Circulating microparticles and the risk of thrombosis in inherited deficiencies of antithrombin, protein C and protein S

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Summary
Many subjects carrying inherited thrombophilic defects will never experience venous thromboembolism (VTE) while other individuals developed recurrent VTE with no known additional risk factors. High levels of circulating microparticles (MP) have been associated with increased risk of VTE in patients with factor V Leiden and prothrombin G20210A mutation, suggesting a possible contribution of MP in the hypercoagulability of mild genetic thrombophilia. The role of MP as additional risk factor of VTE in carriers of natural clotting inhibitors defects (severe thrombophilia) has never been assessed. Plasma levels of annexin V-MP, endothelial-derived MP (EMP), platelet-derived MP (PMP), tissue factor-bearing MP (TF+) and the MP procoagulant activity (PPL) were measured in 132 carriers of natural anticoagulant deficiencies (25 antithrombin, 63 protein C and 64 protein S defect) and in 132 age and gender-matched healthy controls. Carriers of natural anticoagulant deficiencies, overall and separately considered, presented with higher median levels of annexin V-MP, EMP, PMP, TF+MP and PPL activity than healthy controls (p<0.001, <0.001, <0.01, 0.025 and 0.03, respectively). Symptomatic carriers with a previous episode of VTE had significantly higher median levels of annexin-V MP than those without VTE (p=0.027). Carriers with high levels of annexin V-MP, EMP and PMP had an adjusted OR for VTE of 3.36 (95 % CI, 1.59 to 7.11), 9.26 (95 % CI, 3.55 to 24.1) and 2.72 (95 %CI, 1.16 to 6.38), respectively. Elevated levels of circulating MP can play a role in carriers of mild and severe inherited thrombophilia. The clinical implications of this association remain to be defined.

Keywords
Coagulation inhibitors, hypercoagulability, microparticles, thrombophilia, venous thrombosis

Introduction
Inherited deficiencies of antithrombin (AT), protein C (PC), and protein S (PS) are associated with a high risk of venous thromboembolism (VTE) particularly in the presence of triggering conditions such as surgery, trauma, hormonal therapy, pregnancy and puerperium. Although these deficiencies are rather uncommon, with a prevalence of less than 1 % in the general population and a prevalence of 5 % in patients with VTE, they are stronger risk factors for VTE than Factor V Leiden (FVL) and prothrombin mutation G20210A (PTM) (1, 2). Indeed, carriers of AT, PC, or PS defects have a four- to 30-fold increased risk of VTE as compared with non-carriers. The highest incidence of 0.9–4.0 per 100 person-years is observed in carriers of antithrombin deficiency (1–8).

Over the past decades, there have been great advances in the understanding of the pathogenesis of VTE in patients with inherited risk factors. However, a number of questions remain unanswered. In particular, it is well known that some subjects carrying inherited thrombophilic defects will never experience a thrombotic episode while other individuals with the same defects develop recurrent thromboembolic events with no known additional acquired risk factors (9). The reason of this discrepancy remains unknown.

Microparticles (MP) are small cell-derived vesicles that can be released by almost all eukaryotic cell types during cellular stress and cell activation. In recent years their role in promoting the development of VTE (10–13) has been well described. The procoagulant capability of MP is due to both their negatively-charged surface and the presence of Tissue Factor (TF) (14, 15). Two recently published studies by our group (16, 17) showed that carriers of FVL and PTM had increased levels of circulating MP (total, platelet-derived, endothelial-derived and TF-bearing) as compared to healthy controls, and that carriers with a history of VTE presented with higher levels than carriers without previous VTE. In particular, high levels of TF-bearing MP were associated with a four-fold increased risk of VTE in FVL carriers, while in PTM carriers high levels of platelet-derived MP were associated with a five-fold increased VTE risk, suggesting a possible contribution of MP in triggering the global prothrombotic state found in mild genetic thrombophilia.

