Circulating microparticles in carriers of factor V Leiden with and without a history of venous thrombosis

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Summary

Although factor V Leiden (FVL) is a major determinant of thrombotic risk, the reason why less than 10% of carriers eventually develop venous thromboembolic (VTE) events is unknown. Recent observations suggest that circulating levels of microparticles (MP) may contribute to the thrombogenic profile of FVL carriers. We measured the plasma level of annexin V-MP (AMP) platelet-MP (PMP), endothelial-MP (EMP), leukocyte-MP (LMP) and tissue factor-bearing MP (TF+MP), and the MP procoagulant activity (PPL) in 142 carriers of FVL (of these 30 homozygous and 49 with prior VTE), and in 142 age and gender-matched healthy individuals. The mean (± SD) level of AMP was 2,802 ± 853 MP/μl in carriers and 1,682 ± 897 in controls (p<0.0001). A statistically significant difference between homozygous and heterozygous carriers of FVL was seen in the level of PMP, EMP and LMP, but not in that of the remaining parameters. When the analysis was confined to carriers with and without a VTE history, the mean level of AMP was 3,110 ± 791 MP/μl in the former, and 2,615 ± 839 MP/μl in the latter (p<0.005). The mean level of all subtypes of circulating MP showed a similar pattern. The PPL clotting time was 39 ± 9 seconds (sec) in carriers, and 52 ± 15 sec in controls (p=0.003); and was 35 ± 8 sec in carriers with prior thrombosis, and 41 ± 10 sec in thrombosis-free carriers (p<0.005). Our study results suggest that circulating MP may contribute to the development of thrombosis in carriers of FVL mutation.

Keywords

Microparticles, factor V Leiden, tissue factor-bearing microparticles, venous thromboembolism

Introduction

It is well known that in comparison to matched control individuals the carriage of factor V Leiden (FVL), by far the most prevalent inherited thrombophilic abnormality in Western countries, increases the risk of venous thromboembolism (VTE) disorders by three to seven times (1–5). However, although a number of either genetic (6) or acquired (7) determinants of the thrombotic risk have been suggested, the true reason why less than 10% of carriers eventually develop VTE complications during their lifetime is still unknown (8).

In recent years, there has been a growing interest in the role of microparticles (MP), vesicles derived from the budding of membranes of multiple cell types, in promoting the development of VTE (9–11). Indeed, they possess a well-known procoagulant activity due to their ability to bind tissue factor on their surface (12–14), as well as to the presence on their surface of phosphatidyserine, which in turn provides binding sites for different clotting factors including tenase and prothrombinase complexes (15, 16).

Evidence that circulating MP may contribute to the thrombogenic profile of FVL carriers comes from two observations. Firstly, the observation that there is an approximately 10- to 20-fold reduction in the rate of activated protein C (APC) catalysed inactivation of plasma-derived factor Va when bound to synthetic phospholipid vesicles (17). Secondly, the observation that APC can induce endothelial MP production and that APC bound to endothelial MP is no longer capable of FVa inactivation (18). In addition, the level of MP was recently found to be higher in a small cohort of heterozygous carriers of FVL than in matched controls (19).

In order to assess whether carriers of FVL do indeed have a higher level of circulating MP than healthy individuals, whether homozygous present with higher levels than heterozygous carriers, and whether the level of circulating MP in carriers with prior thrombosis differs from that of thrombosis-free subjects, we measured the plasma levels of annexin V (AMP) platelet (PMP), endothelial (EMP), leukocyte (LMP) and tissue factor-bearing MP (TF+MP) as well as the MP procoagulant activity in 142 carriers of FVL (30 homozygous and 49 with a history of VTE), and compared them with those of 142 age and gender-matched healthy individuals free from thrombophilic abnormalities.

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Materials and methods

Study patients

All consecutive patients who referred to the Thromboembolism Unit of the University of Padua between January 2008 and June 2010 with a first episode of objectively proven VTE, as well as their uneventful relatives, were eligible for the current investigation provided that they were identified as carriers of FVL and gave their informed consent. Those subjects who had conditions potentially accounting for increased MP levels (such as acute infections, pregnancy or hormonal therapy, acute or chronic cardiovascular diseases, antiphospholipid syndrome, severe blood hypertension, diabetes mellitus and recent surgery) were excluded from the study, as were carriers of other thrombophilic defects, subjects younger than 18, those with previous VTE and/or conditions requiring indefinite anticoagulation. The study protocol was approved by the Institutional Review Board of the University of Padua.

