ORIGINAL ARTICLE

Increased Levels of Circulating Microparticles Are Associated with Increased Procoagulant Activity in Patients with Cutaneous Malignant Melanoma

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Microparticles (MPs) are known to be increased in various malignancies and are involved in tumor invasion, angiogenesis, coagulation, and metastasis. We investigated the plasma levels of annexin-V MPs (AV$^+$MPs), platelet-derived MPs (PMPs), and endothelial-derived MPs (EMPs) in patients with melanoma ($n = 129$) and in healthy controls ($n = 49$). A functional coagulation test *STA Procoag-PPL* measuring the clotting time was performed on samples containing MPs to evaluate their procoagulant potential. The plasma levels of PMPs, EMPs, and AV$^+$MPs were significantly higher, and the *clotting time-PPL* was significantly lower in melanoma patients than in healthy controls. The plasma levels of PMPs, EMPs, and AV$^+$MPs were higher in stage IV than in the other stages of melanoma, but with no significant difference. In addition, we observed an inverse correlation between PMPs, AV$^+$MPs, and clotting times. Our data suggest that MPs are involved in the progression of melanoma and may be associated to melanoma-associated thrombogenesis.

INTRODUCTION

Microparticles (MPs) are membrane vesicles generated from different cellular compartments and released by any cell type into the vascular compartment during activation or apoptosis processes (Théry *et al.*, 2009). They are secreted by budding or shedding from the plasma membrane. Membrane markers of their originating cell are used to distinguish several circulating MPs, including platelet-derived MPs (PMPs), which are predominant in the bloodstream of healthy donors, or endothelial-derived MPs (EMPs) (Chironi *et al.*, 2009; Italiano *et al.*, 2010; Leroyer *et al.*, 2010). Their size varies from 0.1 to 1 μm diameter. They are secreted following a stimulus yielding an important calcium influx that modifies the activity of phospholipid transmembrane carriers (that is, flippase, floppase, and scramblase), activates calpains, and favors the burgeoning of the plasma membrane. These phenomena lead to the release of MPs carrying phosphatidylserine (PS) on their surface (Martinez *et al.*, 2004). MPs are involved in many tumoral and inflammatory diseases such as systemic lupus erythematosus and psoriasis (Pelletier *et al.*, 2011). In cancer, MPs contribute through their proteolytic properties to angiogenesis, metastatic progression, immune escape, and thrombogenicity (Martínez *et al.*, 2006; Lacroix *et al.*, 2007; Castellana *et al.*, 2010). In cancers, MPs are not only issued from tumor cells but they may also originate from the cells of the host, such as platelets and endothelial cells. MPs have been studied in a number of neoplasia, and there are few studies in melanoma. Most studies have been performed *in vitro* using murine melanoma cell lines. Lima *et al* (2009) have demonstrated that the release of murine melanoma MPs contributed to the formation of metastases. Melanoma MPs are also involved in the production of regulatory T cells and induce apoptosis of melanoma-specific CD8+ T lymphocytes through the expression of Fas ligand or TRAIL (Wieckowski *et al.*, 2009). Moreover, thrombotic events that are a common characteristic of all cancers may be promoted by the procoagulant activity of melanoma MPs (Lima *et al.*, 2011).

To our knowledge, no study has measured the levels of MPs in patients with melanoma. For these reasons, we wished to investigate the presence of MPs in melanoma and their procoagulant properties.

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Abbreviations: AV, annexin-V; EMP, endothelial-derived microparticle; HS, healthy subject; MP, microparticle; PMP, platelet-derived microparticle; PS, phosphatidylserine

RESULTS
Patients
One hundred and twenty-nine patients (74 men and 55 women) with cutaneous melanoma, including 32 stage I, 28

stage II, 42 stage III and 27 stage IV, were investigated. Forty-nine blood donor healthy subjects (HSs) (37 men and 12 women) were included.

