Cell-derived microparticles: composition and function
Biró, É.

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Plasma markers of coagulation and endothelial activation in Fabry disease: Impact of renal impairment

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Abstract

**Background:** In Fabry disease, storage of globotriaosylceramide in arterial walls is thought to underlie the clinical manifestations. Abnormalities of the vessel wall, hemodynamics and pro- and anticoagulant factors may play a role, though the exact pathophysiology is incompletely understood.

**Objectives:** In this study we try to clarify inconsistencies in the literature regarding coagulation activation, fibrinolysis, platelet and endothelial activation in 36 patients with Fabry disease.

**Methods:** Cell-derived microparticles, markers for coagulation activation (prothrombin fragment F$_{1+2}$, thrombin-antithrombin complexes, soluble tissue factor, soluble endothelial protein C receptor), fibrinolysis (D-dimer, tissue plasminogen activator, α$_2$-antiplasmin), platelet activation (β-thromboglobulin, platelet factor-4), endothelial activation (von Willebrand factor) and acute phase response (interleukin-6, C-reactive protein) were studied in relation to renal function and severity of disease, and compared to data from 36 sex- and age-matched healthy controls.

**Results:** Markers for endothelial activation and fibrinolysis were normal. Male patients had elevated levels of soluble tissue factor and β-thromboglobulin, with an association between soluble tissue factor and renal function and severity of disease. In female patients levels of thrombin-antithrombin complexes, β-thromboglobulin, platelet factor-4, CD63 positive platelet-derived microparticles and interleukin-6 were somewhat increased, with no correlation with renal function or disease severity.

**Conclusions:** Only minimal abnormalities in markers for platelet and endothelial activation, coagulation activation and fibrinolysis could be established in a large cohort of Fabry disease patients. The existing laboratory abnormalities are more likely related to renal insufficiency than to Fabry disease itself.
Fabry disease (McKusick 301500) is an X-linked lysosomal storage disorder. Deficient activity of α-galactosidase A leads to accumulation of glycosphingolipids (mainly globotriaosylceramide, Gb₃) in lysosomes in cells throughout the body [1,2]. Extensive storage occurs in arterial walls, in particular in endothelial and smooth muscle cells. This accumulation is believed to underlie the clinical manifestations in Fabry disease: progressive renal insufficiency, cardiac infarction or hypertrophy, arrhythmias and cerebral infarctions [3], although the exact pathophysiology of the disease is still incompletely understood, in particular the occurrence of brain injury. Abnormalities of the vessel wall, blood components (pro- and anticoagulant factors) and abnormal hemodynamics may play a role [4]. A number of studies performed by Moore and colleagues have shown dysfunction of the cerebral circulation [5-12], in particular hyperperfusion, which may be related to abnormal peripheral vascular reactivity [13].

Clinical observations reveal a high incidence of thrombosis in Fabry disease patients [14] and in mouse models of the disease [15,16]. In addition, based upon case histories [17-19] and a study in mice [20], an association between α-galactosidase A deficiency and the early development of atherosclerosis has been suggested, though a more recent study revealed an increased carotid intima-media thickness in the absence of atherosclerosis in Fabry disease patients [21].

Laboratory investigations that have been performed to assess determinants of coagulation or endothelial activation are not always in accordance. Elevated levels of soluble intercellular adhesion molecule (sICAM-1), soluble vascular cell adhesion molecule-1 (sVCAM-1), sP-selectin, and plasminogen activator inhibitor (PAI), and decreased levels of thrombomodulin (TM) [22] suggested a prothrombotic profile in patients with Fabry disease, although only the elevated levels of sVCAM-1 could be confirmed by Demuth et al. [23]. Furthermore, elevated β-thromboglobulin (β-TG) and increased platelet aggregation [24,25], as well as decreased levels of tissue factor pathway inhibitor (TFPI) and increased levels of tissue factor (TF) [26] have been described.