Study controls

Healthy volunteers, referred to our study centre for thrombophilia screening in the same study period, matched for age (±3 years), sex and ethnic origin with the cases qualified as study controls provided that they had not experienced thromboembolic episodes, were free from thrombophilic defects, without conditions potentially accounting for increased MP levels, and gave their informed consent.

Laboratory determination

Procedures

In all patients with VTE, samples were collected at least six months after withdrawal from oral anticoagulant therapy that, in turn, had been administered for at least three months. A total of 9 ml of venous blood was collected from patients and controls with 21-gauge needles, without applying venostasis, into syringes pre-filled with 1 ml of sodium citrate 109 M. Vacuum collection tubes were not used because of possible artificial cellular disruption and formation of MP by this collection method. Cell counts were performed by routine automatic methods (Sysmex Counter XE-2100 Datisi Spa, Milan, Italy). Coagulation tests, including activated partial thromboplastin time (aPTT), prothrombin time (PT), factor VIII (FVIII:C), factor IX (FIX:C) and fibrinogen activity were performed, as previously described (20, 21), by standard methods on the BCT-Analyser (Dade Behring, Marburg, Germany) in our hospital routine coagulation laboratory. Platelet-free plasma (PFP) was obtained by double centrifugation of whole blood (2 x 15 minute [min] at 2,500 g) at room temperature and 1.5-ml aliquots were stored at –80°C until use. All samples were processed within 1 hour (h) after collection and immediately frozen at –80°C until use. Samples were thawed by incubation for 5 min at 37°C and assayed immediately. Patient and control samples were all processed the same way.

FVL mutation

APC resistance was detected using a “home-made” method on the ACL 3000 (Instrumentation Laboratory, Milan, Italy) and DNA analysis for FVL mutations was performed with a standard method, as previously reported (22, 23).

MP assessment and characterisation

MP were identified by size and Annexin V– fluorescein isothiocyanate (FITC) (Bender MedSystems GmbH, Vienna, Austria) labelling. To measure the different populations of MP, the MP were co-labelled with antibodies against cell-type specific antigens and Annexin V. Thirty microliters of freshly thawed PFP were incubated for 15 min with 10 μl of monoclonal antibodies against cell-type specific antigens and 10 μl of Annexin V–FITC. PMP were identified using CD61-PE (phycoerythrin), EMP using CD62e-PC5.
(phycoerythrin-cyanin 5.1) (both from Beckman Coulter, Miami, FL, USA), LMP using CD45-PC5 (BioLegend Europe, Uithoorn, The Netherlands) and TF+MP with CD142-PE (BD, Biosciences, Milan, Italy). The isotype controls used were IgG1-PC5, clone MOPC-21 (BioLegend Europe), IgG1-PE, clone MOPC-21 (BD Biosciences, San Jose, CA, USA); mouse IgG1-FITC, clone MOPC-21 (BioLegend Europe). The samples were diluted in 500 μl of Annexin-V kit binding buffer (Bender MedSystems GmbH, Vienna, Austria) before analysis. A total of 30 μl of counting beads with an established concentration (Flow Count™ Fluorospheres, Beckman Coulter) were added to each sample in order to calculate MP as absolute numbers per microliter of PFP. The MP gate was established using a blend of mono-dispersed fluorescent beads of three diameters (0.5, 0.9 and 3 μm) (Megaminx, BioCytex, Diagnostica Stago, Asnieres, France). MP analyses were performed on a Cytomix FC500 flow cytometer (Beckman Coulter) as previous described (24, 25). All samples were tested blind and the operator did not know the FVL status of any of the samples.

MP procoagulant activity

Procoagulant activity of MP was measured using the STA® Procoagulant Phospholipids assay (PPL, Diagnostica Stago). The assay was performed by measuring the clotting time in an assay system dependent on the procoagulant phospholipids of the sample (26). The assay is performed in phospholipid-depleted substrate plasma to eliminate the influence of any coagulation factors upstream. Factor Xa, in the presence of calcium, triggers the coagulation cascade and a shortening clotting time of the sample indicates an increased concentration of procoagulant phospholipids. PPL activity linearly correlates with the functional activity of MP present in the sample (27).

Table 1: Main demographic and clinical characteristics of the study patients and controls.

