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

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Letter to the editors

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

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Thrombocytes, or blood platelets, regulate thrombosis and haemostasis,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 unknown whether this also holds true for other platelet characteristics. We recently reviewed the available literature on platelet cancer biomarker studies,⁹ and observed that approximately 60% of the published human studies, in which platelet content was investigated, lacked control groups that were matched for age and sex. This suggests that many platelet biomarker studies may have resulted in unreliable interpretation of results. 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 declaration of Helsinki and approved by the local medical ethical committee (Maastricht University Medical Center+ (MUMC⁺) number 114117). All subjects have provided written informed consent. Using a strict protocol,¹⁰ blood from 94 healthy volunteers was collected, 50 men (average age 65.9, range 43.6-85.3) and 44 women (average age 63.7, range 40.9-84.7). Platelet activation was quantified by whole blood flow cytometry of platelet membrane integrin αIIbβ₃ conformational change and expression of P-selectin, before and after stimulation with 2Me-S-ADP, thrombin or convulxin.¹⁰ PDGF (platelet derived growth factor), PF4 (platelet factor 4), CTAPIII (connective tissue activating peptide III), TSP-1 (thrombospondin-1) and VEGF (vascular endothelial growth factor) concentrations in platelets and platelet free plasma (PFP) were measured with human Duo Set ELISA assays (R&D Systems, Abington, United Kingdom).

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 and Figure 1Ai). Hyperreactivity 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). This dissimilarity may be due to higher levels of platelet hydrogen peroxide levels in older individuals.¹² Increased intraplatelet hydrogen peroxide, a critical mediator of integrin αIIbβ₃ increased inside-out activation, leads to hyperactivation of platelets resulting in amplified αIIbβ₃ activation and fibrinogen binding, while it has no effect
on alpha-granule secretion as measured by P-selectin expression. This may also explain why we observed a small, but significant increase in integrin αIIbβ3 activation in resting platelets of older individuals (Figure 1Ai). Concurrently, P-selectin expression decreased with age after stimulation with both low and high concentrations of the strong agonists thrombin and convulxin (Table 1 and Figure 1Aii). It is unclear whether this reduction is due to a decrease in P-selectin density in platelet alpha-granules, a decline in platelet secretory capability or a reduction in 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. Also, no correlation between the VEGF content of platelets and age was found. This may be explained by the fact that VEGF present in platelets is mainly derived from sequestration from plasma and to a lesser extent from synthesis by megakaryocytes. When we divided the groups in individuals younger versus older than 65 years, which was around the average age (Figure 1B), the concentrations of PF4, PDGF and VEGF in platelets were significantly lower in older subjects (Figure 1Bi, -iv and -v). For CTAPIII and VEGF different results were obtained when analyzing the data either for the correlation between age and platelet growth factor content (Table 1) or the difference between individuals younger or older than 65 (Figure 1B). In these cases, we believe that the correlation results from Table 1 are most conclusive, as these are independent from an 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 alpha-granule protein content and age fits the decline of P-selectin expression after stimulation with strong agonists in aged individuals (Table 1 and Figure 1Aii). This could be due to a reduced number of alpha-granules in platelets of older individuals, 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, may have direct or indirect effects on platelets. This hypothesis is supported by the present findings, and other studies, showing that platelet count decreases as age increases 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 (Supplementary Figure 1D), while no substantial differences in platelet concentrations of PF4, CTAPIII, TSP-1 and VEGF were detected (Supplementary Figure 1A, B, C and E). This suggests that the per
platelet concentration 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 calculated as well, and shown 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 233x10⁶ vs. 197x10⁶ platelets/mL, p<0.001). This signifies 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., based on the Italian population, has suggested that reference intervals for platelet count should be age- and sex-specific to allow for better diagnosis of thrombocytopenia and thrombocytosis. Furthermore, the mean platelet volume (MPV) was found to be smaller in women than in men (Table 1), confirming data from earlier studies. No significant gender-related differences in platelet reactivity were detected upon stimulation with thrombin, ADP or convulxin (Supplementary Figure 2). This also holds true for the concentrations of platelet-derived proteins in plasma (Supplementary Figure 3).