The aim of the present study was to evaluate the rate and source of MP and their procoagulant activity in carriers of natural...
Materials and methods

Study participants

Consecutive patients, who had previously experienced a first episode of objectively proven VTE, referred to the Thrombotic Unit of the University of Padua between January 2013 and December 2014 identified as carriers of AT, PC or PS deficiency, were eligible for the current investigation. Patients were only enrolled at least one month after stopping any anticoagulant treatment. Non-VTE carriers of the same thrombophilic defects were recruited by screening of family members. Healthy volunteers, friend or companions unrelated to the cases referred to our Center for thrombophilia screening in the same study period were used as controls. They were matched for age (±3 years), sex and ethnic origin with the cases.

All subjects were free from conditions potentially associated with increased MP levels in the three months before enrolment into this study (i.e. infections, pregnancy/puerperium, hormonal therapy, cardiovascular diseases, severe hypertension (>160/100 mm Hg), diabetes mellitus, surgery and cancer). Carriers of other thrombophilic defects, subjects younger than 18, patients with conditions requiring lifelong anticoagulation were excluded. All patients had been anticoagulated for at least three months. The study protocol was approved by the Institutional Ethical Committee of the University of Padua Hospital and all subjects gave their written informed consent.

Laboratory determinations

Nine ml of venous blood was collected from patients and controls, using 21-gauge needles without any venostasis, directly into syringes pre-filled with 1 ml of sodium citrate 109 mol/L. The first few ml were discarded to avoid the contact phase activation. Platelet-poor plasma (PPP) was prepared within 1 hour after blood collection by double centrifugation (2 x 15 min at 2,500g) at room temperature. Aliquots (1.5 ml) were immediately frozen and then stored at −80°C until use. Samples were thawed by incubation for 5 minutes (min) in a water bath at 37°C immediately before assay. Assays were performed at least one month after storage at −80°C. Samples were analysed only after a single freeze-thaw cycle, and repeated freeze-thaw cycles were avoided. Patient and control samples were all processed in the same way by the same experienced operators.

Coagulation tests for thrombophilia

Activated partial thromboplastin time (aPTT, reference values (r.v.) 24.4–36.5 s) and prothrombin time (PT, r.v. 70–100%) were measured using Dade® Actin® Activated Cephaloplastin and Thromborex® S reagents (Siemens Healthcare Diagnostics, Muenchen, Germany), respectively.

Antithrombin activity (r.v. 80–120%) was detected using a thrombin-based chromogenic substrate assay from Roche Diagnostics GmbH (Mannheim, Germany). Protein C chromogenic (r.v. 70–130%) and coagulometric (r.v. 80–120%) activities were measured using two commercial kits: Berichrom® Protein C and Protein C Reagent (Siemens Healthcare Diagnostics), respectively. The above tests were performed on a BCS XP coagulation analyzer (Siemens Healthcare Diagnostics). Protein S coagulometric activity (r.v. 70–130%) was assessed using the ProS kit (Instrumentation Laboratory) on an ACL TOP 300 CTS coagulation analyzer (Instrumentation Laboratory, Milan, Italy).

AT antigen (r.v. 80–120%) was determined by a home-made enzyme-linked immunosorbent assay (ELISA), as previously described (18). Protein C antigen (r.v. 80–120%) was determined by a home-made sandwich ELISA using a sheep anti-human protein C antibody (Affinity Biologicals, Ancaster, ON, Canada) as the capture antibody and a horseradish peroxidase (HRP)-conjugated sheep anti-human protein C antibody (Affinity Biologicals) as the detection antibody. Total (r.v. 80–120%) and free protein S antigen (r.v. 80–120%) were measured with a home-made ELISA as previously described (19). The criteria used for the classification of AT, PC and PS defects were in accordance with those reported in the current literature (6, 20).