<table>
<thead>
<tr>
<th></th>
<th>FVL carriers with prior VTE (N = 49)</th>
<th>FVL carriers without VTE (N = 93)</th>
<th>Controls (N = 142)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age- years (mean ± SD)</td>
<td>48.7 ± 16.3</td>
<td>50.3 ± 10.7</td>
<td>49.5 ± 13.7</td>
</tr>
<tr>
<td>Male sex- No (%)</td>
<td>19 (38.7)</td>
<td>39 (41.9)</td>
<td>58 (40.8)</td>
</tr>
<tr>
<td>Homozygotes- No (%)</td>
<td>18 (36.7)</td>
<td>12 (12.9)*</td>
<td>-</td>
</tr>
<tr>
<td>Unprovoked VTE- No (%)</td>
<td>35 (71.4)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Family history of VTE- No (%)</td>
<td>5 (10.2)</td>
<td>11 (11.8)</td>
<td>-</td>
</tr>
<tr>
<td>Time from VTE- months (mean ± SD)</td>
<td>15 ± 8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PT- % (mean ± SD)</td>
<td>95 ± 14</td>
<td>95 ± 20</td>
<td>96 ± 9</td>
</tr>
<tr>
<td>aPTT- sec (mean ± SD)</td>
<td>26 ± 4</td>
<td>28 ± 3</td>
<td>29 ± 4</td>
</tr>
<tr>
<td>FVIII:C-% (mean ± SD)</td>
<td>145.5 ± 39.5</td>
<td>140 ± 28.6</td>
<td>137.9 ± 35.1</td>
</tr>
<tr>
<td>FIX:C-% (mean ± SD)</td>
<td>131 ± 38</td>
<td>128 ± 32</td>
<td>124 ± 19</td>
</tr>
<tr>
<td>Fibrinogen- mg/dl (mean ± SD)</td>
<td>310 ± 92</td>
<td>305 ± 76</td>
<td>298 ± 88</td>
</tr>
<tr>
<td>Platelets- x10⁹/L (mean ± SD)</td>
<td>258 ± 59</td>
<td>243 ± 4.5</td>
<td>263 ± 56</td>
</tr>
<tr>
<td>White blood cells- x10⁹/L (mean ± SD)</td>
<td>9.25 ± 3.15</td>
<td>8.98 ± 3.01</td>
<td>8.29 ± 2.0</td>
</tr>
</tbody>
</table>

*p = 0.019 for comparison between FVL carriers with and without history of VTE; not significant difference in the other comparisons.

Statistical analysis

Mean values and standard deviation (SD) as well as median values and interquartile ranges (IQR) were calculated for all data sets. The Student's t-test was used for all parametric data. Non-parametric statistics (Mann-Whitney U test) was used for all other data. Correlation between the AMP level and the procoagulant activity of MP was calculated with the Spearman-test. All tests for statistical significance were two-tailed and p-values less than 0.05 were considered statistically significant. The association between the odds of VTE and levels of circulating MP was evaluated. To this purpose, the 95th percentile of AMP, TF+ MP plasma levels and PPL clotting time measured in the control subjects were used as cut-off points, and the odds ratios (OR) and the 95% confidence interval (CI) were calculated and then adjusted for the genotype (homozygosity or heterozygosity) with the use of a logistic regression model. The OR was considered to be statistically significant when the lower limit of the 95% CI was > 1.0. The statistical analyses were performed using a commercially available statistics software package – SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Patients

Of the 225 eligible carriers of FVL, 83 were excluded because of conditions associated with elevated MP (54), ongoing anticoagulation (16), age younger than 18 (6), carriage of other thrombophilic defects (5) and lack of informed consent (2) (Fig. 1). Thus 142 carriers of FVL were recruited, of whom 30 (21%) were homozygous and 112 heterozygous carriers of FVL. Of these 142
subjects 49 (34.5%) had experienced an episode of VTE (18 homozygous and 31 heterozygous FVL carriers), while the remaining 93 belonged to the group of healthy relative. Out of 150 eligible healthy volunteers, 142 gave their informed consent and were then recruited for the current investigation. The main characteristics of the study patients (FVL carriers with and without a history of thrombosis) and controls are shown in Table 1. As shown in the table, no appreciable differences were seen between cases and controls in any of the demographic, clinical and routine coagulation parameters.

MP in carriers of FVL and in controls

In carriers of FVL the median [IQR] level of AMP, PMP, EMP, LMP and TF+MP was 2,824 [2,167–3,842], 913 [566–1,046], 335 [172–524], 25 [14–30], and 39 [22–69] MP/μl. The corresponding figure in controls was 1,540 [1,076–1,911], 384 [232–554], 244 [173–308], 10 [6–12], and 14 [10–17] MP/μl, respectively. All the differences were highly statistically significant (p< 0.001).

