapy, and<sup>e</sup>Medizinische Klinik III, Campus Grosshadern, Klinikum der Ludwig Maximilians Universität München, München, Germany**Key Words**

Microparticles · Climacteric · Thrombosis · Platelets

**Abstract**

**Background:** Climacteric increases the risk of thrombotic events by alteration of plasmatic coagulation. Up to now, less is known about changes in platelet- (PMP) and endothelial cell-derived microparticles (EMP). **Methods:** In this prospective study, plasma levels of microparticles (MP) were compared in 21 premenopausal and 19 postmenopausal women. **Results:** No altered numbers of total MP or EMP were measured within the study groups. However, the plasma values of CD61-exposing MP from platelets/megakaryocytes were higher in premenopausal women ( $5,364 \times 10^6/l$ , range 4,384–17,167) as compared to postmenopausal women ( $3,808 \times 10^6/l$ , range 2,009–8,850;  $p = 0.020$ ). This differentiation was also significant for the subgroup of premenopausal women without hormonal contraceptives ( $5,364 \times 10^6/l$ , range 4,223–15,916;  $p = 0.047$ ;  $n = 15$ ). Furthermore, in premenopausal women, higher plasma levels of PMP exposing CD62P were also present as compared to postmenopausal women ( $288 \times 10^6/l$ , range 139–462, vs.  $121 \times 10^6/l$ , range 74–284;  $p = 0.024$ ). This difference was also true for CD63+ PMP levels ( $281 \times 10^6/l$ , range 182–551, vs.  $137 \times$

$10^6/l$ , range 64–432;  $p = 0.015$ ). **Conclusion:** Climacteric lowers the level of PMP but has no impact on the number of EMP in women. These data suggest that PMP and EMP do not play a significant role in enhancing the risk of thrombotic events in healthy, postmenopausal women.

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**Introduction**

In premenopausal women with regular menstrual cycles, the hormonal axis is controlled by the hypothalamus and the hypophysis. Follicle-stimulating hormone (FSH) released in the hypophysis stimulates the maturation of ovarian follicles, which in turn produce estradiol. Exerting a positive feedback, the increasing estradiol concentrations lead to elevated levels of luteinizing hormone during the follicular phase of the menstrual cycle triggering ovulation. During treatment with oral contraceptives (OC), the continuous administration of gestagens or the combined administration of gestagens and ethinylestradiol results in constant hormonal levels under which FSH and luteinizing hormone concentrations remain unchanged [1].

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In Europe, women reach the menopause at around the age of 54 years [2]. At this time, there are less than a thousand follicles remaining in the ovaries leading to decreased levels of estradiol [3]. Through a feedback mechanism, the hypophysis increases the production and distribution of FSH in order to stimulate the ovaries. Menopausal symptoms such as hot flushes, sweating, vaginal atrophy and osteoporosis are a consequence of the decreased estradiol levels. However, climacteric is also responsible for a dramatic increase in the risk of adverse thrombotic events such as myocardial infarction, stroke, deep venous thrombosis and pulmonary embolism [4].

Up to now, most investigations regarding the changes in the coagulation system focus on plasmatic factors and show inconstant laboratory findings: plasma concentrations of fibrinogen and fibrin D-dimer, both markers of thrombotic risk, are elevated, and endogenous fibrinolytic activity is reduced in healthy postmenopausal women as compared to younger women [5]; however, inhibitors like protein C, protein S, antithrombin, or tissue factor pathway inhibitor are also increased in menopausal women [6]. In view of these findings, climacteric seems to destabilize the plasmatic coagulation system.

Less is known about changes of platelets and endothelial cells which could also play a role in the observed enhancement of thrombotic risk during aging. One way to determine the activation status of platelets as well as of endothelial cells *in vivo* is to measure microparticle (MP) concentrations in plasma. MP can be released from every blood and endothelial cell into the peripheral circulation. However, in healthy humans, MP are mainly CD61+ and are derived from megakaryocytes or from platelets (platelet-derived microparticles, PMP) with additional exposing of the platelet activation markers P-selectin and CD63 [7]. In particular, the composition and function of PMP have been intensively analyzed [8, 9]. PMP are major players in coagulation [10] as they are highly thrombogenic [11] because of their phospholipid surfaces with binding sites for several different coagulation factors [12, 13]. PMP are important initiators of coagulation and provide a link between the platelet-based hemostatic system and plasmatic coagulation *in vivo*. Patients with Glanzman's disease [14] or Scott Syndrome [15] have reduced counts of PMP leading to bleeding disorders. On the other hand, patients with elevated levels of PMP suffer from diseases like cerebral infarction [16], acute coronary syndrome [17], sepsis [18] and venous thrombosis [19]. In the latter cases, MP from endothelial cells (EMP) are also regularly increased, reflecting injuries to the endothelial surface of the vessels. However, it is not known whether these in-

