Clinical significance of circulating microparticles for venous thromboembolism in cancer patients

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Summary
Cancer patients have a four- to seven-fold increased risk to develop a venous thromboembolic event. Accumulating evidence from experimental and clinical studies indicates that microparticles (MPs), small procoagulant membrane vesicles that are defined by size and a negatively charged phosphatidylserine rich surface, play an important role in the pathogenesis of cancer-related venous thromboembolism (VTE). However, the clinical significance of MPs as a predictive biomarker for VTE in cancer patients has not been fully elucidated yet. This might be due to unresolved methodological problems and a lack of data from large prospective clinical studies that investigate the role of MPs in cancer-related VTE.

It is the aim of this review to give an overview on the most important characteristics of MPs and studies dealing with the role of MPs in cancer-related VTE. Also recent progresses, unresolved problems and future perspectives in this research field will be discussed. In the conclusion we will assess the clinical significance of MPs in cancer-related VTE.

Circulating microparticles (MPs) are small membrane vesicles that are defined by size (0.1–1 μm) and a procoagulant negatively charged phosphatidylserine(PS)-rich surface. MPs are shed from activated or apoptotic cells when the asymmetric phospholipid distribution of the cell membrane is lost and negatively charged PS is redistributed from the endoplasmatic- to the exoplasmatic leaflet (1). MPs circulating in plasma are mainly derived from
- platelets (2),
- erythrocytes (3),
- endothelial cells (4),
- lymphocytes (5),
- monocytes (6), and
- smooth muscle cells (7).

The most abundant MPs in plasma are platelet derived MPs that constitute 70–90% of circulating MPs (8).

MPs act as mediators of cell-to-cell communication. They carry and transfer a broad array of membrane-, cytoplasmatic- and nuclear molecules, that reflect the antigenic profile of the cells they originate from (1). A significant role of MPs (9, 10) has been particularly found in
- inflammation,
- immunity,
- vascular disease, and
- cancer-related VTE.

In 1983 Dvorak et al. demonstrated that PS-bearing MPs are shed from cultured cancer cells (11). They suggested that procoagulant circulating MPs favour tumour growth because MPs promote fibrin deposition in the tumour microenvironment and provide a matrix for tumour angiogenesis (12). In a number of studies this hypothesis has been confirmed (13) and several additional pathways of MP-mediated tumour progression have been found. For example, it

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was shown that cancer cells shed complement-enriched and Fas-ligand expressing MPs into the circulation that suppress the immune response of the host against the tumour (14, 15). Furthermore, it was established that cancer-derived MPs alter the function of non-malignant cells and thereby promote cancer cell migration and invasion (16). Yu et al. demonstrated that two of the most common genetic alterations in human malignancy, namely the inactivation of p53 and the mutation of K-ras, are closely linked to the shedding of TF-bearing MPs from cancer cells (17). They also found strong evidence that TF expression in colorectal cancer cells is not merely an epiphenomenon but required for full manifestation of an aggressive cancer phenotype.

A main hallmark of circulating MPs is their procoagulant activity. It was already shown in 1967 by Wolf et al. that MPs (back then denoted as „platelet dust“) are promoting thrombus formation (18). MPs are prothrombotic because

- their negatively charged PS-rich surface promotes the aggregation and subsequent activation of coagulation factors and
- highly procoagulant MP subpopulations co-express prothrombotic proteins like tissue factor (TF) and P-selectin glycoprotein ligand-1 (PSGL-1) on their surface.

The PS-rich surface of MPs has been reported to be 50- to 100-fold more prothrombotic than the equivalent area on activated platelets (19) and about 25% of the procoagulant potential in blood has been attributed to MPs (20).

Circulating MP sub-populations that express active TF, the main initiator of the coagulation cascade, are particularly procoagulant. TF-bearing MPs were reported to arise from distinct cholesterol-rich parts of the cell membrane, so-called lipid rafts (21) and originate from stimulated monocytes (22), endothelial cells (23), platelets (24, 25) and cancer cells (26).

Elevated levels of TF-bearing MPs were found in different diseases that are associated with a prothrombotic state like gram-negative sepsis (22), diabetes (27) and cancer-related VTE (28).

MP-bound TF was also reported to be present in an encrypted form in the circulation that is not involved in blood coagulation but in cell signaling (29, 30).

**Microparticle detection**

A number of methods and protocols are applied for the quantification of MPs. Published protocols for MP measurement differ widely with regard to analytical and pre-analytical variables such as plasma preparation, blood drawing, storage conditions (freezing and thawing of MPs), labeling of MPs and instrument adjustments (31–33). These differences lead to a high variability of detected MP levels in different studies even when the same detection method is used and hamper the comparison of results. For example, a 40-fold range in the number of platelet derived MPs in healthy subjects was found when studies were compared.