In FVL carriers the PPL clotting time (39 ± 9 seconds [sec]) was significantly shorter than in controls (52 ± 15 sec; p=0.003). In the overall study population, the Annexin V number of MP inversely correlated with PPL (r = – 0.52, p=0.0043).

MP levels in carriers of FVL homozygotes and heterozygotes

As shown in Figure 2, a statistically significant difference between homozygous and heterozygous carriers of FVL was seen in the level of PMP, EMP and LMP, but not in the remaining parameters. The levels of all investigated parameters were consistently higher than in controls in each sub-category.

MP levels in carriers of FVL with and without a history of thrombosis

Table 2 reports the median of the tested parameters in carriers of FVL with and without a history of thrombosis, as well as in the control population.
**OR of VTE**

In comparison to non-carriers, carriers of FVL with high levels of AMP and TF + MP (above the 95th percentile, cut-off point 3435 MP/μl and 31 MP/μl, respectively) had an OR for VTE of 3.08 (95% CI, 1.42 to 6.69) and 2.28 (95% CI, 1.07 to 4.85), respectively. The corresponding figure for PPL (above the 95th percentile, cut-off point 70 sec) was 3.47 (95% CI, 0.79 to 15.30). After adjustment for the genotype (homozygosity or heterozygosity), these ratios became 2.52 (95% CI, 0.83 to 5.18), 1.75 (95% CI, 0.65–3.78), and 2.81 (95% CI, 0.33 to 8.47), respectively.

**Discussion**

Our study results, obtained from a large cohort of carriers of FVL consecutively referred to our study centre, show that they have on average levels of circulating MP that are remarkably higher than those detectable in matched control individuals. The difference between carriers and controls decreases, but remains highly statistically significant, even after removal from the analysis of patients with a history of thrombosis. Our findings are consistent with those of a recent investigation in a smaller cohort of heterozygous carriers of FVL (19). In addition, FVL carriers with prior thrombosis have a significantly higher level of circulating MP than thrombosis-free carriers. It should be noted that care was taken to prevent confounders. Indeed, the blood sample was taken at least nine months after the thrombotic episode and at least six months after withdrawal from oral anticoagulation. Interestingly, in all analyses a consistent pattern was found across all different MP subtypes, and both homozygous and heterozygous FVL patients presented with higher MPs plasma levels than healthy individuals.

Our study results suggest that circulating MP may contribute to the underlying hypercoagulability of otherwise healthy individuals who are carriers of the FVL mutation. They are consistent with the findings obtained from previous studies, which suggested that family members of probands with FVL who are, in turn, carriers of the mutation have a substantially higher risk of thromboembolic complications than family members who are free from the mutation (28). Most importantly, the remarkably high level of circulating MP still detectable at least nine months after the thrombotic episode in carriers of FVL suggests that these MP may have played a key role in promoting the index thrombotic episode, and may place them at a persistently higher risk of recurrent events. The association between the risk of VTE and the levels of circulating MP is indirectly confirmed by the remarkably high OR for VTE we found in carriers of FVL with high levels of AMP or TF + MP in comparison to control individuals, although these ratios became not significant after adjustment for genotype. Our results are consistent with those of a recent study, in which an increased endogenous thrombin potential was demonstrated in carriers of FVL with thrombosis as compared to those without thrombosis (29).

The contribution of MP to the thrombogenic prophile of carriers of FVL is plausible and supported by two recent observations (17, 18). Firstly, the observation that there is an approximately 10– to 20-fold reduction in the rate of APC catalysed inactivation of plasma derived factor Va when bound to synthetic phospholipid vesicles (17). Secondly, the observation that APC can induce endothelial MP production and that APC bound to endothelial MP is no longer capable of FVa inactivation (18). It should be noted, however, that the increase in thrombin generation related to the presence of FVL might interfere with the mechanisms of generation of MP from different cell types (30–32), and elevated levels of MP may reflect the various underlying genetic/acquired triggers for the activation of leukocytes, platelets and/or endothelium in these subjects. In addition, other unknown mechanisms might be responsible for the increase in MP in FVL carriers.