creased levels of PMP and EMP are the consequence or the cause of hypercoagulation.

Within this prospective case-control study, the impact of climacteric on plasma levels of PMP and EMP in healthy women was investigated. As surrogate markers for the activation status of platelets, megakaryocytes and endothelial cells, they may play a possible role in triggering thrombotic events in postmenopausal women.

## Patients and Methods

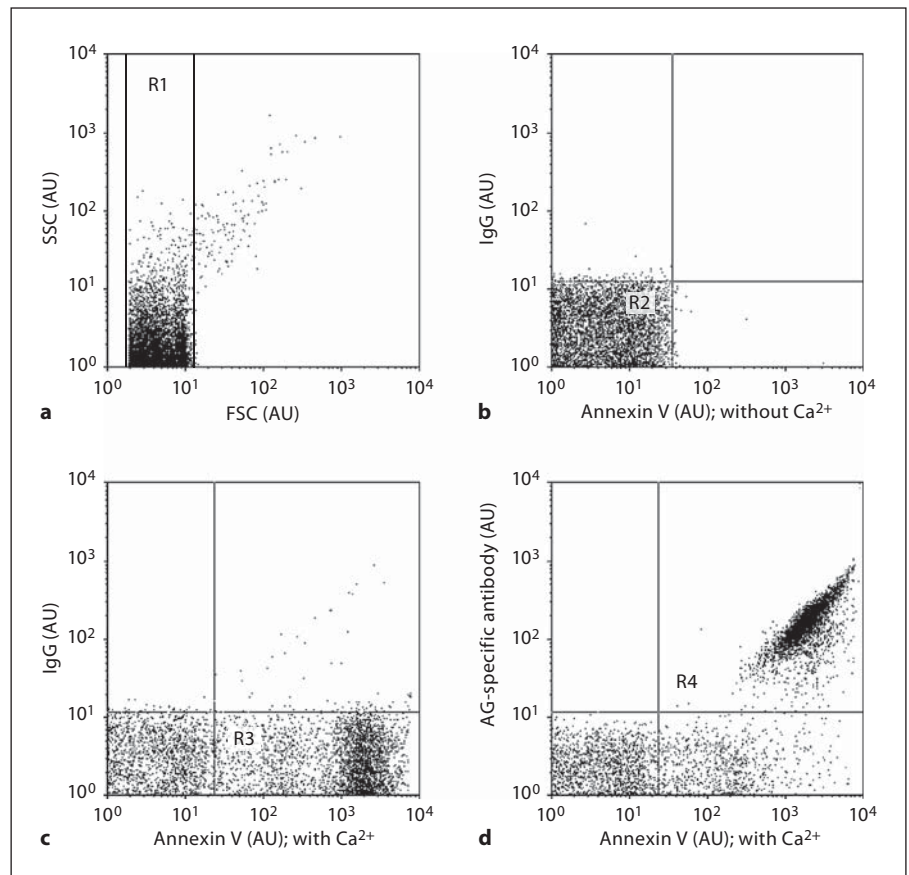
### *Study Population and Trial Design*

In this prospective case-control study, 21 premenopausal women with or without hormone-containing OC were compared with a group of 19 menopausal women. Patients were screened and recruited from the Department of Obstetrics and Gynecology, Ludwig-Maximilians University, Klinikum Grosshadern, Munich, Germany, between November 2007 and July 2009. Women taking other medications except OC were excluded, especially postmenopausal women taking hormone replacement therapy. Participants were asked about their menstrual pattern or, accordingly, their last cycle. Premenopausal women were only enrolled in the study if they have got a normal frequent menstrual cycle between 21 and 35 days within the last year at least. The time point of blood sampling in this group was estimated during women's follicular phase between menses and ovulation. Postmenopausal women were only included in the study if their last menstrual cycle dated back 1 year or longer. Furthermore, the history of venous thromboembolism (VTE) or other disorders was evaluated and women with a history of VTE were excluded. All included participants were Caucasians and had to have normal blood counts as well as normal prothrombin time and activated partial thromboplastin time, normal fibrinogen plasma levels, and negative D-dimer at the time of investigation. To avoid other possible confounders in our study, there were further criteria which the women had to fulfill before they were enrolled: a normal body mass index between 19 and 25 (calculated after measuring height and weight), normal blood pressure <140/90 mm Hg (checked at the time point of blood sampling), non-smoking, no history of disorders and no long-time medication as well as no acute medication within the last week before blood sampling. The Human Investigation Review Board of the Ludwig-Maximilian University Munich approved the study. Signed informed consent was obtained from all women allowing analysis of all clinical and laboratory data mentioned in this paper.