PMP levels

For each stage (I, II, III, and IV), the plasma levels of PMPs $(CD31^+/CD41^+)$ ($2{,}135 \pm 1{,}224$, $1{,}908 \pm 1{,}644$, $1{,}860 \pm 1{,}236$, and $3{,}272 \pm 2{,}300\,\mu l^{-1}$, respectively) were significantly higher ($P < 0.0001$) in melanoma patients than in HSs ($533 \pm 570\,\mu l^{-1}$) (Table 1, Figure 1a). In addition, the plasma levels of PMPs in patients with the American Joint Committee on Cancer stage IV were higher than those measured in stage I, II, and III patients, but with no significant difference. No correlation was found between plasma PMP levels and tumor thickness ($r = 0.09$).

EMP levels

Plasma levels of EMPs $(CD31^+/CD41^-$ phenotype) were significantly ($P < 0.0001$) higher in patients with stage I, III, and IV (50 ± 207, 14 ± 11, and $14 \pm 8\,\mu l^{-1}$, respectively) than in HSs ($7 \pm 4\,\mu l^{-1}$) (Table 1, Figure 1b). EMP levels did not significantly differ between the different stages of melanoma. No correlation was found between plasma EMP levels and tumor thickness ($r = 0.04$).

Annexin-V MP levels

Plasma levels of annexin-V MPs (AV^+MPs) were significantly ($P < 0.0001$) increased in patients with stage II, III, and IV (278 ± 373, 465 ± 604, and $472 \pm 633\,\mu l^{-1}$, respectively) as compared with AV^+MP levels measured in HSs ($76 \pm 77\,\mu l^{-1}$) (Table 1, Figure 1c). Plasma levels of AV^+MPs observed in stage I patients ($328 \pm 855\,\mu l^{-1}$) were higher than those observed in HSs, but with no significant difference. Although plasma levels of AV^+MPs in stage IV and III patients were higher, we did not found any significant difference between the different stages of melanoma. No correlation was found between plasma AV^+MP levels and tumor thickness ($r = 0.10$).

Procoagulant activity of MPs

The procoagulant activity of MPs was measured using the functional coagulation test, *STA Procoag-PPL* (Table 1, Figure 1d).

The variation of STA PPL values was associated with the presence of MPs. As expected, samples S1–S5 demonstrated a procoagulant activity along with high levels of PMPs. In contrast, samples S1′–S5′ demonstrated normal clotting times as measured by STA PPL and low levels of PMPs. The clotting times were significantly longer ($P = 0.04$) in PMP-free plasma (Table 2).

The clotting times were significantly ($P < 0.0001$) lower in stages I, II, III, and IV melanoma patients (68 ± 17, 69 ± 13, 64 ± 10, and 61 ± 14 seconds, respectively) than in HSs (88 ± 8 seconds). However, there was no statistical difference between the different stages of melanoma. No correlation was found between PPL STA and tumor thickness ($r = -0.15$).

We found an inverse correlation between clotting times and PMPs ($r = -0.72$), and AV^+MPs levels ($r = -0.21$) in patients. However, there was no correlation between clotting times and EMP levels ($r = -0.07$).

## Table 1. Plasma levels of EMPs, PMPs, and AV^+ MPs in patients with melanoma and in healthy control subjects

	Healthy controls ($n = 49$)	Stage I ($n = 32$)	P-value stage I/healthy	Stage II ($n = 28$)	P-value stage II/healthy	Stage III ($n = 42$)	P-value stage III/healthy	Stage IV ($n = 27$)	P-value stage IV/healthy
Age[1] (years)	49 ± 10	59 ± 15	NS	69 ± 17	<0.0001	60 ± 12	<0.0001	62 ± 15	<0.0001
Sex (male/female)	37/12	18/14	ND	13/15	ND	26/16	ND	17/10	ND
PMPs[2] $(CD31^+/41^+)$									
Mean number/s.d.	533 ± 570	$2{,}135 \pm 1{,}224$	<0.0001	$1{,}908 \pm 1{,}644$	<0.0001	$1{,}860 \pm 1{,}236$	<0.0001	$3{,}272 \pm 2{,}300$	<0.0001
Range	(92–3,421)	(263–4,762)		(346–8,053)		(595–6,059)		(393–10830)	
AV^+MPs[2]									
Mean number/s.d.	76 ± 77	328 ± 855	NS	278 ± 373	<0.0001	465 ± 604	<0.0001	472 ± 633	<0.0001
Range	(6–298)	(26–4,883)		(47–1,397)		(18–2,855)		(36–2,505)	
EMPs[2] $(CD31^+/41^-)$									
Mean number/s.d.	7 ± 4	50 ± 207	<0.0001	10 ± 7	NS	14 ± 11	<0.0001	14 ± 8	<0.0001
Range	(1–17)	(4–1,182)		(2–33)		(1–47)		(3–38)	
STA PPL[3]									
Mean number/s.d.	88 ± 8	68 ± 17	<0.0001	69 ± 13	<0.0001	64 ± 10	<0.0001	61 ± 14	<0.0001
Range	(68–104)	(43–133)		(46–97)		(42–86)		(39–95)	