In conclusion, we proclaim that platelet features differ between men and women and change with progression of age. The hitherto underestimated association of platelet features with age and gender may have resulted in unreliable interpretation of data and questionable conclusions in earlier studies. This underpins the importance of age- and gender-matched control groups in studies investigating platelet characteristics or platelet-derived biomarkers of disease.

References


Table 1. Correlation of platelet characteristics with age and gender. 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 calculated the combined effect of age and gender on platelet characteristics.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Age as independent predictor (r)</th>
<th>P-value</th>
<th>Gender as independent predictor (r)</th>
<th>P-value</th>
<th>Age and gender as predictors (r)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet count</td>
<td>-0.21</td>
<td>0.042</td>
<td>0.322</td>
<td>0.001</td>
<td>0.368</td>
<td>0.001</td>
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<td>MPV (fl)</td>
<td>0.293</td>
<td>0.004</td>
<td>-0.201</td>
<td>0.026</td>
<td>0.339</td>
<td>0.004</td>
</tr>
<tr>
<td>% αIIbβ3 activation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin 0.1 nM</td>
<td>0.012</td>
<td>0.09</td>
<td>-0.112</td>
<td>0.141</td>
<td>0.112</td>
<td>0.561</td>
</tr>
<tr>
<td>Thrombin 1 nM</td>
<td>0.164</td>
<td>0.104</td>
<td>-0.105</td>
<td>0.158</td>
<td>0.19</td>
<td>0.188</td>
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<tr>
<td>ADP 1 nM</td>
<td>0.431</td>
<td>&lt;0.0001</td>
<td>0.079</td>
<td>0.226</td>
<td>0.433</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ADP 10 nM</td>
<td>0.403</td>
<td>&lt;0.0001</td>
<td>0.159</td>
<td>0.063</td>
<td>0.419</td>
<td>&lt;0.0001</td>
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<tr>
<td>Convulxin 0.5 ng/ml</td>
<td>-0.116</td>
<td>0.133</td>
<td>0.003</td>
<td>0.488</td>
<td>0.116</td>
<td>0.539</td>
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<tr>
<td>Convulxin 50 ng/ml</td>
<td>0.009</td>
<td>0.932</td>
<td>-0.029</td>
<td>0.392</td>
<td>0.029</td>
<td>0.962</td>
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<tr>
<td>% P-selectin expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin 0.1 nM</td>
<td>-0.228</td>
<td>0.013</td>
<td>-0.089</td>
<td>0.197</td>
<td>0.225</td>
<td>0.047</td>
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<tr>
<td>Thrombin 1 nM</td>
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<td>0.026</td>
<td>0.155</td>
<td>0.068</td>
<td>0.242</td>
<td>0.064</td>
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<tr>
<td>ADP 1 nM</td>
<td>-0.03</td>
<td>0.387</td>
<td>-0.002</td>
<td>0.492</td>
<td>0.031</td>
<td>0.958</td>
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<tr>
<td>ADP 10 nM</td>
<td>-0.036</td>
<td>0.366</td>
<td>0.182</td>
<td>0.04</td>
<td>0.182</td>
<td>0.215</td>
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<tr>
<td>Convulxin 0.5 ng/ml</td>
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<td>0.002</td>
<td>0.047</td>
<td>0.325</td>
<td>0.29</td>
<td>0.018</td>
</tr>
<tr>
<td>Convulxin 50 ng/ml</td>
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<td>0.018</td>
<td>0.023</td>
<td>0.413</td>
<td>0.217</td>
<td>0.111</td>
</tr>
<tr>
<td>Platelet content</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PF4 (ng/10⁶ plt)</td>
<td>-0.347</td>
<td>&lt;0.0001</td>
<td>0.189</td>
<td>0.034</td>
<td>0.379</td>
<td>0.001</td>
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<tr>
<td>CTAPIII (ng/10⁶ plt)</td>
<td>-0.222</td>
<td>0.016</td>
<td>0.091</td>
<td>0.193</td>
<td>0.232</td>
<td>0.08</td>
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<tr>
<td>TSP1 (ng/10⁶ plts)</td>
<td>-0.169</td>
<td>0.052</td>
<td>0.108</td>
<td>0.15</td>
<td>0.192</td>
<td>0.182</td>
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<tr>
<td>PDGF (pg/10⁶ plt)</td>
<td>-0.425</td>
<td>&lt;0.0001</td>
<td>0.153</td>
<td>0.071</td>
<td>0.439</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>VEGF (pg/10⁶ plt)</td>
<td>0.122</td>
<td>0.122</td>
<td>0.13</td>
<td>0.106</td>
<td>0.17</td>
<td>0.267</td>
</tr>
</tbody>
</table>