Recently, elevated levels of endothelial cell-derived microparticles in ten untreated pediatric Fabry disease patients have been reported [27]. Microparticles are small vesicles released from the surface membrane of various cell types upon activation or apoptosis. Extensive evidence shows that they play a role in blood coagulation and in inflammatory processes. A thromboembolic tendency is seen in association with increased numbers of microparticles (heparin-induced thrombocytopenia [28], cerebrovascular accidents [29] or acute coronary syndromes [30,31]), while decreased numbers are found in conditions with a bleeding tendency (Scott syndrome [32]). Also, isolated microparticles are capable of initiating and supporting coagulation in vitro [33-35] and are thrombogenic in vivo, as demonstrated in a rat model [36]. They play a role in inflammatory processes via transfer of bioactive molecules or ligand-receptor interactions [37-40], as well as activation of the complement system [41-44].
In the current study we aimed to clarify existing inconsistencies in the literature regarding coagulation activation, fibrinolysis, and platelet and endothelial activation in Fabry patients. We also analyzed cell-derived microparticles and measured markers of the acute phase response. All parameters were correlated to renal function and severity of disease in these patients.

**Methods**

**Patients and healthy controls**

Thirty-six patients [17 males (median age: 47 years, range 24-67), 19 females (median age: 47 years, range 30-73)] with genetically and/or enzymatically confirmed Fabry disease were included in this study. Twenty of these patients (14 males and 6 females) were receiving enzyme replacement therapy (ERT) at the time of the study. Data from the Fabry patients were compared with those from 36 sex- and age-matched healthy controls. Written informed consent was obtained from all patients as a part of previous studies.

Venous blood for all analyses except β-TG and platelet factor-4 (PF4) measurements was collected into 0.1 volume of 105 mmol/L trisodium citrate. Blood cells were removed by centrifugation (1550 × g, 20 min and 20°C) immediately after sample collection. The plasma samples were snap-frozen in liquid nitrogen and stored at –80°C.

Venous blood for β-TG and PF4 analyses was collected into 0.1 volume of anticoagulant containing 10 mmol/L theophylline, 94 mmol/L PGE₁, 90 mmol/L Na₂EDTA·2H₂O, and 0.63 mmol/L Na₂CO₃, without the use of a tourniquet or vacuum. Samples were then placed on melting ice for 15 min, then centrifuged for 60 min at 2000 × g and 4°C. Afterwards, 1 mL from the middle of the plasma layer was carefully aspirated, snap-frozen in liquid nitrogen and stored at –80°C.

**Isolation and flow cytometric analysis of microparticles**

Microparticles were isolated and labeled as described previously [45]. Aliquots (250 μL) of cell-free citrated plasma were thawed on melting ice, and centrifuged for 30 min at 18000 × g and 20°C. Afterwards 225 μL supernatant were removed, and replaced by phosphate-buffered saline (PBS; 154 mmol/L NaCl, 1.4 mmol/L phosphate, pH 7.4) containing 10.5 mmol/L citrate (PBS/citrate). Microparticles were resuspended and pelleted again by centrifugation, after which 225 μL supernatant were again removed and the microparticle pellet resuspended in PBS/citrate.

Microparticle suspensions (5 μL) were labeled in a final volume of 55 μL PBS containing 2.5 mmol/L CaCl₂ (PBS/Ca, pH 7.4) with a fluorescein isothiocyanate (FITC)- and/or a phycoerythrin (PE)-labeled monoclonal antibody (5 μL each). The mixtures were incubated in the dark for 15 min at 20°C. Subsequently, 900 μL of PBS/Ca were added and
the suspensions analyzed on a FACSCalibur flow cytometer with CELLQuest 3.1 software [Becton, Dickinson and Company (BD) Immunocytometry Systems, San José, CA, USA].

TF and the endothelial protein C receptor (EPCR) were stained in an indirect procedure, in which microparticle suspensions were labeled in a final volume of 50 μL PBS/Ca with an antibody against TF or EPCR (5 μL), not conjugated with fluorochromes. After incubation for 30 min at 20°C, the microparticles were washed once with 200 μL of PBS/Ca. Then, F(ab’)2-PE (5 μL) directed against the primary antibody was added, and the samples incubated for 15 min at 20°C, in the dark. Finally, 900 μL PBS/Ca were added and the samples analyzed on the flow cytometer.