MP assessment and characterisation

The MP gate was established by preliminary standardisation experiments using a blend of mono-dispersed fluorescent beads of three diameters (0.5, 0.9 and 3 µm) (MegaMix, BioCytex, Diagnostica Stago, France). The upper and the outer limit of the MP gate was established just above the size distribution of the 0.9 µm beads in a forward (FS) and side scatter (SS) setting (log scale). The lower limit was the noise threshold of the instrument and an absolute minimum threshold of 1 was set at the SS parameter (discriminator) to limit the high background noise. This threshold excluded the smallest-sized events. All assays were performed on a Cytomics FC500 flow cytometer (Beckman Coulter, Miami, FL, USA). Thirty µl of counting beads with an established concentration (Flow Count TM Fluorospheres, Beckman Coulter) were added to each sample in order to calculate MP as absolute numbers per microliter of PPP.

MP expressing phosphatidylserine (PS) were identified by size and annexin V-fluorescein isothiocyanate (FITC) (Bender MedSystems GmbH, Vienna, Austria) labelling (annexinV-MP) as previously described (16, 17). To measure the different populations of MP, the MP were co-labelled with antibodies against cell-type specific antigens and annexin V. Thirty µl of freshly thawed PPP were incubated for 15 min with 10 µl of monoclonal antibodies against cell-type specific antigens and 10 µl of annexin V-FITC. Endothelial-derived MP (EMP) were identified using CD62E-PC5 (phycoerythrin-cyanin 5.1), Platelet-derived MP (PMP) using CD61-PE (phycoerythrin) (both Beckman Coulter), and tissue factor positive (TF+MP) with CD142-PE (BD Biosciences, Milan, Italy).

The samples were diluted in 500 µl of annexin-V kit binding buffer (Bender MedSystems GmbH) before analysis. Thirty µl of counting beads with an established concentration (Flow Count
TM Fluorospheres, Beckman Coulter) were added to each sample in order to calculate MP as absolute numbers per µl of PPP.

**MP procoagulant activity**

Procoagulant activity of the MP was measured using the STA’Procoag PPL assay (Diagnostica Stago), as previously described (16, 17). The PPL activity linearly correlates with the functional activity of MP present in the sample (21).

**Statistical analysis**

Statistical analysis was performed using the PASW Statistics 17.0.2 (SPSS Inc., Chicago, IL, USA) for Windows. The results were calculated as median values and interquartile ranges (IQR) and differences between cases and controls were evaluated using the non-parametric Mann-Whitney U test. For multiple comparisons (differences between the three groups of cases) Kruskall-Wallis test was used. Spearman’s correlation analysis was used to detect significant associations between the MP numbers and PPL test. A p-value <0.05 was considered statistically significant. The association between the odds of VTE and levels of circulating MP was evaluated. For this purpose, the 95th percentiles of all MP subtypes plasma levels measured in the control subjects were used as cut-off points, and the odds ratio (OR) and the 95% confidence interval (CI) were calculated and then adjusted for the possible confounders (age, sex, body mass index [BMI]) with the use of a logistic regression model.

**Results**

**Patients**

Of the 132 carriers of natural anticoagulants deficiency enrolled into the study, 25 (19%) were carriers of antithrombin deficiency, 63 (48%) and 44 (33%) were carriers of protein C and protein S deficiency, respectively. The main characteristics of the study patients and controls (n = 132) are shown in Table 1. In particular, no appreciable differences were seen between cases and controls in age, sex, BMI and routine coagulation parameters (PT and aPTT). Among cases, 10 (40%) carriers of AT defect, 22 (35%) carriers of PC deficiency, and 20 (45%) carriers of PS deficiency were carriers of a type III defect. The 95th percentiles of all MP subtypes plasma levels measured in the control subjects were used as cut-off points, and the odds ratio (OR) and the 95% confidence interval (CI) were calculated and then adjusted for the possible confounders (age, sex, BMI) with the use of a logistic regression model.