### *Blood Sampling and Isolation of MP*

Blood samples were taken by puncture of the antecubital vein without tourniquet through a 20-gauge needle. For isolation of MP, blood samples were centrifuged at 1,550 g for 20 min within 15 min after sampling and stabilized in liquid nitrogen for 15 min. Samples were stored at -80°C until assayed. Samples were defrosted carefully over a period of approximately 1 h. After centrifugation of 250 µl plasma at 18,890 g for 30 min, 225 µl of plasma/supernatant was removed. MP were resuspended after the addition of 225 µl phosphate-buffered saline (PBS) containing citrate (0.32% trisodiumcitrate) and centrifuged again for 30 min

**Fig. 1.** Identification of MP by flow cytometry. AU = Arbitrary units. **a** MP can be found in region 1 (R1) by their dimension, as described by Nieuwland et al. [12]. SSC = Sideward scatter; FSC = forward scatter. **b** For negative control, MP were double stained with unspecific antibody IgG plus annexin V in a Ca<sup>2+</sup>-free buffer (R2). **c** Annexin V in Ca<sup>2+</sup>-containing buffer identifies phosphatidylserine on the MP surface with high specificity and sensitivity (R3). **d** Double staining of annexin V plus antigen (AG)-specific antibody (here: anti-CD61) determines the cell origin of MP (R4).



at 18,890 g. After removal of the supernatant (225 ml), 75  $\mu$ l of PBS/citrate buffer was added, and the MP pellet was resuspended. Five microliters of the MP suspension was diluted in PBS containing 35  $\mu$ l CaCl<sub>2</sub> (2.5 mmol/l) or, alternatively, 35  $\mu$ l citrate for annexin-V-negative controls. Finally, 5  $\mu$ l annexin V was added plus 5  $\mu$ l of each cell-specific monoclonal antibody or isotype-matched control antibodies and incubated in the dark at room temperature for 15 min. The reaction was stopped with 900  $\mu$ l of PBS containing calcium (2.5 mmol/l).

#### Reagents

EMP were identified via double staining with APC-labeled annexin V (Immuno Quality Products, Groningen, The Netherlands) and a fluorescein isothiocyanate (FITC)-labeled anti-CD144 antibody (Acris, Hiddenhausen, Germany). PMP were detected via allophycocyanin (APC)-labeled annexin V, FITC-labeled anti-CD61 antibody (Immuno Quality Products), and anti-CD62P antibody (Immunotech, Marseille, France) or anti-CD63 antibody (Immunotech) as a platelet activation marker. IgG-FITC and IgG-phycoerythrin (IgG-PE), both from BD Biosciences (Heidelberg, Germany), were used as antibody negative controls. All antibodies and annexin V were diluted with PBS (154 mmol/l NaCl, 1.4 mmol/l phosphate, pH 7.4). Final dilutions were: annexin-V-APC 1:300 (v/v), anti-CD144-FITC 1:20, anti-CD61-FITC 1:100, anti-CD62P-PE 1:100, anti-CD63-PE 1:20, anti-IgG-FITC 1:100, and anti-IgG-PE 1:100.