Acquisition was performed for 1 minute per sample, during which the flow cytometer analyzed 60 μL of the suspension. Forward scatter and side scatter were set at logarithmic gain. To identify marker positive events, thresholds were set based on microparticle samples incubated with similar concentrations of isotype-matched control antibodies. Calculation of the number of microparticles per liter plasma was based upon the particle count per unit time, the flow rate of the flow cytometer, and the net dilution during sample preparation of the analyzed microparticle suspension.

IgG1-FITC and IgG1-PE were obtained from BD (both clone X40) and from Dako (Glostrup, Denmark; both clone DAK-G01). CD4-PE (clone SK3) and CD8-PE (clone SK-1) were from BD, CD14-FITC (clone CRIS-6) from Biosource (Camarillo, CA, USA), CD20-PE (clone L27) from BD, CD61-PE (clone VI-PL2) from PharMingen (San José, CA, USA), CD62P-FITC (clone CLB-Thromb/6) and CD63-FITC (clone CLB-gran/12) from Immunotech (Marseille, France), CD66E-PE (clone CLB-gran/10, IH4Fc) from Sanquin (Amsterdam, the Netherlands), CD144-FITC (cat. no. BMS158FI) from Bender MedSystems (Vienna, Austria), and anti-glycophorin A-PE (clone JC159) from Dako. IgG1 (clone MOPC-31C) was obtained from BD, anti-TF (clone TF9-10H10) from American Diagnostica Inc. (Stamford, CT, USA), and rabbit anti-mouse F(ab’)2-PE (cat. no. R0439) was obtained from Dako. Anti-EPCR 1495 monoclonal antibody was a kind gift of dr. C.T. Esmon, Oklahoma Medical Research Foundation (OK, USA) and dr. B. Woodhams, Diagnostica Stago (Gennevilliers, France).

Coagulation activation

Prothrombin fragment F1+2 and thrombin-antithrombin complexes (TAT) in plasma samples of patients and controls were measured by ELISA (Enzygnost F1+2 cat. no. OPBD03, and Enzygnost TAT micro, cat. no. OWMG15, Dade Behring GmbH, Marburg, Germany).

TF and EPCR in microparticle-containing and microparticle-free plasma were also quantified by ELISA (IMUBIND® TF ELISA cat. no. 845, American Diagnostica Inc.; Asserachrom sEPCR cat. no. 00264, Diagnostica Stago). For this, plasma samples were thawed on melting ice to preserve microparticle structure, and either measured directly, or after pelleting of the microparticles. In the latter case, plasma (350 μL) was centrifuged for
60 min at 18000 × g and 20°C, after which the upper layer of the supernatant (150 μL) was carefully aspirated and used for measurements. Microparticle-bound TF and microparticle-bound EPCR were calculated by subtracting the values obtained in microparticle-free plasma from the values obtained in microparticle-containing plasma.

**Fibrinolysis**
D-dimer was analyzed on the automated clinical chemistry analyzer Modular Analytics P800 (Roche Diagnostics, Basel, Switzerland) using Tina-quant reagents [cat. no. 11556487, Roche Diagnostics]. Tissue plasminogen activator (tPA) was measured by ELISA (tPA antigen ELISA, cat. no. TC 11000, Technoclone GmbH, Vienna, Austria), and α2-antiplasmin (α2-AP) was measured on a STA-R coagulation analyzer (Diagnostica Stago) using the STA antiplasmin kit (cat. no. 1776916, Roche Diagnostics).

**Platelet activation**
β-TG and PF4 were measured in the plasma samples by ELISA (Asserachrom β-TG cat. no. 11875370011, and Asserachrom PF4 cat. no. 11875353011, Roche Diagnostics).