Abbreviations: AV, annexin-V; EMP, endothelial-derived microparticle; MP, microparticle; ND, not determined; NS, not significant; PMP, platelet-derived microparticles.
[1]Results are expressed as the mean number ± s.d. of year.
[2]Results are expressed as the mean number ± s.d. of MPs per plasma µl (range).
[3]Results are expressed as the mean number ± s.d. of time clotting per seconds.
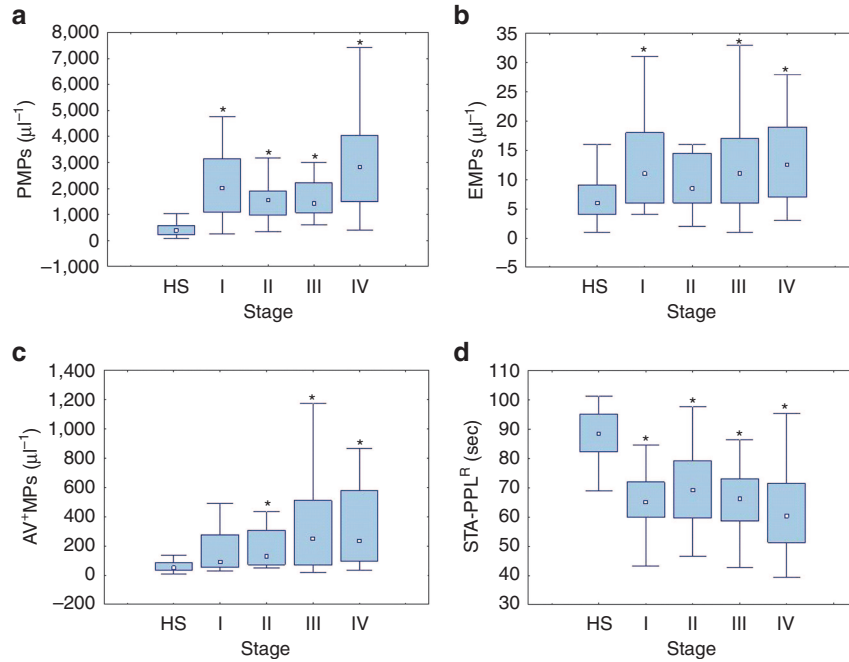
Figure 1. Microparticle (MP) levels and thrombogenic activity of MPs in HSs and in the different American Joint Committee on Cancer stages in melanoma patients. (**a**) Platelet-derived MP (PMP) levels (CD31$^+$/CD41$^+$), (**b**) endothelial-derived MP (EMP) levels (CD31$^+$/CD41$^-$), (**c**) annexin-V MP (AV$^+$MP) levels, and (**d**) thrombogenic activity of MPs were assessed using the functional coagulation test *STA Procoag-PPL*. *Indicates significant difference between the corresponding stage of melanoma and healthy subject (HS).

Table 2. The variation of STA PPL values (expressed in s) was associated with the presence of MPs (μ^{-1})

	PMPs (μl^{-1})	STA PPL (s)
S1	6,174	57.4
S1′	697	118.4
S2	3,320	65.8
S2′	405	117.7
S3	4,482	55.4
S3′	525	104.1
S4	4,095	63.1
S4′	604	119.9
S5	8,302	56
S5′	549	120.4

Abbreviations: MP, microparticle; PMP, platelet-derived microparticle. After an ultracentrifugation (20,000 *g*) during 90 min, clotting times were normal, and PMPs were low in MP-free plasma (S1′–S5′) compared with initial plasmas (S1–S5) ($P = 0.04$ and 0.01, respectively).