#### Analysis of Microparticle

The samples were analyzed in a FACScan flow cytometer (BD Biosciences, Heidelberg) running Cell Quest Software (BD Biosciences, San Jose, Calif., USA) which was calibrated daily using beads from BD Biosciences (Heidelberg). FACScan was run on high pressure. Forward scatter, sideward scatter and fluorescence channels were set at logarithmic gain. All samples were analyzed for 1 min. The volume analyzed in 1 min (V; approximately 50–80  $\mu$ l) was measured daily before analysis by taking the weight of a sample (aqua dest) before and after analysis. The gating strategy is described in figure 1. The number of MP/ $\mu$ l of plasma was determined by using the following formula:  $MP/l = n \times (100 \mu l / 5 \mu l) \times (950 \mu l / V) \times (106 / 250 \mu l)$  according to Berckmans et al. [20], where n = absolute number of MP determined by FACS analysis; 100  $\mu$ l = total volume of washed microparticle suspension; 5  $\mu$ l = pellet used for analysis; 950  $\mu$ l = total volume before analysis (pellet + antibodies + buffer); 250  $\mu$ l = original volume of the sample before isolation of MP.

#### Statistical Methods

Results are given as median values with interquartile ranges, if not otherwise indicated. Differences between the groups were analyzed by the non-parametric Mann-Whitney test. All statistical tests were two-tailed. p values <0.05 were regarded as statistically significant. Data were analyzed with SPSS for Windows, release 18.0.

**Table 1.** MP in pre- and postmenopausal women

|                               | Total MP             | CD61+ MP              | CD62P+ PMP     | CD63+ PMP      | EMP           |
|-------------------------------|----------------------|-----------------------|----------------|----------------|---------------|
| Premenopausal women (n = 21)  | 6,159 (4,427–15,023) | 5,364 (4,384–17,167)* | 288 (139–462)* | 281 (182–551)* | 347 (207–499) |
| Without OC (n = 15)           | 6,159 (4,325–13,577) | 5,364 (4,223–15,916)* | 209 (108–455)  | 276 (140–535)* | 376 (265–539) |
| With OC (n = 6)               | 7,168 (4,045–16,730) | 7,303 (4,096–18,465)  | 323 (178–536)  | 314 (250–658)* | 289 (173–502) |
| Postmenopausal women (n = 19) | 6,484 (3,639–8,419)  | 3,808 (2,009–8,850)   | 121 (74–284)   | 137 (64–432)   | 495 (198–711) |

Data are median MP  $\times 10^6/l$ , with interquartile ranges in parentheses. \*  $p < 0.05$  compared to postmenopausal women.

## Results

### Study Population

The median age of the 21 premenopausal women was 27 years (Q25: 23 years, Q75: 35 years, range 18–40). All had regular menstrual cycles. Fifteen women had not been taking OC or other hormone-containing contraceptives for at least 1 year prior to the time of analysis. Six premenopausal women were taking OC containing 0.03 mg ethinylestradiol and 2 mg dienogest (Valette®). Nineteen women were postmenopausal for at least 1 year with a median age of 61 years (Q25: 56 years, Q75: 66 years, range 53–70). None of the pre- and postmenopausal women had a history of VTE or any other diseases.

### Microparticle Analysis

Counts of total annexin-V-positive MP did not differ between pre- and postmenopausal women. Similarly, no differences were measured in the numbers of CD144-exposing endothelial cell-derived MP (table 1). In contrast, the value of platelet-derived CD61-exposing MP was significantly higher in the premenopausal group ( $p = 0.02$ ) compared to postmenopausal women (fig. 2). This difference was also significant for the subgroup of premenopausal women without contraception ( $p = 0.047$ ). The highest values were measured in premenopausal women taking OC but without statistical significance because of the small number of women investigated. The plasma levels of PMP exposing the activation markers P-selectin and CD63 were also increased in women before the menopause ( $p = 0.024$  and  $0.015$ , respectively). However, the percentage fraction of PMP-exposing activation markers was similar in all investigated (sub-)groups: a median value of 4.1% was observed in premenopausal women (4.1% without OC, 3.7% with OC) versus 4.1% in postmenopausal women (CD62P+ PMP), along with 4.3% CD63+ PMP in premenopausal women (4.3% without OC and 4.3% with OC vs. 4.9% in postmenopausal women).

