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REVIEW ARTICLE

Contributing role of extracellular vesicles on vascular endothelium haemostatic balance in cancer

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Extracellular vesicles (EVs) generated during tumourigenesis are thought to play a major role in the hypercoagulant state observed in cancer patients. They exhibit negatively charged phospholipids and tissue factor (TF) that promote coagulation cascade activation. In addition, they contain surface proteins and cytoplasmic molecules, both originating from the producing cell that can impact target cells' expression. By targeting endothelial cells of blood vessels, these EVs could disturb the physiological anticoagulant properties of these cells and be partly responsible for the vascular endothelium activation observed in cancer patients. Indeed, vascular endothelium naturally exhibits heparin-like proteoglycan, TF pathway inhibitor and protein C anticoagulant pathway that prevent thrombosis in physiological condition. An overexpression of TF and a decreased expression of coagulation cascade inhibitors have been reported after EVs' treatment of endothelial cells. The induction of apoptosis and an increased expression of platelet adhesion molecules have also been highlighted. These events may promote thrombus formation in cancer. The aim of this paper is to provide a targeted review on the current evidence and knowledge of roles and impact of EVs on endothelial surface anticoagulant and procoagulant factors and cellular adhesion molecules expression.

Keywords: *cancer*; *endothelium*; *haematology*; *extracellular vesicles*; *thrombosis*

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The relationship between cancer and thrombosis began with Trousseau's researches in 1865. Currently, thrombosis is the second leading cause of death in cancer patients after malignancy itself (1). The overall procoagulant state leading to thrombosis is variable depending on cancer type, stage and treatment. A total of 18–29% of patients diagnosed with thromboembolism actually suffer from cancer (2). The underlying pathophysiological mechanism of thrombotic events in cancer patients is multifactorial. Among the contributing factors, extracellular vesicles (EVs) produced by tumour cells and their microenvironment generate considerable interest since the discovery of their pro- and anticoagulant properties, their fibrinolytic activity (3,4) and their ability to contribute to thrombosis in vivo (5). The involvement of EVs in the prothrombotic state in cancer is not limited to their haemostatic phenotype. Indeed, EVs are known to be a way of communication between cells and are able to impact remote target cells' phenotype (6). In this context, it is anticipated that EVs target endothelial cells and contribute to their activation and the disturbance of their physiological anticoagulant properties.

This review aims at providing the latest evidence on the contribution of EVs to the haemostatic balance in cancer patients. A more specific discussion on their contributing roles on the prothrombotic state in haematological malignancies is also proposed.

Generated EVs in cancer

EVs are vesicles ranging from 0.03 to 1 μm generated by almost all cell types, including endothelial cells, and they play a role in intercellular communication (7). EVs are heterogeneous and depending on their tissue of origin, size or intracellular origin have been known by different names, such as microparticles, oncosomes, microvesicles, ectosomes, exosomes, and so on. Since the distinction

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between the different types of EVs is still challenging, considering the various generation and isolation methods used in the publications cited in this review, here we chose to use only the generic term EVs (8). Cells communicate via several mechanisms: secreted molecules (9), cell-to-cell direct contacts (10) and nanotubules formation (11). The communication by EVs is a more recently discovered mechanism representing a major interest in the scientific world. Cells in reaction to various stresses and to apoptosis perform the EVs production. The generated EVs exhibit surface proteins and can contain cytoplasmic molecules, both originating from the producing cell (6). Thus, the analysis of surface proteins of EVs can reveal the original cell. EVs are found in all biological fluids such as saliva (12), urine (13), cerebrospinal fluid (14) and blood (6).

Cancer cells are known to produce large amounts of EVs contributing to the tumour microenvironment. In addition, various cells in this microenvironment also produce EVs in reaction to various stresses caused by the tumour itself or the pharmacological treatment. To support this later hypothesis, a study highlighted an in vitro chemotherapy-induced endothelial cell death triggering a massive release of EVs (15). EVs produced during tumourigenesis have been associated with a lot of cancer features such as angiogenesis (16), inflammation (17), chemoresistance (18), metastasis (16) and coagulation (19). To support the roles of EVs on angiogenesis, Grange et al. have demonstrated that EVs stimulate endothelial cells to form neo-tubes and induce angiogenic factors production by stromal cells (16). By interfering with immune cells, EVs also contribute to create a favourable inflammatory niche for tumour cells (17). EVs could also transfer multidrug resistance from resistant cancer cells to sensitive ones, partly due to miRNA transfer (18). In addition, EVs enable the formation of a pre-metastatic niche favourable to the implantation of a circulating tumour cell (16,20). These characteristics are due to their content in nucleic acids or in proteins but also to the lipids and proteins present in their phospholipid membrane. These various molecules enable EVs to interact with target cells and modify their phenotype (6).

