LETTER TO THE EDITOR

Age- and gender-matched controls needed for platelet-based biomarker studies

Thrombocytes, or blood platelets, regulate thrombosis and hemostasis,[1] but also play a key role in inflammation, tumor angiogenesis, and cancer progression.[2-4] Consequently, platelets are considered a valuable source of biomarkers of disease. It is known that platelet count and volume are dependent on both age and sex;[5-8] however, it is not known whether this is also the case for other platelet characteristics. We recently reviewed the available literature on studies of platelet cancer biomarkers[9] and observed that approximately 60% of the published studies on humans in which platelet content was investigated lacked control groups matched for age or sex. This suggests that the interpretation of the results of many platelet biomarker studies may be unreliable. Therefore, we examined the relationship of both age and gender with phenotypic and functional platelet characteristics. We present the association of age and gender with platelet growth factor content, state of activation, and response to stimulation.

This study was performed in accordance with the principles of the Declaration of Helsinki and was approved by the local medical ethical committee (Maastricht University Medical Center+ [MUMC+], n. 114117). All study subjects provided written informed consent. Using a strict protocol,[10] we collected blood from 94 healthy volunteers: 50 men (average age 65.9 years, range 43.6-85.3) and 44 women (average age 63.7 years, range 40.9-84.7). Platelet activation was quantified by whole blood flow cytometry

Table 1. Correlation of platelet characteristics with age and gender.

Variables	Age as independent predictor (r)	*P*	Gender as independent predictor (r)	*P*	Age and gender as predictors (r)	*P*
Platelet count	-0.21	0.042	0.322	0.001	0.368	0.001
MPV fL	0.293	0.004	-0.201	0.026	0.339	0.004
% αIIbβ3 activation						
Thrombin 0.1 nM	0.012	0.09	-0.112	0.141	0.112	0.561
Thrombin 1 nM	0.164	0.104	-0.105	0.158	0.19	0.188
ADP 1 nM	0.431	<0.0001	0.079	0.226	0.433	<0.0001
ADP 10 nM	0.403	<0.0001	0.159	0.063	0.419	<0.0001
Convulxin 0.5 ng/mL	-0.116	0.133	0.003	0.488	0.116	0.539
Convulxin 50 ng/mL	0.009	0.932	-0.029	0.392	0.029	0.962
% P-selectin expression						
Thrombin 0.1 nM	-0.228	0.013	-0.089	0.197	0.225	0.047
Thrombin 1 nM	-0.201	0.026	0.155	0.068	0.242	0.064
ADP 1 nM	-0.03	0.387	-0.002	0.492	0.031	0.958
ADP 10 nM	-0.036	0.366	0.182	0.04	0.182	0.215
Convulxin 0.5 ng/mL	-0.289	0.002	0.047	0.325	0.29	0.018
Convulxin 50 ng/mL	-0.217	0.018	0.023	0.413	0.217	0.111
Platelet content						
PF4 ng/10^6 plt	-0.347	<0.0001	0.189	0.034	0.379	0.001
CTAPIII ng/10^6 plt	-0.222	0.016	0.091	0.193	0.232	0.08
TSP-1 ng/10^6 plt	-0.169	0.052	0.108	0.15	0.192	0.182
PDGF pg/10^6 plt	-0.425	<0.0001	0.153	0.071	0.439	<0.0001
VEGF pg/10^6 plt	0.122	0.122	0.13	0.106	0.17	0.267

Pearson correlation was used to calculate the regression coefficient (r) between independent variables and age and gender. A multivariable linear regression analysis was used to calculate the combined effect of age and gender on platelet (plt) characteristics. MPV: mean platelet volume; ADP: adenosine biphosphate; PF4: platelet factor 4; CTAPIII: connective tissue activating peptide III; TSP-1: thrombospondin 1; PDGF: platelet-derived growth factor; VEGF: vascular endothelial growth factor.

of platelet membrane integrin αIIbβ3 conformational change and expression of P-selectin, before and after stimulation with 2Me-S-adenosine biphosphate (ADP), thrombin, or convulxin.[10] Platelet-derived growth factor (PDGF), platelet factor 4 (PF4), connective tissue activating peptide III (CTAPIII), thrombospondin-1 (TSP-1), and vascular endothelial growth factor (VEGF) concentrations in platelets and platelet-free plasma (PFP) were measured with human Duo Set ELISA assays (R&D Systems, Abington, UK).