## Discussion

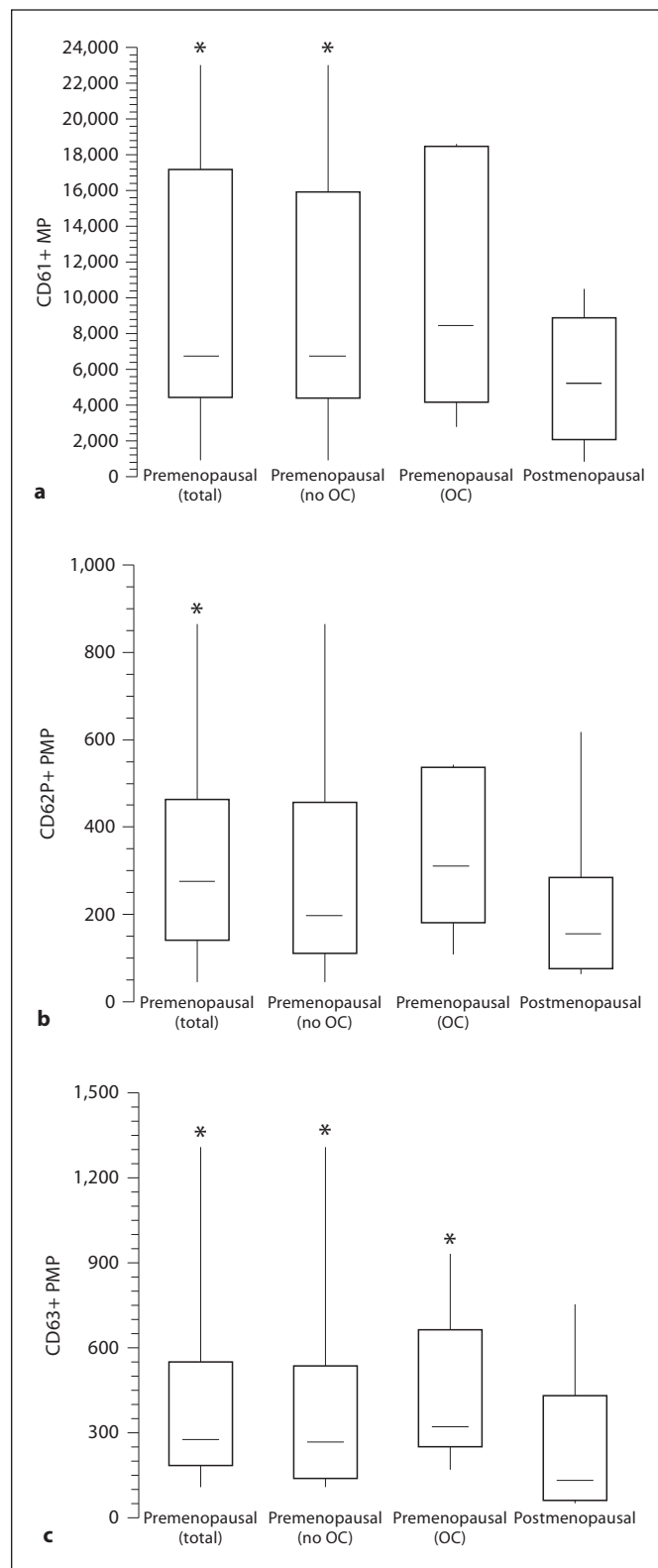
Our data demonstrate lower plasma levels of CD61-exposing MP in postmenopausal women. These results may seem surprising as PMP are prothrombogenic and potent stimulators of coagulation. Therefore, higher CD61+ MP in postmenopausal women would be expected, possibly explaining the increased risk of thrombotic events in this population. Indeed, patients suffering from acute deep venous thrombosis or pulmonary embolism showed elevated counts of PMP [21, 22]. Ramacciotti et al. [23] could even show a positive correlation between thrombus weight and level of PMP in a mouse model. However, the elevated PMP levels reported in these other studies were present in patients with manifest thrombotic events.