A first step towards standardization was recently done when a new protocol for flow cytometry based MP detection was published. In this protocol a set of calibrated synthetic submicrometric beads was used for the calibration of different types of flow cytometers and a low coefficient of variation of platelet MP levels was achieved when measurements were compared (34).

The most frequently used technique for the investigation of MPs in experimental and clinical studies is flow cytometry (35).

It allows the determination of MP levels, definition of the cellular origin of MPs and the investigation of MP-bound surface proteins, but no information regarding functional activity of proteins expressed on MPs can be obtained.

Although the potential of flow cytometry for MP research seems to be compelling, there are major limitations of this method, which need to be addressed. First of all, small MPs cannot be investigated with flow cytometry because the lower detection limit of flow cytometers is restricted by the wavelength of the laser light applied for MP measurement and ranging between 0.3 and 0.5 μm (36).

Notably, the median size of MPs in plasma was reported to lie at 0.3 μm and MPs smaller than 0.2 μm were shown to be responsible for 50% of the endogenous thrombin potential in plasma (37).

Therefore, evidence indicates that a significant number of small procoagulant MPs cannot be detected with standard flow cytometry.

**Impedance-based flow cytometry**

Recently Zwicker et al. developed a new method, the impedance-based flow cytometry, to investigate TF-bearing MPs in cancer patients with- and without VTE. They found 10000-fold higher levels of TF-bearing MPs with this new method than with standard flow cytometry (45). The huge difference in the number of detected TF-bearing MPs is startling and also questions most results from previous studies applying standard flow cytometry. However, we believe that data based on impedance-based flow cytometry still need to be confirmed by other research groups who should also systematically investigate the accuracy of impedance-based flow cytometry compared to other methods.

**Chromogenic assays**

Another approach for MP detection is the indirect quantification of MPs by the determination of the procoagulant activity of MPs with chromogenic assays (36). Two frequently used chromogenic assays are the prothrombinase assay and the MP-associated TF (MP-TF) activity assay (28). The prothrombinase assay uses thrombin generation to indirectly quantify levels of procoagulant MPs (38). This test is mainly dependent on the exposure of procoagulant phosphatidylserine on MPs that binds to annexin V and gives a measure of the overall number of procoagulant MPs in plasma. The MP-TF activity assay quantifies levels of circulating highly procoagulant TF-bearing MPs by measuring the MP-TF dependent factor Xa generation. This assay has been suggested to be of particular interest for the investigation of the VTE risk in...
cancer patients. It is a drawback of both assays that in contrast to flow cytometry no direct information about the number of circulating MPs and their cellular source can be obtained.

**New techniques**

To overcome inherent limitations of the aforementioned rather well established and widely used methods new techniques like dynamic light scattering (37) and atomic force microscopy (39) have been developed or adopted for MP quantification. However, they are still in their infancy and future studies with appropriate design will show whether they allow more accurate, reproducible and robust measurement of MPs than the more established methods.

### MPs and cancer-related venous thromboembolism

VTE is a common complication in cancer patients and a frequent cause of cancer-related mortality (40). Up to 20% of cancer patients develop VTE during the course of disease (41).

An association between elevated MP levels and acute VTE in cancer patients has been well established for TF-bearing MPs and was first described in a seminal study by Tesselaar et al. (28). In this study procoagulant properties of TF-bearing MPs were investigated in blood samples from 50 patients with breast- or pancreatic cancer, of whom seven had VTE at study inclusion. Also 37 healthy subjects and seven patients who presented with idiopathic VTE were included. MP levels were measured with flow cytometry and a MP-TF activity assay. MP-TF activity levels were significantly higher in advanced breast- and pancreatic cancer patients than in primary breast cancer patients, patients with idiopathic VTE and healthy subjects. Those cancer patients with a VTE event had the highest MP-TF activity levels, which lied above the 99th percentile of healthy subjects. The authors concluded that they found evidence for a significant role of TF-bearing MPs in the development of cancer related VTE. They also stated that the precise mechanism underlying the observed association still remained to be elucidated.

Levels of TF-bearing MPs were also investigated in a quite similarly designed subsequent study by Manly et al. that also used a chromogenic MP-TF activity assay (42). Patients with different types and stages of cancer were included. Thirteen cancer patients with VTE were compared to 53 without VTE. Like in the aforementioned study levels of TF-bearing MPs were also investigated. Like in the aforementioned study.