**Table 1: Main demographic and clinical characteristics of the study population.**

<table>
<thead>
<tr>
<th></th>
<th>AT deficiency (N = 25)</th>
<th>PC deficiency (N = 63)</th>
<th>PS deficiency (N = 44)</th>
<th>Controls (N = 132)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex, n (%)</td>
<td>9 (36)</td>
<td>20 (31)</td>
<td>17 (38)</td>
<td>46 [34]</td>
</tr>
<tr>
<td>Previous VTE, n (%)</td>
<td>14 (56)</td>
<td>26 (43)</td>
<td>18 (40)</td>
<td></td>
</tr>
<tr>
<td>Unprovoked VTE, n (%)</td>
<td>10 (71)</td>
<td>18 (69)</td>
<td>11 (61)</td>
<td></td>
</tr>
<tr>
<td>PT, %</td>
<td>99.5 [90–102.7]</td>
<td>96.8 [89.6–103]</td>
<td>94.3 [86.7–99.2]</td>
<td>99.5 [89–105.5]</td>
</tr>
<tr>
<td>AT activity, %</td>
<td>49 [46.2–61.2]**</td>
<td>-</td>
<td>-</td>
<td>102.5 [98.2–107]</td>
</tr>
<tr>
<td>Antigen, %</td>
<td>50 [42.5–66.5]</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Type I defect, n (%)</td>
<td>20 (80)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC coagulometric activity, %</td>
<td>-</td>
<td>49.5 [43–58.2]**</td>
<td>-</td>
<td>102 [92–113]</td>
</tr>
<tr>
<td>Chromogenic activity, %</td>
<td>55 [45.7–62.2]**</td>
<td>-</td>
<td></td>
<td>112 [100–125]</td>
</tr>
<tr>
<td>Antigen, %</td>
<td>54.5 [47–64.2]**</td>
<td>-</td>
<td>-</td>
<td>100 [96–118]</td>
</tr>
<tr>
<td>Type I defect, n (%)</td>
<td>54 (86)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS activity, %</td>
<td>-</td>
<td>-</td>
<td>49.5 [37.2–60.2]**</td>
<td>101 [88–115]</td>
</tr>
<tr>
<td>Free antigen, %</td>
<td>-</td>
<td>-</td>
<td>56 [41–64]**</td>
<td>98 [87–112]</td>
</tr>
<tr>
<td>Total antigen, %</td>
<td>-</td>
<td>-</td>
<td>75 [65–91.2]*</td>
<td>99 [91–117]</td>
</tr>
<tr>
<td>Type I defect, n (%)</td>
<td>-</td>
<td>-</td>
<td>20 (45)</td>
<td>-</td>
</tr>
<tr>
<td>Type III defect, n (%)</td>
<td>24 (54)</td>
<td></td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

Data are expressed by median and interquartile range; p are calculated vs controls. * p < 0.05; ** p < 0.01. n, number; BMI, body mass index; VTE, venous thromboembolism; PT, prothrombin time; aPTT, activated partial thromboplastin time; AT, antithrombin; PC, protein C; PS, protein S.
PC defect and 14 (32%) of PS defect had experienced a previous episode of VTE. About 60–70% of VTE were unprovoked.

**MP levels in carriers of thrombophilic defects and in controls**

Overall carriers of natural anticoagulant deficiencies showed statistically significant higher median levels of total MP than healthy controls (p < 0.001) (Table 2). Overall thrombophilic carriers showed higher levels of annexin V-MP (2786 [1844–3935] MP/µl), EMP (303 [133–445] MP/µl), PMP (436 [205–809] MP/µl) and TF+MP (51 [37–92] MP/µl) than healthy controls (1845 [982–2122], 111 [95–159], 325 [229–529] MP/µl and 31 [20–72] MP/µl, respectively). The difference was statistically significant for all the comparisons (p < 0.001, < 0.001, < 0.01 and 0.025, respectively) (Table 2). In the Table 2 were also provided the MP count as percentage of total MP events. It is important to notice that only 51.4% of total MP obtained from thrombophilic patients and 46.6% of total MP obtained from healthy controls were labeled with annexin V.