**Endothelial activation and the acute phase response**
Von Willebrand factor antigen (vWF) was measured by ELISA as described previously [46]. C-reactive protein (CRP) was analyzed on the Modular Analytics P800 using Tina-quant reagents [CRP (Latex) HS cat. no. 11972855, Roche Diagnostics]. Interleukin-6 (IL-6) in the plasma samples was measured by ELISA (Quantikine® HS Human IL-6, cat. no. HS600B, R&D Systems, Minneapolis, MN, USA).

**Clinical evaluation**
Laboratory parameters that were abnormal in patients with Fabry disease were studied in relation to their renal function and overall severity of disease. Renal function was estimated by calculating the creatinine clearance from plasma creatinine and total urinary creatinine in a representative 24-hour urine collection. Overall severity of disease was assessed using the Mainz Severity Score Index (MSSI) [47]. In brief, the MSSI is composed of four sections that cover the general, neurological, cardiovascular, and renal signs and symptoms of the disease. Within the general section, items such as facial appearance, angiokeratoma, edema, and diaphoresis are listed, while in the neurological section the presence or absence of tinnitus, vertigo, acroparesthesia and pain, as well as cerebrovascular events are scored. The cardiovascular section encompasses left ventricular hypertrophy, ECG abnormalities, valve insufficiencies, presence or absence of a pacemaker and hypertension, while the renal component scores proteinuria up to end stage renal failure. The total scores are reported to represent mild (<20), moderate (20–40), or severe (>40) Fabry disease.
Statistical analysis
Data were analyzed with GraphPad PRISM 3.02 (GraphPad Software, Inc., San Diego, CA, USA). Differences between patients and controls were analyzed with the Wilcoxon matched pairs signed rank test. Differences between male and female patients were analyzed with the Mann-Whitney U test. Correlations were determined using Spearman’s correlation test. Differences and correlations were considered significant at $P < 0.05$. Data are presented as median (range).

Results

Renal function and MSSI in Fabry patients
Renal function was assessed in all patients, revealing a median creatinine clearance of 110 mL/min (range 16–136 mL/min) in males and 109 mL/min (range 64–141 mL/min) in females. According to the K/DOQI (kidney disease outcomes quality initiative chronic kidney disease) classification [48] 10 males and 14 females suffered from stage 1 chronic kidney disease (CKD), 3 males and 5 females stage 2 CKD, 2 males stage 3 CKD and 2 males stage 4 CKD. Data to calculate MSSI were obtained through medical history and clinical evaluation. MSSI in males: 26 (2–59) and in females: 20 (0–32).

Coagulation activation
Results of the analyses are shown in Table 1. Prothrombin fragment F$_{1+2}$ did not differ between patients and controls, though levels in male patients were significantly lower as compared to female patients. TAT complexes were somewhat increased in the female patients. Levels of sTF, i.e. TF in microparticle-free plasma, were increased in male patients but did not correlate with levels of TAT ($P = 0.620$). Levels of microparticle-bound TF determined by ELISA did not differ between patients and controls. Similarly, concentrations of TF positive microparticles detected by flow cytometry were very low in both groups, with no differences. Also, sEPCR (EPCR in microparticle-free plasma), microparticle-bound EPCR and concentrations of EPCR positive microparticles did not differ between patients and controls.

No correlation between levels of TAT complexes and renal function or MSSI could be found in the patients. The levels of sTF did show a strong correlation with the two clinical parameters in both male (renal function: $r = -0.846; P = 0.002$, MSSI: $r = 0.682; P = 0.025$) and female patients (renal function: $r = -0.850; P = 0.006$, MSSI $r = 0.787$, $P = 0.017$), as shown in Figure 1.
Table 1. Markers of coagulation activation, fibrinolysis, platelet and endothelial cell activation, as well as cell-derived microparticles and markers of the acute phase response in Fabry disease patients.