Age was found to be an independent predictor for platelet activation (Table 1). Integrin αIIbβ3 activation increased with age after stimulation with low and high concentrations of ADP (Table 1, Figure 1Ai). Hyper-reactivity at older age was also observed when platelets were stimulated with a low dose of thrombin (Figure 1Ai), although correlation analysis showed a non-significant trend (Table 1). Our findings may explain the earlier observation of increased platelet aggregation in response to ADP with age.[11] ADP is considered a weak platelet agonist with limited effect on platelet secretion, which explains the difference between integrin αIIbβ3 activation (Figure 1Ai) and P-selectin expression (Figure 1Aii). These differences may be due to higher levels of platelet hydrogen peroxide in older individuals.[12] Increased intraplatelet hydrogen peroxide, a critical mediator of the increased inside-out activation of integrin αIIbβ3, leads to hyperactivation of platelets resulting in amplified αIIbβ3 activation and fibrinogen binding, while it has no effect on α-granule secretion as measured by P-selectin expression.[12] This may also explain

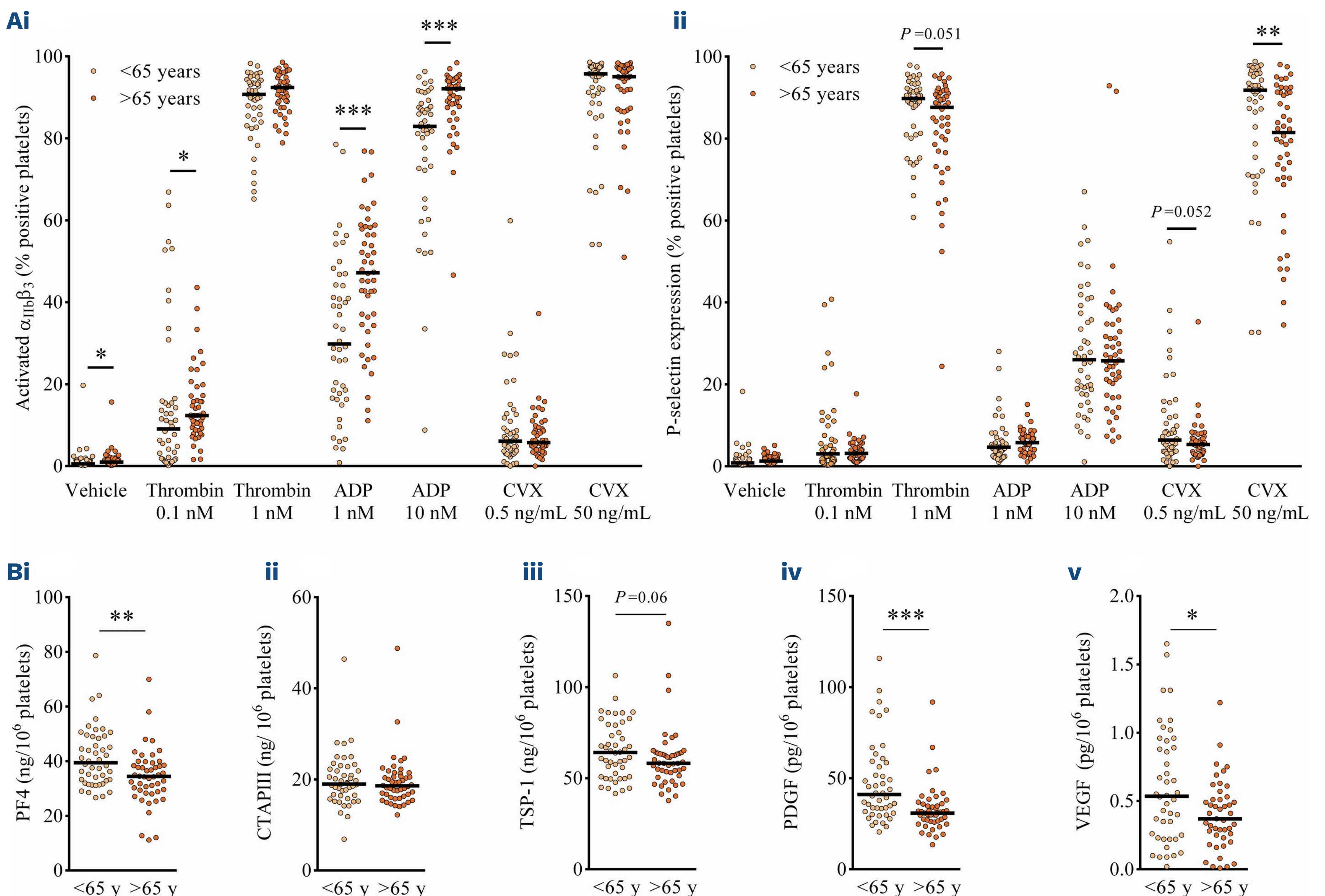

Figure 1. Platelet activation and growth factor content changes with age. (A) Effect of thrombin, adenosine biphosphate (ADP) and convulxin (CVX) on activation of αIIbβ3 (i) and expression of P-selectin (ii) on platelets of healthy individuals. Whole blood flow cytometry was used to measure platelet activation upon stimulation with vehicle (hepes), thrombin (0.1 and 1 nM), ADP (1 and 10 nM) and convulxin (0.5 and 50 ng/ml). Individuals aged <65 years (n=46) are shown with light colored circles; individuals aged >65 years (n=48) are shown with dark colored circles. Scatter plots show the effect on binding of FITC-conjugated monoclonal antibody PAC-1 which binds to activated αIIbβ3 (i) and expression of P-selectin (ii). (B) Platelet growth factor content changes with age. Concentrations of (i) platelet factor 4 (PF4), (ii) connective tissue activating peptide III (CTAPIII), (iii) thrombospondin 1 (TSP-1), (iv) platelet-derived growth factor (PDGF), and (v) vascular endothelial growth factor (VEGF) were determined by ELISA in platelets of healthy individuals. Individuals aged <65 years are shown with light colored circles; individuals aged >65 years are shown with dark colored circles. Data are presented as scatterplot with a horizontal line as median. *$P<0.05$; **$P<0.01$; ***$P<0.0001$.