DISCUSSION

Our study demonstrate that circulating PMPs are significantly increased in melanoma at any stage compared with controls. It is to our knowledge previously unreported. EMPs and AV$^+$MPs were also significantly increased in melanoma patients at stages I, III, and IV and II, III, and IV, respectively. However, although MP levels were much higher in stage IV melanoma, we did not find any significant difference between the different stages. In addition, we demonstrated that the

procoagulant activity of MPs was significantly increased at each stage of melanoma and was correlated to the levels of PMPs and AV$^+$MPs.

Our data confirm the predominance of circulating plasma PMPs in melanoma patients. The increase of PMPs has already been found in other forms of cancer, such as gastric cancer (Kim *et al.*, 2003) and prostatic cancer (Helley *et al.*, 2009). Kim *et al* have shown that PMP rates were better correlated with the severity of gastric cancer than blood dosages of vascular endothelial growth factor, interleukin-6, and RANTES. Kim also observed that PMP levels were correlated with the presence of metastasis in these patients. Furthermore, Helley *et al.* (2009) demonstrated that the overall survival of patients with hormone-resistant prostate cancer varied inversely according to the rate of PMPs.

The exact mechanisms inducing platelet activation and then formation of PMPs in cancer are not well understood, but it seems that there is a direct activation by the tumoral cells releasing procoagulant molecules such as the tissue factor, as well as an indirect activation by immune cells, which in response to the tumoral cells release tumor necrosis factor-alpha or interleukin-1β (Chironi *et al.*, 2009). Circulating MPs may have different roles in carcinogenesis. First, PMPs are involved in angiogenesis through the secretion of prostacyclin by monocytes and endothelial cells (Barry *et al.*, 1999) and the release of growth factors (vascular endothelial growth factor and fibroblast growth factor 2) and lipids, mainly sphingosin 1-phosphate (Varon and Shai 2009). It has been demonstrated that the angiogenic potential of PMPs is as important as the angiogenic potential of platelets (Brill *et al.*, 2005). PMPs also

have a part in tumoral invasion, through plasminogen activation and the production of metallopreoteases (Varon and Shai 2009). Furthermore, they are also involved in thrombogenicity by the presence of anionic phospholipids, particularly PS and the procoagulant tissue factor (Varon and Shai 2009, Suades *et al.*, 2012).

High levels of EMPs have been already reported in cardiovascular diseases (acute coronary syndromes, stroke, metabolic syndrome, and hypertension), autoimmune diseases (idiopathic thrombocytopenic purpura, antiphospholipid syndrome, systemic lupus erythematosus, vasculitis, and psoriasis) (Angelot *et al.*, 2009; Pelletier *et al.*, 2011), but not in cancers (Toth *et al.*, 2008; Rank *et al.*, 2012a). However, EMPs can be involved in tumoral angiogenesis. Several studies have indeed shown the involvement of EMPs at different stages of angiogenesis (Kim *et al.*, 2004), including matricial degradation, recruitment and differentiation of endothelial progenitors, and proliferation and migration of endothelial cells. Besides, EMPs are involved in cellular survival through the induction of cytoprotector and anti-inflammatory responses (Zernecke *et al*, 2009). They also demonstrate procoagulant properties through the expression of tissue factor and phospholipids (Leroyer *et al.*, 2010). EMPs can also generate thrombin *in vivo* and *in vitro* and transfer their procoagulant activity to other cellular types, amplifying the procoagulant response (Sabatier *et al.*, 2011).