Up to now, platelets were thought to be the major cell source of CD61+ MP. Accordingly, circulating CD61+ MP are commonly called ‘platelet-derived microparticles’ in the literature. Nevertheless, more than 10 years ago, Cramer et al. [24] showed via electronic microscopy in cell cultures that megakaryocytes constrict not only platelets but also CD61+ MP simultaneously. As megakaryocytes eject platelets directly into the blood through cytoplasmic processes penetrating the sinus wall into the sinus lumen [25], this may also result in the concurrent release of megakaryocyte-derived MP into the circulation. Although the regulation of megakaryopoiesis is not yet exactly understood, the female steroid hormone estrogen has been identified as an endogenous activator of differentiation, proliferation and proplatelet formation as well as of the constriction of megakaryocytic MP. The estrogen receptor is expressed on megakaryocytes [26]. Estradiol and progesterone are synthesized in male as well as female megakaryocytes indicating an autocrine stimulation loop of megakaryopoiesis [27]. Furthermore, addition of extracellular estradiol enhances proplatelet formation in a dose-dependent manner and an estrogen

receptor antagonist inhibits platelet production in vivo. This would explain the higher levels of CD61-exposing MP in premenopausal women compared to those detected in postmenopausal women who are known to have no relevant estrogen level any more. In line with this conclusion, we found the highest counts of CD61-exposing MP in premenopausal women who took estrogen-containing contraceptives, even though the number of investigated women is small and limits the validity of our data. But the idea of a relevant influence of female hormones on the plasma level of CD61+ MP is supported by recent published data from our group: this subtype of plasma MP is higher in postmenopausal women with hormone replacement therapy compared to those without hormone replacement therapy [28]. The general relevance of estrogens becomes evident by the fact that women have higher mean counts of platelets as well as of CD61-exposing MP – probably mainly derived from megakaryocytes – than men [29].

Unfortunately, it is not possible to distinguish MP released from platelets and megakaryocytes using common detecting antigens like fibrinogen (CD41/CD61), or von Willebrand receptor (CD42a and CD42b), as they are expressed on the surface of both cell types. Recently, however, by measuring full-length filament A and, accordingly, lysosome-associated membrane glycoprotein 1 in CD41/CD42b-exposing MP, it was shown that indeed ‘PMP’ without activation markers originate largely from megakaryocytes in mice [30]. Data from our own investigations indicate that this is also true in humans: killing megakaryocytes by total body irradiation in patients undergoing stem cell transplantation leads to a faster decline in CD61-exposing MP than in platelets [31]. Thus, the main population of CD61+ MP seems to be directly derived from megakaryocytes and only a small fraction, around 3–5% in healthy humans [32], are released from platelets after cell activation. This fraction of ‘real PMP’ is released from activated platelets because of various reasons like high shear stress around a vessel stenosis [33, 34] in patients with myocardial infarction, intermittent claudicatio, or ischemic events [35, 36]. In our study population without a history of such disorders, the percentage of platelet activation marker-positive MP in pre- and postmenopausal women was comparable with data from the literature.

We also investigated EMP levels in pre- and postmenopausal healthy women. As a specific and general marker for MP derived from vascular endothelium we used vascular endothelium-cadherin (CD144) which is thought to play an important role in endothelial cell biol-



**Fig. 2.** Platelet-derived MP exposing CD61 (a), CD62P (b) and CD63 (c) ( $\times 10^6/l$ ; data presented as the median and box plot). \*  $p < 0.05$  compared to postmenopausal women.

ogy by controlling the cohesion and organization of the intercellular junctions [37]. Endothelium injury raises the level of circulating, CD144-exposing EMP as shown in patients with atherosclerosis and especially in those with plaque instability [38, 39]. We did not find different levels of CD144 positivity in any investigated (sub-)group. This indicates vessel wall integrity without any damage or activation of endothelial cells within our study population.

Summing up, postmenopausal women who are known to be at high risk for vascular events showed lower PMP, probably because of female hormone deficiency. Furthermore, climacteric does not seem to have an influence on the number of EMP in healthy women. From a clinical point of view, our data show that neither PMP nor EMP are responsible for enhancing the risk of venous or arterial thromboembolism in menopausal women. Our findings are the first published impression of the impact of the climacteric on MP serum levels, but a higher number of participants is necessary to confirm our results. Fur-

thermore, in a next step, we will also go more into detail of qualitative MP changes, e.g., the frequency of tissue factor-bearing MP.

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### Disclosure Statement

The authors state no conflict of interest.

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