#### Table 1

<table>
<thead>
<tr>
<th>first author (ref.)</th>
<th>study design</th>
<th>method of MP quantification</th>
<th>patients</th>
<th>main results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tesselaar (28)</td>
<td>retrospective</td>
<td>MP-TF activity assay</td>
<td>43 patients without VTE (18 with pancreatic cancer, 25 with breast cancer), 7 patients with VTE (5 with pancreatic cancer, 2 with breast cancer), 7 patients with idiopathic VTE, 37 healthy subjects</td>
<td>higher MP-TF activity levels in cancer patients with VTE compared to healthy subjects and patients with idiopathic VTE</td>
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<tr>
<td>Manly (42)</td>
<td>retrospective</td>
<td>MP-TF activity assay</td>
<td>66 patients with different malignancies: 53 with VTE and 13 without VTE</td>
<td>higher MP-TF activity in cancer patients with VTE compared to cancer patients without VTE</td>
</tr>
<tr>
<td>Campello (43)</td>
<td>case-control/retrospective</td>
<td>flow cytometry</td>
<td>60 patients with different malignancies: 30 with VTE and 30 without VTE, 30 patients with idiopathic VTE, 90 age- and sex matched healthy controls</td>
<td>higher levels of EMPs, PMPs and TF-bearing MPs in patients compared to healthy controls</td>
</tr>
<tr>
<td>Zwicker (46)</td>
<td>case-control/prospective</td>
<td>impedance-based flow cytometry</td>
<td>30 patients with different malignancies and VTE, 22 patients with idiopathic VTE, 60 age-, sex-, stage- and diagnosis matched cancer patients (controls) without VTE, controls were followed for 2 years for future VTE</td>
<td>significantly higher TF-bearing MPs in cancer patients with VTE than in controls, elevated TF-bearing MPs predicted an increased risk of future VTE, cancer surgery eliminated TF-bearing MPs</td>
</tr>
<tr>
<td>Auwerda (47)</td>
<td>prospective</td>
<td>MP-TF activity assay</td>
<td>122 multiple myeloma patients: 15 with VTE at follow up, 20 healthy controls</td>
<td>no association between future VTE and MP-TF activity levels in multiple myeloma patients</td>
</tr>
<tr>
<td>Thaler (48)</td>
<td>case-control/prospective</td>
<td>prothrombinase assay</td>
<td>728 patients with different malignancies, 65 age- and sex matched healthy controls, 2-year follow up for future VTE</td>
<td>significantly elevated MP levels in cancer patients compared to healthy controls, no association between MP levels and risk of future VTE in cancer patients</td>
</tr>
</tbody>
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EMPs: endothelial microparticles; PMPs: platelet microparticles; MP: microparticles; TF: tissue factor; VTE: venous thromboembolism
by Tesselaar et al. MP-TF activity levels were significantly higher in cancer patients with VTE compared to the others. Also in this study the question remained unanswered whether TF-bearing MPs were primarily involved in the pathogenesis of cancer-related VTE or just the result of a VTE event.

Campello et al. applied flow cytometry to measure levels of platelet MPs (PMPs), endothelial MPs (EMP) and TF-bearing MPs in 90 patients (30 with active cancer, 30 with active cancer and VTE and 30 with idiopathic VTE) and in a group of 90 healthy controls (43). PMP-, EMP- and TF-bearing MP levels were significantly higher in cancer patients with/without VTE and in patients with idiopathic VTE compared to healthy controls. They also found that TF-bearing MP levels were significantly higher in cancer patients with VTE than in cancer patients without VTE. Surprisingly, in multivariate analysis they failed to detect a significant association between elevated TF-bearing MPs (>95\textsuperscript{th} percentile of healthy controls) and VTE in cancer patients. The authors did not provide an explanation for this result, which might be regarded as inconsistent with the findings of Tesselaar et al.

Evidence for an increased propensity for future VTE in cancer patients with elevated levels of TF-bearing MPs arises from two recently published prospective studies. In one study multiple MP-TF activity measurements were performed during the course of disease in pancreatic cancer patients. In another study patients with different cancer types were prospectively followed for future VTE after an initial MP-TF activity measurement. No predictive role of MP-TF activity levels for future VTE was found in multiple myeloma patients.