Hypercoagulability is well documented in all types of cancers, and is the second leading cause of death in cancer patients (Prandoni *et al.*, 2005). It has been shown that MPs, especially tumor-derived MPs, activate coagulation through the tissue factor among other factors (Davila *et al.*, 2008). Our data demonstrated a statistically significant difference between melanoma patients, irrespective of the American Joint Committee on Cancer stage, and HSs regarding the functional dosage of procoagulant activity of MPs. We also observed an inverse correlation between PMP and AV^+MP levels and the clotting time measured by the functional coagulation test *STA Procoag-PPL*. These data reflect the functional procoagulant activity of circulating MPs that is linked to the phospholipids (PS and phosphatidylethanolamin) present at the MP surface. Tas *et al.* (2012) recently showed that there was a significant difference of several coagulation tests (prothrombin time, activated partial thromboplastin time, prothrombin activity, D-dimer, and fibrinogen levels) between melanoma patients and healthy subjects (HSs) and that there was a significant difference between fibrinogen level and survival. Suades *et al.* (2012) recently demonstrated that an increased level of PMPs induced platelet deposition and thrombus formation, even in HSs. Dvorak *et al.* (1983) showed that phospholipids of MPs issued from different tumor cell lines induced the activation of both tissue factor and prothrombinase complex. Several previous studies have demonstrated high levels of procoagulant MPs in patients with neoplasia, which were correlated with the risk of thrombosis (Rak, 2010). Thaler *et al.* (2012) found a correlation between the rates of MPs carrying tissue factor and mortality in pancreatic cancer. The presence of circulating MPs positive for tissue factor in cancer patient has been correlated with the

progression of cancer (Leroyer *et al.*, 2010). The origin of MPs is still discussed. Lima *et al.* (2011) have shown that melanoma tumor-derived MPs have a procoagulant activity linked to the expression of the tissue factor on their membrane. In our study, we only studied the phospholipidic component of coagulation but not the tissue factor. The *STA Procoag-PPL* test is a specific test that measures the specific procoagulant activity of phospholipids but not the activity of the tissue factor. PS expression provides a common signature for the presence of AV^+MPs. AV is considered as an useful marker to determine general levels of MPs, although it is not present on all MPs. However, AV^+ is neither specific nor sensitive for MPs. AV may bind with other negatively charged phospholipids from cell fragments other than MPs, or PS may bind to other marker than AV, for example, lactadherin. In addition, we were not able to identify melanoma-derived MPs, despite the use of different antibodies directed against melanoma tumor antigens (Melan-A, MART-1, and MCSP). It was therefore impossible to conclude on the possible involvement of melanoma MPs in the procoagulant activity.

In our study, we cannot exclude an effect of the age of the melanoma patients because they were significantly older than HSs. However, it has been demonstrated that both PMPs and EMPs did not increase with age in pre- and postmenopausal women (Rank *et al.*, 2012b). Furthermore, we only investigated PMPs, EMP, and AV^+MPs, and we cannot exclude the presence of MPs from other cell origin, that is, inflammatory, immune, or tumor cells.

In conclusion, our data demonstrate higher levels of circulating PMPs, EMPs, and AV^+MPs in melanoma patients as compared with healthy controls in association with a significant procoagulant activity. Further studies are required to assess both the prognosis and the thrombogenic value of MP levels in these patients and to determine whether these MPs can also originate from other cell types.

MATERIALS AND METHODS
Study population
The study design was approved by the local research ethics committee, and written informed consent was provided before enrolment. The study adhered to the Declaration of Helsinki principles. This prospective study was conducted between April and September 2012 in the Department of Dermatology, Besançon University Hospital, France. Patients with melanoma were included at different clinical stages graded according to the latest American Joint Committee on Cancer staging classification (Balch *et al.*, 2009). Patients with cardiovascular diseases, diabetes, chronic renal failure, or chronic inflammatory disease were excluded. Forty-nine HSs were enrolled in a control group (blood donors, Etablissement Français du Sang, Bourgogne Franche-Comté, Besançon, France). HS had no known pathology according to the criteria imposed for blood donation.