**MP levels in AT, PC, PS deficiency and in controls**

Carriers of AT defect showed significantly increased median levels of annexin V-MP (3915 [2584–4498] MP/µl), EMP (420 [302–501] MP/µl) and PMP (750 [396–894] MP/µl) than controls (p < 0.01 for all comparisons). Similarly, carriers of PC defects presented with higher median levels of annexin V-MP (2999 [2491–4083] MP/µl), EMP (479 [313–462] MP/µl) and PMP (770 [215–1355] MP/µl) as compared to controls (p < 0.01 in all comparisons). No significant difference was found in TF+MP levels between AT and PC defects carriers and controls. Carriers of PS defects showed significantly increased median levels of annexin V-MP (2901 [2305–4090] MP/µl), EMP (401 [324–529] MP/µl) and TF+MP (65 [36–129] MP/µl) than controls (1845 [982–2122], 111 [95–159], 31 [20–72] MP/µl, respectively; p < 0.01, < 0.001 and < 0.05, respectively). No significant difference was found in PMP median levels between PS defects carriers and controls.

**PPL clotting time in carriers of thrombophilic defects and in controls**

In the thrombophilic population PPL clotting time was significantly shorter (57 [43–74] s) as compared to healthy controls.

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**Table 2: Circulating levels of microparticles (MP) and procoagulant phospholipid activity (PPL) in natural anticoagulant deficiency subjects and controls.**

<table>
<thead>
<tr>
<th>MP subtype, MP/µl</th>
<th>Thrombophilic patients (N=132)</th>
<th>Controls (N=132)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total MP</td>
<td>5149 [3987–6004]***</td>
<td>3955 [2241–4788]***</td>
</tr>
<tr>
<td>Annexin V-MP (%)</td>
<td>2786 [1844–3935] (54.1%)</td>
<td>1845 [982–2122] (46.6%)</td>
</tr>
<tr>
<td>EMP (%)</td>
<td>303 [133–445] (5.8%)</td>
<td>111 [95–159] (2.8%)</td>
</tr>
<tr>
<td>PMP (%)</td>
<td>436 [205–809] (8.5%)</td>
<td>325 [229–529] (8.2%)</td>
</tr>
<tr>
<td>TF+MP (%)</td>
<td>51 [37–92] (0.99%)</td>
<td>31 [20–72] (0.78%)</td>
</tr>
<tr>
<td>PPL, s</td>
<td>57 [43–74]</td>
<td>72 [61–77]</td>
</tr>
</tbody>
</table>

Data are expressed by median and interquartile range; p are calculated vs controls. * p < 0.05; ** p < 0.01; *** p < 0.001. MP, microparticles; EMP, endothelial-derived microparticles; PMP, platelet-derived microparticles; TF+MP, tissue factor-bearing microparticles; PPL, phospholipid clotting time.
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In the overall study population (cases and controls), the annexin V-MP and EMP number of MP inversely correlated with PPL (r = −0.71, p = 0.009 and r = −0.31, p = 0.002, respectively). In carriers of single thrombophilic defects, the median PPL clotting time was significantly shorter (45 (37–93) s in AT defects, 61 (45–85) s in PC defects and 54 (44–69) s in PS defects) than in controls (72 (61–77) s; p < 0.001 for all comparisons).

MP levels in the three groups of thrombophilic defects

Figure 1 shows the median levels of MP in the three groups of thrombophilic defects considered. In particular, carriers of AT defects showed statistically significant higher median levels of EMP and TF+MP than carriers of PC defects (p = 0.006 and 0.005, respectively). Carriers of AT defect had significant increased median annexin V-MP, EMP, and PMP levels as compared to PS carriers (p = 0.04, 0.007, and 0.007, respectively). Finally, carriers of PC defect presented with significantly higher median levels of PMP and significantly lower median levels of TF+MP as compared to carriers of PS defect (p = 0.002 and 0.001, respectively).

Table 3: Circulating levels of microparticles (MP) in natural anticoagulant deficiency subjects with and without previous VTE.