<table>
<thead>
<tr>
<th></th>
<th>Male patients</th>
<th>$P^m$</th>
<th>$N^m$</th>
<th>Male controls</th>
<th>$P^{mf}$</th>
<th>Female patients</th>
<th>$P^f$</th>
<th>$N^f$</th>
<th>Female controls</th>
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</thead>
<tbody>
<tr>
<td><strong>Coagulation</strong></td>
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<tr>
<td>$F_{1+2}$ (pmoVL)</td>
<td>151 (67-519)</td>
<td>N.S.</td>
<td>16</td>
<td>137 (85-370)</td>
<td>**</td>
<td>315 (67-576)</td>
<td>N.S.</td>
<td>19</td>
<td>264 (90-507)</td>
</tr>
<tr>
<td>TAT (μg/L)</td>
<td>3.5 (2.4-10.9)</td>
<td>N.S.</td>
<td>17</td>
<td>3.5 (2.2-6.3)</td>
<td>N.S.</td>
<td>4.4 (1.9-7.3)</td>
<td>$^*$</td>
<td>19</td>
<td>3.2 (2.4-6.2)</td>
</tr>
<tr>
<td>sTF (ng/L)</td>
<td>144 (78-348)</td>
<td>*</td>
<td>11</td>
<td>106 (68-212)</td>
<td>N.S.</td>
<td>117 (73-241)</td>
<td>N.S.</td>
<td>9</td>
<td>111 (60-130)</td>
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<tr>
<td>MP-bound TF (ng/L)</td>
<td>5 (0-39)</td>
<td>N.S.</td>
<td>11</td>
<td>11 (4-32)</td>
<td>N.S.</td>
<td>0 (0-19)</td>
<td>N.S.</td>
<td>9</td>
<td>5 (0-19)</td>
</tr>
<tr>
<td>TF pos. MP ($\times 10^6$/L)</td>
<td>73 (0-775)</td>
<td>N.S.</td>
<td>16</td>
<td>123 (0-533)</td>
<td>N.S.</td>
<td>62 (0-347)</td>
<td>N.S.</td>
<td>19</td>
<td>48 (0-265)</td>
</tr>
<tr>
<td>sEPCR (μg/L)</td>
<td>130 (72-467)</td>
<td>N.S.</td>
<td>11</td>
<td>111 (86-383)</td>
<td>N.S.</td>
<td>183 (67-538)</td>
<td>N.S.</td>
<td>9</td>
<td>128 (9-317)</td>
</tr>
<tr>
<td>MP-bound EPCR (μg/L)</td>
<td>0 (0-6)</td>
<td>N.S.</td>
<td>11</td>
<td>0 (0-18)</td>
<td>N.S.</td>
<td>0 (0-3)</td>
<td>N.S.</td>
<td>9</td>
<td>0 (0-97)</td>
</tr>
<tr>
<td>EPCR pos. MP ($\times 10^6$/L)</td>
<td>16 (0-202)</td>
<td>N.S.</td>
<td>12</td>
<td>12 (0-66)</td>
<td>N.S.</td>
<td>18 (0-223)</td>
<td>N.S.</td>
<td>16</td>
<td>10 (0-197)</td>
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<tr>
<td><strong>Fibrinolysis</strong></td>
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<td>D-dimer (mg/L)</td>
<td>0.1 (0.1-1.9)</td>
<td>N.S.</td>
<td>15</td>
<td>0.1 (0.1-0.3)</td>
<td>N.S.</td>
<td>0.1 (0.1-0.6)</td>
<td>N.S.</td>
<td>19</td>
<td>0.1 (0.1-0.8)</td>
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<tr>
<td>tPA (μg/L)</td>
<td>0.8 (0.3-2.4)</td>
<td>N.S.</td>
<td>17</td>
<td>0.8 (0.2-8.9)</td>
<td>***</td>
<td>1.8 (0.8-3.0)</td>
<td>N.S.</td>
<td>19</td>
<td>1.8 (0.8-2.9)</td>
</tr>
<tr>
<td>$\alpha_2$-AP (%)</td>
<td>95 (79-112)</td>
<td>N.S.</td>
<td>15</td>
<td>97 (83-114)</td>
<td>*</td>
<td>100 (89-126)</td>
<td>N.S.</td>
<td>19</td>
<td>103 (88-119)</td>
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<td><strong>Platelet activation</strong></td>
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<tr>
<td>β-TG (IU/mL)</td>
<td>63.9 (22.1-284.5) * 16 33.8 (15.0-114.9) N.S. 76.6 (23.5-400.0) ** 19 38.7 (20.0-86.1)</td>
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<tr>
<td>PF4 (IU/mL)</td>
<td>7.9 (1.0-94.2) N.S. 16 4.3 (1.4-31.3) * 16.4 (3.9-200.0) * 19 7.3 (1.0-24.1)</td>
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<tr>
<td>Platelet-derived MP (× 10⁶/L)</td>
<td>4519 (223-14889) N.S. 17 4048 (1599-15625) N.S. 3362 (248-16800) N.S. 19 3067 (831-8441)</td>
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<tr>
<td>P-selectin pos. MP (% of platelet-derived MP)</td>
<td>38 (35-52) N.S. 17 40 (26-48) N.S. 38 (29-55) N.S. 19 38 (25-49)</td>
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<tr>
<td>CD63 pos. MP (% of platelet-derived MP)</td>
<td>49 (39-66) N.S. 17 48 (32-56) N.S. 51 (36-69) ** 19 43 (31-56)</td>
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<thead>
<tr>
<th><strong>Endothelial activation</strong></th>
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<tbody>
<tr>
<td>vWF antigen (%)</td>
<td>76 (57-150) N.S. 17 80 (55-140) N.S. 98 (52-150) N.S. 19 75 (52-150)</td>
</tr>
<tr>
<td>Endothelial cell-derived MP (× 10⁶/L)</td>
<td>49 (15-369) N.S. 15 76 (3-480) N.S. 128 (0-487) N.S. 16 53 (20-250)</td>
</tr>
</tbody>
</table>