Figure 1. **Platelet activation and growth factor content changes with age.** (A) Platelet activation changes with age. (A) Effect of thrombin, 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). Persons younger than 65 years (n=46) are depicted with light colored circles, while individuals older than 65 years (n=48) are depicted 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 PF4 (i), CTAPIII (ii), TSP-1 (iii), PDGF (iv) and VEGF (v) were determined with ELISA’s in platelets of healthy individuals. Persons younger than 65 years are depicted with light colored circles, while individuals older than 65 years are depicted with dark colored circles. Data are presented as scatterplot with a horizontal line as a median. *p < 0.05; **p < 0.01; ***p < 0.0001.

Figure 2. **Total circulating platelet-derived growth factor concentrations are higher in women compared to men.** Concentrations of PF4 (A), CTAPIII (B), TSP-1 (C), PDGF (D) and VEGF (E) were determined in platelets of healthy men (n=50) and women (n=44). The total circulating platelet concentrations of these proteins was 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 median. *p < 0.05; **p < 0.01; ***p < 0.001.
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Supplemental Figure 1. The concentration of some, but not all platelet-derived factors per platelet are gender-dependent. Concentrations of PF4, CTAPIII, TSP-1, PDGF and VEGF were determined in platelets of healthy men (n=50) and women (n=44). Data are presented as scatterplot with a median. *p < 0.05

Supplemental Figure 2. Platelet activation does not differ between men and women. Effect of thrombin, ADP and convulxin on activation of αIIbβ3 and expression of P-selectin on the platelet surface of healthy men (gray circles, n=50) and women (black circles, n=44). Whole blood flow cytometry was used, platelet activation was measured by PE-conjugated P-selectin antibody and FITC-conjugated monoclonal antibody PAC-1 which binds to activated αIIbβ3. Scatter plots and medians show the effect of vehicle (hepes), ADP (1 and 10 nM), thrombin (0.1 and 1 nM) and convulxin (0.5 and 50 ng/ml) on activation of αIIbβ3 (A) and expression of P-selectin (B) on the platelet surface.

Supplemental Figure 3. The concentrations of platelet-derived proteins in platelet free plasma (PFP) does not significantly differ between genders. Concentrations of PF4 (A), CTAPIII (B), TSP-1 (C), PDGF (D) and VEGF (E) were determined with ELISA’s in platelet free plasma of healthy men (n=50) and women (n=44). Data are presented as scatter plots with a median.
Supplemental figure 1

A  PF4 (ng/10⁶ platelets)

B  CTAPIII (ng/10⁶ platelets)

C  TSP-1 (ng/10⁶ platelets)

D  PDGF (pg/10⁶ platelets)

E  VEGF (pg/10⁶ platelets)
Supplemental figure 2

A

Vehicle  Thrombin  Thrombin  ADP  ADP  Convolxin  Convolxin
0.1 nM  1 nM   10 nM  0.5 ng/ml  50 ng/ml

0  20  40  60  80  100
αIIβ3 positive platelets (%)

B

Vehicle  Thrombin  Thrombin  ADP  ADP  Convolxin  Convolxin
0.1 nM  1 nM   10 nM  0.5 ng/ml  50 ng/ml

0  20  40  60  80  100
P-selectin positive platelets (%)
Supplemental figure 3

A

B

C

D

E

PF4 (µg/ml PFP)

CTAPIII (µg/ml PFP)

TSP-1 (ng/ml PFP)

PDGF (ng/ml PFP)

VEGF (pg/ml PFP)

Men    Women

Men    Women

Men    Women

Men    Women

Men    Women