The strengths of our study lie in the recruitment of a large cohort of consecutive carriers of FVL (of whom one third with a hist-

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**Table 2: Circulating microparticle (MP) levels for Annexin V (AMP), platelet (PMP), endothelial (EMP), leucocyte (LMP), tissue factor-bearing (TF+MP) and procoagulant phospholipid activity (PPL) in the study population.**

<table>
<thead>
<tr>
<th>MP subtype (MP/μl) median [IQR]</th>
<th>Factor V Leiden carriers</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Previous VTE (p-value)*</td>
<td>Without VTE (p-value)^†</td>
</tr>
<tr>
<td>AMP</td>
<td>3284 [2725–3742] (&lt; 0.005)</td>
<td>2668 [1974–3080] (&lt; 0.001)</td>
</tr>
<tr>
<td>PMP</td>
<td>1061 [573–1268] (&lt; 0.05)</td>
<td>855 [310–1021] (&lt; 0.001)</td>
</tr>
<tr>
<td>EMP</td>
<td>480 [240–560] (&lt; 0.05)</td>
<td>349 [155–467] (&lt; 0.05)</td>
</tr>
<tr>
<td>LMP</td>
<td>28 [23–32] (&lt; 0.001)</td>
<td>28 [13–28] (&lt; 0.001)</td>
</tr>
<tr>
<td>TF+MP</td>
<td>51 [32–114] (&lt; 0.001)</td>
<td>35 [18–70] (&lt; 0.001)</td>
</tr>
<tr>
<td>PPL (sec.)</td>
<td>37 [34–51] (&lt; 0.005)</td>
<td>43 [32–47] (&lt; 0.01)</td>
</tr>
</tbody>
</table>

* p for comparison between FVL carriers with and without history of VTE. † p for comparison between carriers of FVL without history of VTE and controls. IQR, interquartile range; sec., seconds.
ory of thrombosis), and in the comparison with an identical number of gender- and age-matched healthy individuals. Potential confounders were avoided by excluding carriers of thrombophilic defects other than FVL and subjects with conditions known to be associated with high levels of circulating MP; and by including as thrombosis-free carriers of the mutation only relatives of the probands. As a result, we were able to gather a highly homogeneous population of FVL carriers, who differed from each other only in the presence or absence of a previous thrombotic episode.

A few limitations in the study warrant proper comment. Firstly, it should be considered that the methods of MP detection currently available are still suboptimal. Indeed, flow-cytometric assays may not be sensitive enough to detect all MP, as many of them fall below the detection limit of these assays (33, 34). Moreover, it has to be noted that the results of flow cytometry measurements depend on several pre-analytical variables including time between venepuncture and centrifugation, the number of centrifugation steps, freezing/long-term storage and temperature of thawing (35). However, we used the procedure that was recently recommended by the ISTH SSC Working Group on Vascular Biology (36). In addition, the findings of the PPL assay—which is regarded as a functional measurement of the phospholipid dependent procoagulant activity of MP irrespective of particle size—reproduced exactly the same pattern obtained with the flow-cytometry. Secondly, we based our evaluations on a single determination of circulating MP. Elevated levels of circulating MP are observed in a variety of diseases, and can occasionally be found even in healthy individuals (37, 38). However, we carefully excluded all conditions accounting for spurious increases in MP levels. Indeed, the average values we obtained reproduced a highly consistent pattern across the three study populations, the highest levels being in FVL carriers with prior thrombosis, and the lowest in healthy individuals. Thirdly, because of the relatively low number of recruited patients we may not have been able to identify a substantially different pattern between homozygous and heterozygous carriers of FVL. Finally, there exists no cut-off that helps discriminate between normal and abnormal circulating levels of MP. Therefore, the OR of thrombosis, which we calculated using the 95th percentile of AMP and TF-MP plasma levels as cut-off in control subjects, should be regarded as an estimated indicator of the risk, whose proportion needs to be evaluated in prospective studies.

At present, our study results do not apparently have any practical implications. There is a need for prospective long-term investigations in which carriers of FVL—with or without prior thrombosis—are followed-up over time to detect the development of (recurrent) thrombosis and its association with the MP level. Indeed, it is at present virtually impossible to predict the thrombotic risk in carriers of this mutation. In addition, while the carriage of FVL has been consistently reported to increase by approximately 50% the risk of recurrent thromboembolism in comparison to non-carriers (39, 40), no definite risk stratification strategy using laboratory testing has yet been proved to be clinically useful for prediction of recurrent events.

In conclusion, carriers of FVL show much higher levels of circulating MP than healthy individuals. These levels are particularly high in carriers with prior thrombosis, irrespective of the genotype. Circulating MP may, therefore, contribute to the development of thrombosis in carriers of this mutation. Development of equipments that can more accurately determine the levels of MP in plasma, refinement of standardisation of functional clotting says, and identification of reliable cut-offs for discriminating normal from abnormal values will make further progress in the field.

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Conflicts of interest

Barry Woodhams is Scientific Director at Diagnostica Stago. None of the other authors declare any conflicts of interest.

References


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