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<thead>
<tr>
<th><strong>MP derived from</strong></th>
<th></th>
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<tbody>
<tr>
<td>T helper cells (× 10⁶/L)</td>
<td>9 (0-32) * 17 17 (3-37) N.S. 13 (0-29) N.S. 19 12 (3-59)</td>
</tr>
<tr>
<td>Cytotoxic T cells (× 10⁶/L)</td>
<td>28 (0-189) N.S. 15 21 (4-55) N.S. 18 (0-84) N.S. 19 15 (5-70)</td>
</tr>
<tr>
<td>Monocytes (× 10⁶/L)</td>
<td>15 (0-109) N.S. 17 26 (0-1008) N.S. 7 (0-348) N.S. 19 5 (0-50)</td>
</tr>
<tr>
<td>B cells (× 10⁶/L)</td>
<td>12 (0-50) N.S. 17 31 (2-93) N.S. 26 (0-59) N.S. 19 14 (2-273)</td>
</tr>
<tr>
<td>Granulocytes (× 10⁶/L)</td>
<td>6 (0-456) N.S. 16 13 (0-459) N.S. 8 (0-993) N.S. 19 13 (2-435)</td>
</tr>
<tr>
<td>Erythrocytes (× 10⁶/L)</td>
<td>244 (89-808) N.S. 17 184 (74-660) N.S. 296 (61-682) N.S. 19 210 (73-677)</td>
</tr>
<tr>
<td><strong>Total concentration of MP (× 10⁶/L)</strong></td>
<td>6678 (1945-16634) N.S. 17 5622 (2997-18510) N.S. 6337 (1930-19515) N.S. 19 4441 (2268-9776)</td>
</tr>
</tbody>
</table>
### Acute phase response

<table>
<thead>
<tr>
<th></th>
<th>Median (Range)</th>
<th>N.S.</th>
<th>Median (Range)</th>
<th>N.S.</th>
<th>P-value</th>
<th>N.S.</th>
<th>Median (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-6 (ng/L)</strong></td>
<td>1.4 (0.6-10.0)</td>
<td></td>
<td>1.0 (0.3-3.3)</td>
<td></td>
<td></td>
<td>1.9 (0.6-10.2)</td>
<td><strong>19</strong> 1.1 (0.2-3.9)</td>
</tr>
<tr>
<td><strong>CRP (mg/L)</strong></td>
<td>1.7 (0.3-3.9)</td>
<td></td>
<td>1.0 (0.3-13.9)</td>
<td></td>
<td></td>
<td>1.0 (0.3-11.3)</td>
<td>N.S. 18 0.8 (0.3-8.9)</td>
</tr>
</tbody>
</table>