Contribution of EVs to the haemostatic balance in cancer

The link between hypercoagulation and cancer is still an interesting field of investigation even if several explanations have already been discovered. Inflammatory state linked to neoplasia, changes in protein metabolism and venous stasis associated with cancer contribute to this procoagulant phenotype (21). Nevertheless, a procoagulant phenotype of tumour cells has also been highlighted (22). First, an overexpression of tissue factor (TF) has been observed on cancer cells surface (23). This major member of coagulation cascade can activate this one by binding to the serine protease FVII/VIIa to form an activator complex. In this way, TF can promote thrombosis formation (24). In addition, tumour cells exhibit higher levels of phosphatidylserine (PS) on their plasma membrane compared to normal cells (25). These negatively charged phospholipids form a catalytic surface for the accumulation of coagulation cascade members (26) and increase TF procoagulant activity (27). This procoagulant phenotype of cancer cells may be responsible for the formation of a thrombus close to the tumour. However, the occurrence of thrombosis far from the tumour site has led to the hypothesis of another cause of thrombus formation in cancer. Indeed, EVs generated by tumour cells also exhibit the same negatively charged phospholipids and TF (3,28) and thus are thought to be partly responsible for this distant thrombosis (19). In vivo mice experiments demonstrated that cancer cell–derived EVs play a major role in thrombus formation (5). Nevertheless, some anticoagulant or fibrinolytic activities of endothelial and cancer EVs have also been reported. This led to the hypothesis that EVs reflect the haemostatic balance of their original cell. In a normal healthy situation, there would be an equilibrium between pro- and antithrombotic EVs. In cancer, this balance would be disturbed and would favour prothrombotic EVs, thus increasing the thrombotic risk (4). By analysing EVs from plasma of healthy and cancer patients with a fluorescence-activated cell sorting, Aharon and co-workers showed a decrease in tissue factor pathway inhibitor (TFPI) expression on EVs in cancer patients, while no significant difference was observed for TF expression (29). TFPI inhibits TF's capacity to initiate blood coagulation and thus represents an antithrombotic protein (30). The increased ratio of TF-positive EVs to TFPI-positive EVs (TF/TFPI) observed in cancer patients would reflect the disturbed balance towards pro-thrombotic EVs in these patients (29). Heterogeneity in EVs' phenotype is also observed for PS expression. A study performed on blood from normal healthy individuals by flow cytometry showed that only 80% of platelet EVs express PS (31). Consistent with these results, a recent cryo-Transmission Electron Microscopy study concluded that about only 50% of EVs are PS-positive (32). Considering the synergy between TF and PS exposure for TF activity (27), future researches should analyse this heterogeneity in cancer patients.

The ability of EVs to impact various target cells could also disrupt the natural barriers against hypercoagulation including the anticoagulant properties of blood vessels' endothelium.

Contribution of EVs to vascular endothelial cells activation

Because of the major role of blood vessels' endothelium as natural anticoagulant surface, tiny modifications of

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endothelial cells (named endothelial activation) could contribute to thrombosis occurrence. Indeed, 3 mechanisms of coagulation cascading inhibition by blood vessels' endothelium have been highlighted. First, heparin-like proteoglycans, located on endothelial cells surface, enhance the inhibition capacity of anti-thrombin and therefore contribute to thrombin inhibition (Fig. 1a) (33). Second, endothelial cells exhibit the coagulation inhibitor TFPI (30). TFPI binds to factor VIIa when this one interacts with TF. This inhibition is strengthened by the binding of factor Xa also neutralized by TFPI (Fig. 1b) (30). Thirdly, the endothelium is where the protein C anticoagulant pathway takes place. In this cascade, thrombin procoagulant activity is inhibited by thrombomodulin (TM) enabling the activation of the protein C by thrombin via the endothelial cell protein C receptor (EPCR). The activated protein C (APC) detaches from the EPCR and interacts with the protein S to inactivate factor Va and VIIIa of the coagulation cascade (Fig. 1c) (34,35).