why we observed a small, but significant increase in integrin αIIbβ3 activation in resting platelets of older individuals (Figure 1Ai). At the same time, P-selectin expression decreased with age after stimulation with both low and high concentrations of the strong agonists thrombin and convulxin (Table 1, Figure 1Aii). It is unclear whether this reduction is due to a decrease in P-selectin density in platelet α-granules, a decline in platelet secretory capability, or a reduction in the number of secretory granules in platelets of older individuals.

Age was also found to be an independent predictor for platelet growth factor content (Table 1). Age appeared to be strongly and negatively correlated with intraplatelet concentrations of PF4, CTAPIII and PDGF, whereas the relation with TSP-1 levels was not significant. In addition, no correlation between the VEGF content of platelets and age was found; this may be explained by the fact that the VEGF present in platelets is mainly derived from sequestration from plasma and to a lesser extent from synthesis by megakaryocytes.[13] When we divided the groups into individuals aged <65 *versus* >65 years (this being approximately the average age of the study cohort) (Figure 1B), the concentrations of PF4, PDGF and VEGF in platelets were significantly lower in older subjects (Figure 1Bi, iv, v). Different results were obtained for CTAPIII and VEGF when analyzing the data either for the correlation between age and platelet growth factor content (Table 1) or the difference between individuals aged <65 or >65 years (Figure 1B). In these cases, we believe that the correlation results from Table 1 are the most conclusive, as these are independent of any age limit. No significant associations were found between plasma concentrations of any of these proteins and age (*data not shown*). The negative correlation between platelet α-granule protein content and age fits with the decline of P-selectin expression after stimulation with strong agonists in older individuals (Table 1, Figure 1Aii). This could be due to a reduced number of α-granules in platelets in elderly subjects, a decline in protein synthesis by megakaryocytes, reduced uptake of proteins from plasma by megakaryocytes and platelets, or a combination of these effects. Little research has been done on the effects of aging on megakaryocytes and platelets, although one can hypothesize that age-related changes in bone marrow activity, such as a reduction in the amount and functional activity of the hematopoietic stem cells,[14] may have direct or indirect effects on platelets. This hypothesis is supported by the present findings, and other studies,[5,7,8] showing that platelet count decreases with age in both men and women ($r=-0.210$, $P<0.05$), while mean platelet volume (MPV) increases ($r=0.293$, $P<0.01$).