Khorana et al. performed the first prospective study that included eleven pancreatic cancer patients who underwent chemotherapy (44). Consecutive blood samples were drawn from these patients at regular intervals and MP-TF activity levels were investigated during the course of disease. In nine patients, of whom none developed a VTE event, MP-TF activity levels stayed low during the observation period. In two patients MP-TF activity levels were progressively rising until both patients develop a VTE event. One patient had a massive and lethal pulmonary embolism, which was confirmed by autopsy, and one patient had a peroneal/distal deep vein thrombosis, which was confirmed by ultrasound. The results from this study indicated a predictive role of rising MP-TF activity levels for future VTE during the course of disease in patients with pancreatic cancer. Given the small sample size these results were regarded as preliminary by the authors and it was suggested that larger similarly designed studies should be performed to either confirm or disprove these results. Surprisingly, no such study was published in the last two years.

The second prospective study was published by Zwicker et al. who developed a new method, the aforementioned impedance-based flow cytometry, for detection and quantification of TF-bearing MPs (45). Thirty patients with a number of different cancer types and VTE and 60 without VTE were included. TF-bearing MPs were detected in 60% (18 of 30) of patients with VTE compared to 27% (16 of 60) without VTE. The number of patients with detectable TF-bearing MPs was significantly higher in patients with VTE. In 60 patients without VTE the occurrence of VTE was analyzed during the two years following enrolment. Four out of 16 patients with measurable TF-bearing MPs and one out of 44 patients without measurable TF-bearing MPs developed VTE after enrolment. The presence of TF-bearing MPs predicted a seven-fold increased risk for future VTE compared to cancer patients who were negative for TF-bearing MPs. In three pancreatic cancer patients TF-bearing MPs were measured before and after cancer resection. In all three patients a massive decrease in TF-bearing MP levels was observed after cancer resection, indicating that the TF-bearing MPs were tumour-derived. The authors concluded from these results that the presence of TF-bearing MPs predicted an increased risk of VTE in cancer patients and suggested that TF-bearing MPs might be used as a biomarker for the identification of patients that would benefit from primary thromboprophylaxis. Consequently, the authors of this study recently initiated a randomized, controlled trial to evaluate the benefit of primary thromboprophylaxis in cancer patients with elevated levels of TF-bearing MPs (46).

In the third prospective study Auwerda et al. measured MP-TF activity levels in 122 newly diagnosed multiple myeloma patients before and after chemotherapy and in 20 healthy subjects (47). During the observation period 15 patients developed VTE. MP-TF activity levels were significantly higher in multiple myeloma patients than in controls but MP-TF activity measurements were not higher in patients who developed VTE compared to those who did not develop VTE at follow up. The authors stated also in this paper that the pathogenetic role of MP-TF activity levels in cancer-related VTE remains to be elucidated.

The currently largest study that investigated the predictive role of MPs for VTE in cancer patients was recently published by our group (48). We used a chromogenic prothrombinase assay to quantify the overall number of circulating procoagulant MPs in plasma and measured MP levels in 728 patients with different malignancies and in 65 age- and sex-matched healthy individuals. At study inclusion a blood sample for MP measurement was drawn and cancer patients were followed prospectively for VTE. During a two-year observation period 53 (7.3%) patients developed VTE. We found that MP levels in cancer patients were significantly higher than in healthy individuals but we did not find a statistically significant difference between cancer patients who had developed VTE compared to those who had not developed VTE.

Therefore, we did not find a predictive role for future VTE of procoagulant MPs determined by the prothrombinase assay.

**Conclusion, perspectives**

Taking the aforementioned data together, it might be suggested that MPs play an important role in the development of cancer-related VTE. Yet, we believe that the clinical significance of MPs as a biomarker for cancer-related VTE must be assessed as low due to a number of reasons.

First of all, there is a lack of large prospective clinical studies that clearly show a predictive role of MPs for future VTE in cancer patients. Only two rather small studies observed a predictive role of TF-bearing MPs for future cancer-related VTE.
while another prospective study did not find such an association. In addition, it was suggested in a large study, that procoagu-
la nt MPs (assessed with a well-established prothrombinase assay) are not predicting an increased VTE risk in cancer patients.

Furthermore, there is a lack of standardization of methods and protocols applied for MP detection that hamper the comparison between published data. It is another impor-
tant point that the feasibility of MP quantifi-
cation in routine laboratories has not been assessed yet, a precondition for every biom-
ar ker to be clinically significant. Therefore, as a first step to improve MP research, it would be of utmost importance to further promote efforts to standardize protocols and methods for MP assessment. Also additional large prospective studies should be perform-
ed to elucidate whether MPs are predictive of cancer related VTE or not. Only then it would be possible to answer thoroughly whether MPs play a clinical significant role for the prediction of VTE in cancer patients.

Conflict of interest
The authors declare that they have no conflicts of interest.

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