<table>
<thead>
<tr>
<th>MP/µl</th>
<th>Thrombophilic patients with previous VTE (N=46)</th>
<th>Thrombophilic patients without previous VTE (N=86)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total MP</td>
<td>5714 [2955–6941]</td>
<td>5397 [2338–6100] *</td>
</tr>
<tr>
<td>Annexin V-MP</td>
<td>2884 [1919–3927] (50.4%)</td>
<td>2407 [1518–3541] * (44.5%)</td>
</tr>
<tr>
<td>EMP</td>
<td>356 [213–464] (6.2%)</td>
<td>292 [115–419] (5.4%)</td>
</tr>
<tr>
<td>PMP</td>
<td>491 [234–884] (8.6%)</td>
<td>375 [177–702] (6.9%)</td>
</tr>
<tr>
<td>TF+MP</td>
<td>68 [43–101] (1.2%)</td>
<td>46 [31–85] (0.8%)</td>
</tr>
<tr>
<td>PPL, s</td>
<td>55 [40–73]</td>
<td>59 [44–70]</td>
</tr>
</tbody>
</table>

Data are expressed by median and interquartile range; p-values are calculated vs controls. * p < 0.05. MP, microparticles; VTE, venous thromboembolism; n, number; EMP, endothelial-derived microparticles; PMP, platelet-derived microparticles; TF+MP, tissue factor-bearing microparticles; PPL, phospholipid clotting time.

Carriers of thrombophilic defects with a previous episode of VTE had significantly higher median levels of total MP (5714 (2955–6941) MP/µl) and annexin-V MP (2884 (1919–3972) MP/µl) than carriers without a history of VTE (5397 (2338–6100) MP/µl and 2407 (1518–3541) MP/µl, respectively; p < 0.01 for both comparisons). The median levels of EMP, PMP and TF+MP were higher in thrombophilia carriers with a previous VTE episode than in carriers without VTE, but the difference was not statistically different (p > 0.05, for all comparisons) (Figure 2).

Carriers of thrombophilic defects without a previous episode of VTE showed significantly higher median levels of annexin-V MP (2407 (1518–3541) MP/µl) and EMP (292 (115–419) MP/µl) than healthy controls (p < 0.001 or both comparisons). No significant difference was shown in the median levels of PMP and TF+MP (p > 0.5) between these two groups (Figure 2). In the Table 3 we also provid the MP count as percentage of total MP events.

Figure 2: MP median levels in carriers of natural anticoagulants deficiency with and without a history of VTE. A) Annexin-V MP; B) Endothelial-derived MP; C) Platelet-derived MP; D) TF+MP. Grey area refers to MP plasma levels found in healthy controls. Data are expressed by median and interquartile range; p-values are calculated vs controls. * p < 0.01; ** p < 0.001.
Notably, only 50.4% of total MP in thrombophilic patients with previous VTE and 44.5% of total MP in thrombophilic patients without previous VTE were labelled with annexin V.

Using the 95% percentile value of the control group as the cut-off point, carriers of natural anticoagulant deficiencies with high levels of annexin V-MP (above the 95th percentile, cut-off point 2734 MP/µl) had an adjusted OR for VTE of 3.36 (95% CI, 1.59 to 7.11, p=0.0012) as compared to carriers with annexin V-MP lower than the cut-off. Those carriers with EMP higher than the cut-off point (171 MP/µl) had an adjusted OR for VTE of 9.26 (95% CI, 3.55 to 24.1, p<0.0001) and those carriers with PMP higher than 791 MP/µl (cut-off point) had an adjusted OR for VTE of 2.72 (95% CI, 1.16 to 6.38, p=0.019). The OR for VTE in carriers with TF+MP higher than 101 MP/µl (cut-off point) did not reach statistical significance [1.63 (95% CI, 1.02 to 2.60, p=0.058)].