In isolated platelets, the concentration of PDGF (per 10^6 platelets) was significantly higher in women compared to men (*Online Supplementary Figure S1D*), while no substantial differences in platelet concentrations of PF4, CTAPIII, TSP-1, or VEGF were detected (*Online Supplementary Figure S1A, B, C, E*). This suggests that the concentration per platelet of some platelet-derived growth factors are gender-dependent. As platelets are the major circulating source of these factors in blood, the total circulating platelet concentrations of PF4, CTAPIII, TSP-1, PDGF and VEGF (platelet content multiplied by platelet count per milliliter) was also calculated, and was found to be significantly higher in women (Figure 2); this difference remained statistically significant when adjusted for age (*data not shown*). This gender-related effect appeared to be primarily due to the higher platelet count in women compared to men (median 233×10^9/mL *vs.* 197×10^9/mL; $P<0.001$). This underlines the importance of matching for gender and correcting for platelet count in studies where potential biomarkers are platelet-derived. The study of Biino *et al.* on the Italian population suggested that reference intervals for platelet count should be age- and sex-specific to allow for better diagnosis of thrombo-

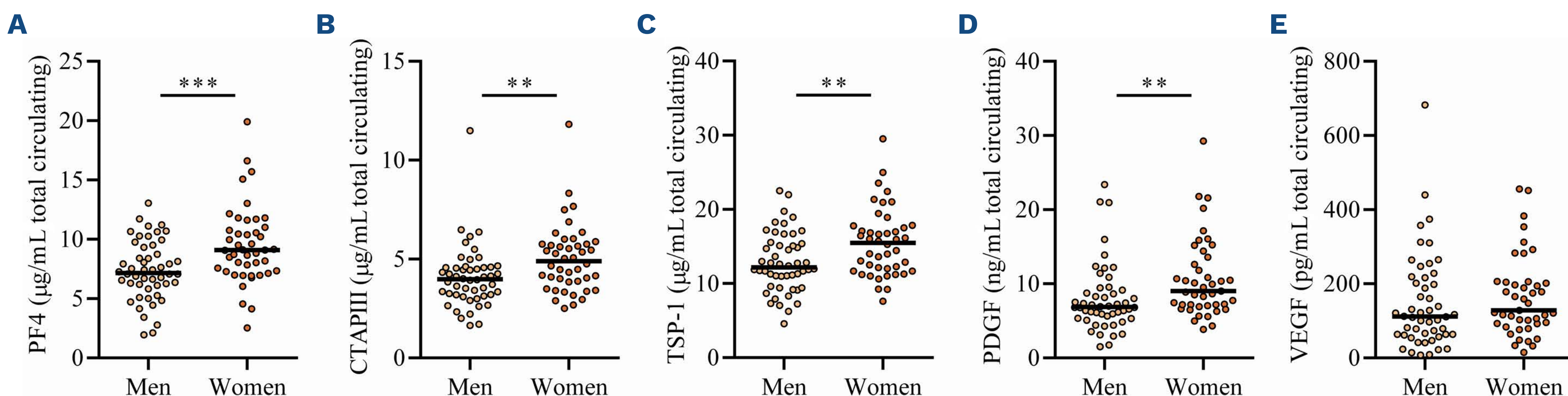

Figure 2. Total circulating platelet-derived growth factor concentrations are higher in women compared to men. Concentrations of (A) platelet factor 4 (PF4), (B) connective tissue activating peptide III (CTAPIII), (C) thrombospondin 1 (TSP-1), (D) platelet-derived growth factor (PDGF), and (E) vascular endothelial growth factor (VEGF) were determined in platelets of healthy men (n=50) and women (n=44). The total circulating platelet concentrations of these proteins were calculated by multiplying the concentrations per platelet by the number of circulating platelets per milliliter of whole blood. Data are presented as scatterplot with a horizontal line as median. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

cytopenia and thrombocytosis.[8] Furthermore, the mean platelet volume (MPV) was found to be smaller in women than in men (Table 1), confirming data from earlier studies.[5,6] No significant gender-related differences in platelet reactivity were detected upon stimulation with thrombin, ADP or convulxin (*Online Supplementary Figure S2*). This was also the case for the concentrations of platelet-derived proteins in plasma (*Online Supplementary Figure S3*).

In conclusion, we confirm that platelet features differ between men and women, and change with increasing age. The hitherto underestimated association of platelet features with age and gender may mean the interpretation of data in earlier studies was unreliable and questions their conclusions. This emphasizes the importance of age- and gender-matched control groups in studies investigating platelet characteristics or platelet-derived biomarkers of disease.

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Contributions

SS, MK, AG and MoE were involved in study design, sample collection, and writing the manuscript. SvK performed statistical analysis and helped write the manuscript.

Data-sharing statement

The data that support the findings of this study are available upon reasonable request to the corresponding author.

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