In this context, various studies suggested vascular endothelial activation in cancer (36) and the EVs generated during tumourigenesis could contribute to this activation and the disturbance of their haemostatic balance.

Induction of TF expression and decrease of TFPI and TM

Various studies have assessed the potential impact of EVs on endothelial cells' anticoagulant activity. The first highlighted effect was the induction of TF expression by endothelial cells (Fig. 2c). In vitro studies showed an increased antigenic expression of TF on endothelial cells treated with leukocytes' EVs (37,38) and tumour cells' EVs, inducing an increased procoagulant activity of endothelial cells (39). This increased surface expression is probably due partly to a direct transfer of TF from EVs surface to cellular cytoplasmic membrane (39). However, several studies also highlighted an increased TF mRNA expression after EVs treatment (37,38). These experiments suggest a mechanism of action not limited to a direct transfer of TF but comprising an impact on target cells' gene expression. EVs' content in nucleic acids or proteins could contribute to this increased gene expression in endothelial cells. A study suggests that leukocytes' EVs could act on gene expression by the induction of a sustained tyrosine phosphorylation of c-Jun NH_2-terminal kinase (JNK1) in endothelial cells (37). JNK1 phosphorylation is especially observed in cells undergoing stress conditions (40). JNK1 phosphorylation leads to activator protein 1 (AP1) activation, a transcription factor complex known to induce TF transcription (41). One in vivo study on TF-low mice also revealed this increased TF expression. Human tumour cells were transplanted to the mice presenting a very low TF expression. Immunofluorescence analysis showed an important human TF expression on blood vessels endothelial cells. These results argue in favour of a TF transfer from the xenograft to the vascular endothelial cells via EVs (39).

The expression of anticoagulant proteins located at the endothelial surface in normal conditions could also be

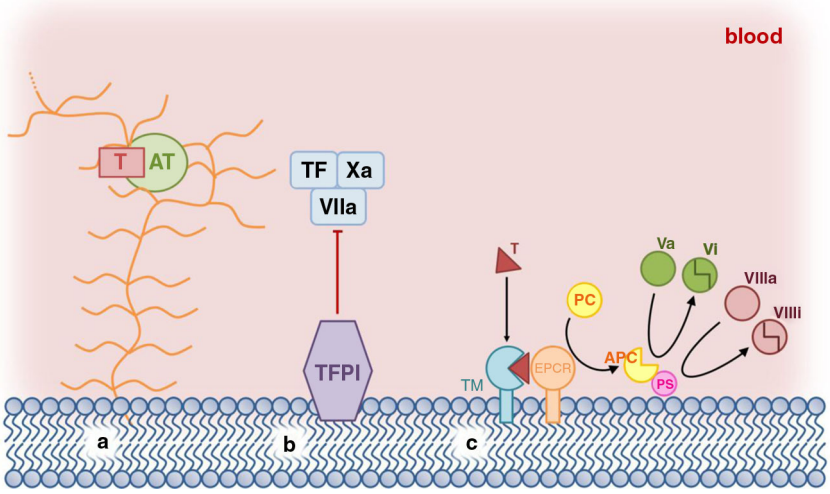

Fig. 1. Physiological anticoagulant characteristics of blood vessels on endothelial cells. (a) Heparin-like proteoglycans located on endothelial cells' surface enhance the inhibition capacity of anti-thrombin (AT). (b) Tissue factor pathway inhibitor (TFPI) inhibits tissue factor (TF) ability to initiate blood coagulation. TFPI actually binds to factor VIIa when this one interacts with TF. This inhibition is strengthened by the binding of factor Xa also neutralized by TFPI. (c) The protein C anticoagulant pathway takes place on endothelial cells surface. In this cascade, thrombin (T) procoagulant activity is inhibited by thrombomodulin (TM) enabling the activation of the protein C (PC) by thrombin via the endothelial cell protein C receptor (EPCR). The activated protein C (APC) detaches from the EPCR and interacts with the protein S (PS) to inactivate factor Va and VIIIa of the coagulation cascade.