**Discussion**

We showed that carriers of the deficiency of natural anticoagulants have increased levels of circulating MP, much higher than those detectable in matched control individuals. Not only the total number of MP, but also the different MP subtypes considered (annexin V-MP, EMP, PMP and TF+) were significantly higher in thrombophilic carriers than in healthy individuals. When we considered every single thrombophilic defect, we found that AT and PC deficiency carriers presented with significantly higher levels of annexin V-MP, EMP and PMP, but not TF-MP as compared to controls. PS deficiency carriers showed significantly higher levels of annexin V-MP, EMP and TF+MP, but not PMP, as compared to controls. The PCL clotting time mirrored the same trend of annexin V-MP. Notably, the thrombophilic carriers with prior thrombosis showed significantly higher levels of annexin V-MP than the non-VTE carriers. As regard MP subtypes, the differences between the VTE and non-VTE thrombophilic groups did not reach significance, although the non-VTE thrombophilic group median values were lower.

These results are similar to our earlier findings in carriers of PTM and FVL (16, 17). In particular, we found that heterozygous and homozygous carriers of PTM (17) and FVL (16) showed significantly increased levels of annexin V-MP, EMP, PMP and TF+MP than healthy controls. In carriers of PTM no significant difference was seen between carriers with and without a history of VTE. In contrast, in the FVL study all differences between the VTE and non-VTE groups reached significance.

This study in carriers of natural anticoagulant deficiencies further confirms a possible role of circulating MP in the underlying hypercoagulability of otherwise healthy individuals who are carriers of hereditary thrombophilia.

In the literature there is consistent evidence for a risk gradient for VTE, which is higher in carriers of AT, PC, PS deficiency and those homozygous or carriers of multiple defects (severe thrombophilia), and moderate in heterozygous carriers of FVL or PTM (mild thrombophilia). In our previous studies (16, 17) we indirectly demonstrated a significant association between the risk of VTE and the levels of circulating MP in carriers of prothrombin mutation and FVL by the significant OR for VTE associated with high levels of annexin V-MP or PMP and annexin-V MP or TF+MP, respectively. Compared to the previous report (16, 17), in the present study we found a higher significant association between MP levels and risk of VTE, with an OR from three-fold for annexin-V MP to nine-fold for EMP. Thus, the strong association between MP levels and VTE in carriers of AT, PC and PS defects may reflect the higher prothrombotic potential in these severe thrombophilic conditions. Moreover, the highest OR for VTE was seen for EMP levels, suggesting a possible predominant role of endothelial dysfunction or remodelling or impairment as initial triggering mechanism for the MP release and increased prothrombotic potential. Thrombophilic carriers with no history of VTE presented with significantly higher annexin V-MP and EMP levels than healthy controls. This increase in MP count and activity could contribute to the hypercoagulability responsible for the development of VTE.

When a subgroup analysis of single anticoagulant defects was performed, carriers of AT defect showed significantly higher median levels of annexin V-MP, EMP and PMP than controls and higher TF+MP levels than carriers of PC defect. We can speculate that the higher thrombin potential due to the lack of antithrombin inhibition (22) may determine a global hypercoagulable condition that impairs the endothelial function (EMP), the platelets activation (PMP) and also the activation of the coagulation cascade (TF+MP). However, the prevalent mechanism involved in the prothrombotic state of PC defects seems to be endothelium and platelet-mediated (higher levels of EMP and PMP as compared to controls and PS defect). Finally, PS defect carriers presented with significantly higher median levels of TF+MP as compared to controls and to PC defect and similar levels to AT defect. It seems that PS deficiency impaired coagulation factors with activation of the extrinsic pathway. It is well known that PS is a cofactor for tissue factor pathway inhibitor (TFPI) accelerating the inhibition of activated factor X (23). The deficiency of PS may result in a poor anticoagulant response to TFPI (24), and consequently a higher activation of TF mediated hypercoagulability (TF+MP) and a higher thrombin generation.

Similar findings in both this and our previous studies (16, 17) indicate that MP could play a role in the development of VTE in all different inherited thrombophilic abnormalities, and that there is an increasing thrombotic potential due to MP with the change from a mild to a severe thrombophilic states. Needless to say, MP activity may be only part of the explanation of the increased thrombotic risk among different thrombophilic conditions and different clinical manifestations in the carriers of the same thrombophilic defect. In fact, it is well known that VTE is a multifactorial disease.