Data are presented as median (range). Differences between patients and controls (\(P^m\) male patients versus male controls, \(P^f\) female patients versus female controls) were analyzed with the Wilcoxon matched pairs signed rank test. Differences between male and female patients (\(P^{mf}\)) were analyzed with the Mann-Whitney test. N.S., not significant (\(P > 0.05\)); *\(P < 0.05\); **\(P < 0.01\); ***\(P < 0.001\).

\(\alpha_2\)-AP, \(\alpha_2\)-antiplasmin; \(\beta\)-TG, \(\beta\)-thromboglobulin; CRP, C-reactive protein; (s)EPCR, (soluble) endothelial cell protein C receptor; F\(_{1+2}\), prothrombin fragment F\(_{1+2}\); II-6, interleukin-6; MP, microparticles; \(N\), number of matched female pairs analyzed; \(N^m\), number of matched male pairs analyzed; PF4, platelet factor-4; pos., positive; TAT, thrombin-antithrombin complexes; (s)TF, (soluble) tissue factor; tPA, tissue plasminogen activator; vWF, von Willebrand factor.
Regarding levels of D-dimer, no differences could be found between patients and controls. Levels of tPA as well as α2-AP were higher in female patients when compared with male patients, but neither male nor female patients differed from their healthy controls (Table 1).

Platelet activation
Parameters of platelet activation are also shown in Table 1. In male patients, levels of PF4 were lower than in female patients, while levels of β-TG, platelet-derived microparticles, and percentages of P-selectin and CD63 positive platelet-derived microparticles were similar (Table 1). Male patients had higher levels of β-TG when compared with their healthy controls, but similar levels of PF4, platelet-derived microparticles, and percentages
of P-selectin and CD63 positive platelet-derived microparticles. On the other hand, female
patients had higher levels of β-TG, PF4, as well as CD63 positive platelet-derived
microparticles compared with their controls, and similar levels of platelet-derived
microparticles and percentages of P-selectin positive platelet-derived microparticles.

The levels of β-TG, increased in both male and female patients, and the levels of PF4
and CD63 positive platelet-derived microparticles, increased in female patients only, did
not correlate to renal function or MSSI.

**Endothelial activation**
Levels of vWF did not differ between patients and controls, and neither did levels of
endothelial cell-derived microparticles. Also, no differences were observed between male
and female patients in this regard (Table 1).

**Microparticles derived from cells other than platelets or endothelial cells**
When comparing concentrations of microparticles derived from various types of leukocytes
in patients and controls, we only found a small difference regarding those derived from T
helper cells, which were present at lower concentrations in the male patients. The levels of
microparticles derived from erythrocytes and the total concentration of microparticles did
not differ between patients and controls (Table 1).

**Acute phase response**
Levels of IL-6 were significantly increased in female patients as compared with their
controls, while levels of CRP did not differ. Male patients did not differ from their controls
regarding these two analytes.

No correlation between elevated levels of IL-6 and renal function or MSSI was
established in female patients.

**Discussion**
Fabry disease has been viewed as a disorder of generalized vasculopathy induced by
accumulation of glycosphingolipids in endothelial cells, which, in the presence of abnormal
hemodynamics or blood flow, leads to endothelial cell activation and subsequently to low
grade activation of coagulation as well as fibrinolysis. Outcomes reported in previous
studies support the assumption that a prothrombotic state is present in Fabry disease. This is
substantiated by the finding of increased sICAM-1, sVCAM-1, sP-selectin and
plasminogen activator inhibitor (PAI), as well as decreased TM concentrations in the
circulation of the patients [22]. Also, more recently, decreased levels of α2-AP were
detected in patients with Fabry disease, which might indicate overconsumption as a result
of enhanced fibrinolysis. However, D-dimer and fibrinogen degradation products were not
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Elevated in these patients [49]. Finally, elevated levels of endothelial cell-derived microparticles have been found in untreated pediatric Fabry patients [27].