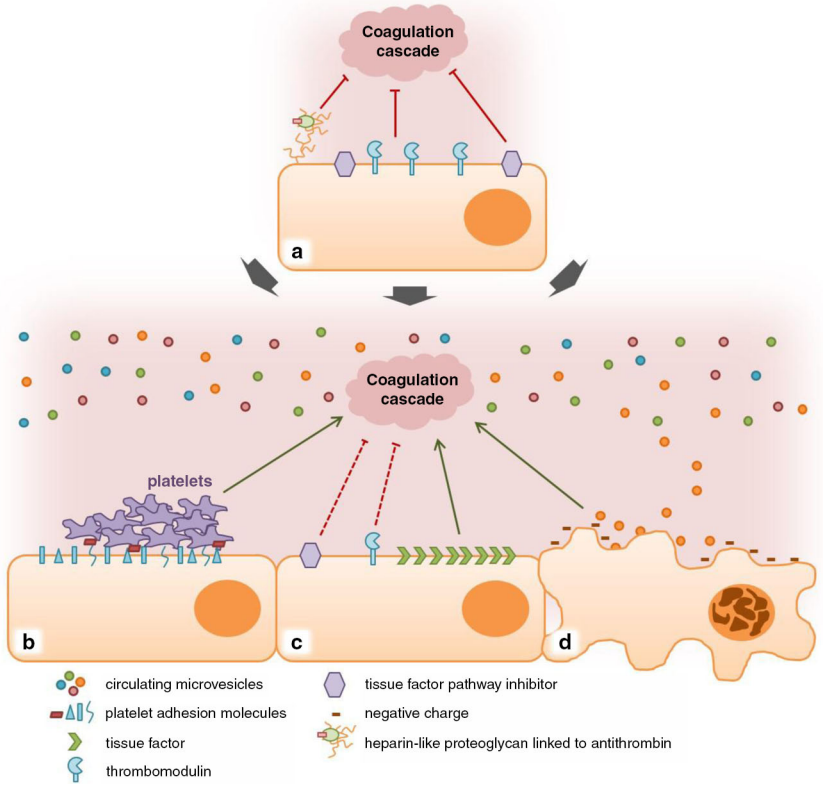

Fig. 2. Impact of extracellular vesicles on endothelial cells' anticoagulant properties. (a) In a physiologic state, blood vessels' endothelium exhibits anticoagulant properties. (b) Extracellular vesicles generated during tumourigenesis could increase platelet adhesion molecules' expression by endothelial cells and thus, favour a procoagulant state of endothelium. (c) A decrease of tissue factor pathway inhibitor and thrombomodulin expression could be induced by extracellular vesicles. Extracellular vesicles could also trigger an increase in tissue factor on endothelial cells, contributing to a procoagulant phenotype. (d) Extracellular vesicles would induce endothelial cells' apoptosis. Apoptotic endothelial cells exhibit negatively charged phospholipids and produce extracellular vesicles both contributing to a procoagulant phenotype of endothelial cells.

affected by EVs. Indeed, TFPI and TM expressions have been shown to decrease in endothelial cells after EVs treatment (Fig. 2c) (38).

Induction of endothelial apoptosis

Another impact of EVs on endothelium would be the induction of endothelial cells apoptosis (Fig. 2d). This has been shown in vitro with monocytic (38) or platelet EVs (42). Apoptotic endothelial cells exhibit a procoagulant phenotype due in part to the exposure of PS at the plasma membrane during apoptosis process. These exposed negative phospholipids represent a catalytic surface for coagulation cascade. Apoptotic human umbilical vein endothelial cells (HUVECs) also present a decreased expression of coagulation inhibitors such as TM, heparan sulphates and TFPI (43). Several mechanisms of endothelial apoptosis induction by EVs have been proposed. First, TF presented at EVs surface could play a role in this cell death induction. A bifunctional role of TF was proposed by Pradier et al. At low concentration, this protein induces in vitro tube formation and confirms the known pro-angiogenic role of TF. An apoptosis induction was however highlighted at high concentration of TF. Physiologically, these dose-dependent effects may play a major role in tissue homeostasis. TF favours apoptosis of strongly damaged cells, while inducing reparation of less injured tissues (44). No in vivo data suggests a local sufficient concentration of EVs in cancer enabling endothelial apoptosis induction. However, platelet EVs have been shown to induce angiogenesis both in vitro and in vivo (45). The underlying mechanism probably involves not only TF.

Other EVs' contents could induce endothelial apoptosis. Platelet EVs have been shown to contain miRNAs and some of them are upregulated during inflammatory response. In this context, Pan and co-workers showed that miR-223 is transmitted to endothelial cells via platelet EVs. miR-223 was able to increase in vitro advanced glycation end products-induced apoptosis. This outcome is probably due in part to the targeting of insulin-like growth factor 1 receptor (IGF-1R) by this non-coding RNA (42). Indeed, IGF-1R is known to be involved in apoptosis protection of cancer cells (46).