The strengths of our study lie in the recruitment of a large cohort of consecutive carriers of AT, PC and PS deficiencies- known to be rare inherited diseases in the general population- and in the comparison with gender- and age-matched healthy individuals. Potential confounders were avoided by excluding subjects with conditions known to be associated with high levels of circulating
MP, and by adjusting the OR for VTE for known prothrombotic confounders (age, sex and BMI).

A few methodological limitations have to be acknowledged. First, currently available methods for detecting MP are still suboptimal. Flow cytometric assays are not sensitive enough to detect all size of MP, given that many of them fall below the method’s detection threshold. We followed the recommended procedure of the ISTH SSC Working Group on Vascular Biology for the analytical and pre-analytical issues (25–27). Second, we only considered MP expressing phosphatidylserine; however, it has been well demonstrated that only a minority of circulating microvesicles expose phosphatidylserine and annexin V binding is highly dependent on Ca\(^{2+}\) concentrations (28, 29). We provided annexin V-MP count as percentage of total MP events detected and showed that only about 50 % of total MP were labeled with annexin V. This means that also the cellular origin of MP was evaluated only in less than 50 % of total MP population (double staining with annexin V). Thus we could only account for a minority percentage of MP and this may lead to interpretation bias. Despite these limitations, annexin V is considered a valuable marker of MP of different origin, with the advantages of having a relatively high density of the target molecules on MP and excellent reproducibility of measurements (29). To better characterise the measurement of these MP we also used a functional MP activity assay. The PPL clotting time assay exactly reflected the flow cytometry results. Third, there exists no cut-off that helps discriminate between normal and abnormal circulating levels of MP. Therefore, the OR for VTE, which we calculated using as cut-off the 95\(^{th}\) percentiles of MP plasma levels as cut-off in control subjects, should be regarded as an estimate of the risk, whose accuracy needs to be assessed in prospective studies. Finally, a new model has been recently hypothesized where MP are complex ambivalent structures that express both activators and inhibitors of coagulation and also convey fibrinolytic properties (30).

In our study, we could only explore the presence of MP with procoagulant properties and no information is available on their potential anticoagulant and fibrinolytic role. In particular, we couldn’t evaluate the possible role of MP-TFPI levels role in modulating the expression of TF-MP in carriers of PS deficiency compared to controls.

In conclusion, carriers of natural anticoagulant deficiencies show much higher levels of circulating MP than healthy individuals. These levels are particularly higher in carriers with previous VTE, with a clear association between high MP levels and VTE development. AT defects show higher EMP, PMP and TF-MP that other defects, PC seems to impair mostly endothelial and platelet functions, PS determine a more pronounced TF-dependent hYpecoagulability. As shown in milder thrombophilic conditions (factor V Leiden and prothrombin mutation), circulating MP may contribute to the development of VTE also in carriers of the more severe inherited thrombophilia. Larger, prospective studies are needed to better clarify the clinical utility of measuring MP and which subtypes of MP can be important to be assessed in hereditary thrombophilia.

What is known about this topic?
- Many subjects carrying inherited thrombophilic defects will never experience venous thromboembolism (VTE) while other individuals developed recurrent VTE with no known additional risk factors.
- Circulating microparticles (MP) play a role in promoting the development of venous VTE in different prothrombotic states.
- High levels of circulating MP have been associated with increased risk of VTE in patients with factor V Leiden and prothrombin G20210A mutation, suggesting a possible contribution of MP in the hypercoagulability of mild genetic thrombophilia.

What does this paper add?
- Carriers of natural anticoagulant deficiencies show much higher levels of circulating MP than healthy individuals.
- MP levels are particularly higher in carriers with previous VTE, with a statistically significant association between high MP levels (particularly for endothelial-derived-MP) and VTE development.
- As shown in milder thrombophilic conditions (factor V Leiden and prothrombin mutation), circulating MP may contribute to the development of VTE also in carriers of the more severe inherited thrombophilia.

Conflicts of interest
Barry Woodhams acts as a consultant, via HaemaCon, for Diagnostica Stago. None of the other authors declares any conflicts of interest.

References