Despite these earlier observations, we established only minimal abnormalities compared to sex- and age-matched healthy controls in the present study. It is possible that ERT may have influenced the results in the treated patients, since it has been reported that such therapy may reverse the abnormalities in the cerebral circulation [5,8,9] or regarding endothelial cell-derived microparticles [27], for example. Analysis of untreated patients only did not lead to a different outcome (data not shown), but the untreated group represented a subset of milder patients, leaving the possibility open that minimal abnormalities may not have been detected. Markers for endothelial activation such as vWF antigen and endothelial cell-derived microparticles were not elevated. Fibrinolytic activity, as reflected by levels of D-dimer, tPA and α2-AP, was also normal. The only abnormalities that were established in Fabry patients compared with controls, specifically in the more severely affected male patients, were a variable elevation in sTF and β-TG, a marker for platelet activation, with minimal alterations in PF4. In addition, we found evidence of enhanced coagulation in female patients with Fabry disease as shown by increased levels of TAT complexes. In four females there was significant platelet activation (β-TG and PF4). These females were not different from the others with respect of severity of disease or use of co-medication. Only one patient received ERT. In all, renal function was still well preserved (creatinine clearance > 100 ml/min, proteinuria < 0.6 g/24h). Two other female patients had relatively higher levels of IL-6. Although not clear from their clinical records, they may have experienced an infection or event that triggered an acute phase reaction. However, even after exclusion of these two outliers, female patients still had elevated levels of IL-6 compared with their controls.

Earlier studies have described elevated levels of β-TG [24,25], as well TF [26] in patients with Fabry disease. In these studies a possible relationship with decline in kidney function was not investigated. Although there was no correlation between the levels of β-TG, PF4 and creatinine clearance in the present study, this was clearly established for sTF. An association between renal insufficiency and increased levels of TF in plasma has been described in several studies [50-53]. In the study by Mercier et al. [53], an association between renal insufficiency and platelet and endothelial activation was also found. Another study in patients with chronic renal insufficiency revealed elevated levels of adhesion molecules, which was hypothesized to reflect inadequate clearance as well as enhanced synthesis and/or release [54]. The authors of this study concluded that in renal failure patients, there is evidence of endothelial cell injury and a high degree of hypercoagulation relative to healthy subjects. In light of this, it may also be possible that the previously observed increases in factors that reflect endothelial activation such as ICAM-1 and VCAM-1 [22] are the result of advanced kidney disease rather than Fabry disease itself. Interestingly, in the study by Fedi et al. [26], in addition to the increased levels of TF, elevated homocysteine levels were reported. Again, renal function is a major determinant of
homocysteine levels [55], and in patients with renal impairment or on dialysis, increased levels of both TF and homocysteine have been reported to correlate with each other, suggesting a similar mechanism for their elevation [56]. Thus, decreased renal function may also be the cause of the elevated homocysteine levels in Fabry patients, despite normal levels of vitamins B₆, B₁₂ and folic acid. Whether the higher levels of β-TG and PF₄, presumed to reflect platelet activation, are also elevated due to reduced urinary clearance of these small proteins in advanced kidney disease, is unclear: in our cohort, we could not establish a relationship with creatinine clearance or with disease severity in general.

In summary, in a large cohort of Fabry patients we found only minimal abnormalities regarding indicators of coagulation, fibrinolysis and platelet as well as endothelial activation, with the exception of more severely affected patients with renal impairment. We conclude that the laboratory abnormalities in Fabry disease are probably better explained by the renal insufficiency than by the underlying disorder itself.

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**Conflicts of Interest**

Anouk C. Vedder, Johannes M.F.G. Aerts and Carla E.M. Hollak receive reimbursement of expenses and small honoraria for lectures on the management of lysosomal storage diseases, including Fabry disease, from Genzyme Corporation and Shire. All honoraria are donated to the Gaucher Stichting, a national foundation that supports research in the field of lysosomal storage disorders. The other authors have nothing to declare.

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