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Apoptosis being a mechanism of EVs production, this process would represent an amplification loop in the context of coagulation (Fig. 2d). Indeed, endothelial EVs are also involved in cancer-associated procoagulant phenotype (15) and have been shown to trigger thrombin generation in vitro and thrombus formation in vivo (47). Another mechanism could also be involved in an increased EVs production by endothelial cells. Pasquier and co-workers recently proposed the hypothesis of an induction of protein kinase B (PKB) phosphorylation in endothelial cells triggered by breast and ovarian cancer cell lines' EVs. This activation of PKB would lead to ADP-ribosylation factor 6 (Arf6) increased expression. By acting on cytoskeleton, Arf6 would then induce EVs budding (48).

Increased expression of platelet adhesion molecules

Activated endothelium has been shown to initiate platelets rolling in vitro (49) and in vivo (50). This feature could contribute to the establishment of a procoagulant state by the adherence of platelets on activated endothelial cells. In some particular pathophysiological conditions, the exposure of subendothelial matrix by endothelium damage is not necessary for platelet adhesion to vascular endothelium. In cancer, the activation of endothelial cells by EVs would also induce the adhesion of platelets and the formation of platelet aggregates. Terrisse et al. observed the formation of platelets strings on HUVECs after treatment with endothelial, monocytic or platelet EVs (49). This acquired pro-adhesive phenotype is probably due to an increased expression of platelet adhesion molecules in endothelial cells after EVs treatment (Fig. 2b).

Several adhesion molecules are implicated in platelet–endothelium interaction. First, P-selectin expressed on activated endothelial cells by Weibel-Palade bodies' exocytosis, can mediate platelet adhesion via the P-selectin glycoprotein ligand-1 (PSGL-1) expressed on platelets (51,52). Experiments conducted on EVs-treated endothelial cells have shown the importance of P-selectin for platelet attachment to endothelium after EVs treatment. In the same experiment, a member of the glycoprotein 1b–IX–V, glycoprotein 1b (GP1b) has been highlighted for its contribution in platelet adhesion because of the observed reduced platelet strings formation after the addition of an anti-GP1b antibody (49). This research group also demonstrated the major role of von Willebrand factor (VWF) on platelet adhesion after EVs treatment. An increased VWF surface expression at endothelial cells membrane has been highlighted after EVs treatment and the addition of an anti-VWF antibody strongly reduces platelet rolling. The induction mechanism of VWF surface expression by EVs implicates a reactive oxygen species (ROS) accumulation in endothelial cells. Indeed, endothelial and monocytic EVs trigger ROS generation in endothelial cells in vitro (53,54). The expression of VWF and the resulting platelet adhesion was decreased by inhibition of ROS (49). The actual interaction between GP1b, VWF and platelets remains to be clarified. An indirect link between GP1b and platelets via a stabilization of VWF strings at endothelial cells surface by GP1b is the current hypothesis (55). However, a direct binding of GP1b to a platelet receptor is not excluded. This increased level of VWF at endothelial cell surface after EVs treatment could impact on endothelial EVs composition. Indeed, VWF has been detected on endothelial EVs and would induce platelet aggregation in vitro, thus contributing to a procoagulant state in cancer patients (56).

The induction of intercellular adhesion molecule-1 (ICAM-1) in endothelial cells after platelet EVs treatment has been observed in vitro (57). Even if ICAM-1 is well known for its ability to bind leukocytes during inflammation and diapedesis process, this molecule is also able to interact with platelets and would promote platelet aggregation to endothelial cells (58). In this context, ICAM-1 expression in endothelial cells after EVs stimulation could promote platelet aggregation in vivo in blood vessels and favour thrombosis occurrence.

Hypothesis supporting the role of EVs in prothrombotic state in haematological malignancies

At first, solid tumours were thought to induce more thrombotic events than haematological malignancies (59). However, comparable thrombotic rates between these 2 types of cancer have been reported (60). Furthermore, new treatments with immunomodulatory drugs such as thalidomide and the use of central vein catheter have increased the incidence of thrombotic events in haematological cancers. The use of oral contraceptives before diagnosis was also highlighted as a risk factor (61). The prevalence of thrombosis in haematological cancers differs depending on the malignancy and ranges from 2 to 12% (59). Patients with haematological malignancies have a 26-fold increased risk of developing thrombosis compared to the general population (62). Incidence of thrombosis in acute leukaemia ranges between 2 and 12% in the literature (63–65). Similar incidences have been highlighted for other haematological malignancies. A 10% incidence of thrombotic events was described for myeloma patients (66) and from 5 to 10% for lymphoma patients (59,67).

Several processes would contribute to the procoagulant phenotype observed in haematological cancers. The procoagulant phenotype of tumour cells and the expression changes of host cells due to a stressful microenvironment are involved in the coagulopathy of haematological malignancies as in cancer in general (68). A resistance to APC was also highlighted in solid tumours (69) as in haematological cancers (70). APC resistance is characterized by a poor anticoagulant response of plasma after the addition

of APC. In a non-cancer situation, APC resistance is often due to a factor V Leiden mutation (71). However, a decreased level of protein S, the cofactor of APC and a reduced level of TFPI in haematological cancers seem to be more likely responsible for the resistance observed (70).

EVs have also been shown to contribute to this thrombosis occurrence in haematological cancers. A procoagulant phenotype of leukaemic EVs has been highlighted by Gheldof et al. in vitro. TF and PS expression by these EVs was shown to be involved in this procoagulant potential (3). A recent clinical study conducted on 7 patients suffering from acute myelocytic leukaemia seems to correlate these results (72). A clinical study also observed an increased EVs–TF activity in multiple myeloma (73). A potential involvement of EVs in a TM resistance has also been suggested in a study analysing plasma from patients suffering from myeloproliferative neoplasms (MPN). Plasma from MPN patients presented a higher procoagulant activity by thrombin generation assay than plasma from healthy patients. By the addition of TM to plasma, a lower inhibition of the thrombin generation was observed with plasma from MPN patients. The filtration of plasma through a 0.22-μm filter enhanced the effect of TM on thrombin generation suggesting an involvement of EVs in this observed TM resistance (74).

Vascular endothelial activation probably contributes to the procoagulant phenotype in haematological malignancies. Indeed, some in vivo results suggest an endothelium activation in haematological malignancies. Increased levels of circulating P-selectin and VWF have been observed in patients' blood compared to controls and would reflect this endothelial activation (75). A procoagulant activity of endothelial cells after daunorubicin, doxorubicin or epirubicin treatment has also been highlighted (76) and an increased release of procoagulant endothelial EVs was observed after daunorubicin treatment (77).

In this context, EVs generated during tumourigenesis could contribute to this endothelium activation by the previously described mechanisms. This new insight into endothelial activation mechanism in cancer might be an interesting field of investigation for the discovery of new prophylactic treatments in haematological malignancies. EVs might represent a new prophylactic target. Indeed, thrombosis prophylaxis is still challenging due to an increased bleeding risk of oncohaematological patients that could suffer from thrombocytopenia for a prolonged time (59). The American Society of Clinical Oncology recommends the use of prophylaxis in cancer patients undergoing surgical procedures or hospitalization. For multiple myeloma patients, the use of low molecular weight heparin or low-dose aspirin is recommended for patients treated with antiangiogenesis agents combined with chemotherapy and/or dexamethasone (78). For ambulatory patients, the lack of validated markers to define high-risk patients of thrombotic events still hampers the use of anticoagulants in these patients (59). In this context, endothelial EVs might serve as biomarkers describing endothelial activation that would contribute to procoagulant phenotype. Soluble proteins produced by activated endothelial cells could also have the same purpose. Members of the selectin family such as E-selectin, specifically expressed by endothelial cells, or members of the integrin or adhesion molecules families represent potential new biomarkers (79).

Conclusion

To sum up, EVs have been shown to contribute to the procoagulant phenotype observed in cancer patients. On one hand, they bear TF and PS that directly promote thrombosis by acting on coagulation cascade. On the other hand, they can target and activate endothelial vascular cells becoming procoagulant in cancer. This new insight into the mechanism of procoagulant phenotype acquisition in cancer would be of particular interest for haematological malignancies. Indeed, the prophylaxis treatment to avoid thrombosis in oncohaematological patients remains challenging due to the increased risk of bleeding in these patients. Endothelial EVs might serve as biomarkers of vascular endothelial status and would help in identifying high-risk patients for thrombosis. EVs might also represent new targets for prophylactic treatment of haematological cancers by directly inhibiting their procoagulant phenotype or by interfering with their targeting of endothelial cells.

Conflict of interest and funding

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