MP isolation from plasma
We adapted the MPs quantification method developed by the International Society on Thrombosis and Haemostasis (Robert *et al.*, 2009) in 2010. Peripheral blood was drawn from healthy controls and patients into two commercially available 5-ml citrate–theophylline–
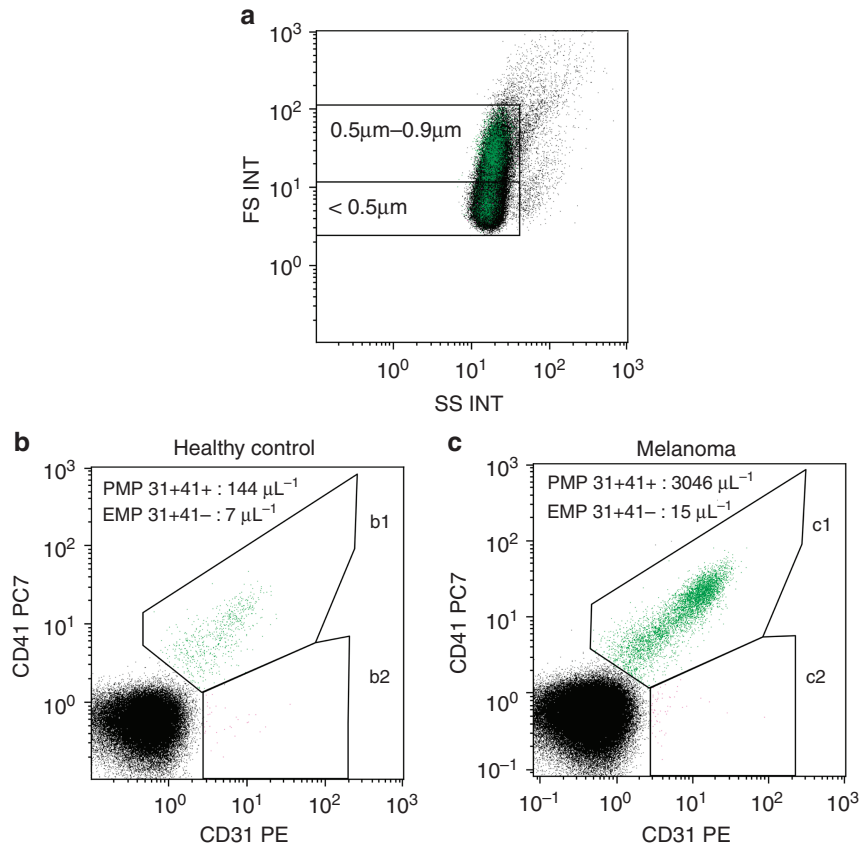
Figure 2. Analysis of circulating microparticles (MPs) using a NAVIOSTM cytometer. (**a**) Analysis of a sample from a healthy subject. Standardized MP-detected areas (0.5–0.9 and <0.5 μm) are represented in side scatter (SS)/forward scatter (FS) dot plot. (**b**) Platelet-derived MPs (PMPs) (CD31$^+$/CD41$^+$) and endothelial-derived MPs (EMPs) (CD31$^+$/CD41$^-$) from a HS are quantified in b1 and b2 areas, respectively. (**c**) PMPs (CD31$^+$/CD41$^+$) and EMPs (CD31$^+$/CD41$^-$) from a melanoma patient in stage IV are quantified in c1 and c2 areas, respectively.

adenosine–dipyridamole Vacutainer tubes (Becton Dickinson, Le Pont de Claix, France). They correspond to the second and third tubes of collected blood.

A first centrifugation of peripheral blood was performed at 2,500 g during 15 minutes, and 1 ml of platelet poor plasma was collected. A second centrifugation of this platelet poor plasma was then performed at 2,500 g during 15 minutes, and 500 μl of cell-free plasma was collected and frozen at $-80\,^{\circ}C$ (Pelletier *et al.*, 2011; Lacroix *et al.*, 2012).

MP quantification by flow cytometry

Flow cytometry was performed with a NAVIOS Cytometer (Beckman Coulter Immunotech, Villepinte, France) (Figure 2). MPs were identified with the following antibodies: phycoerythrin-conjugated CD31 (clone 1F11), phycoerythrin–cyanin7-conjugated CD41 (clone P2), and fluorescein isothiocyanate-conjugated AV (Beckman Coulter Immunotech). To 30 μl of plasma were added 5 μl of AV, 5 μl of CD31, and 3 μl of CD41. After a 20-minute incubation and addition of 150 μl of AV-binding buffer and 30 μl of beads of counting (Cytocount; Dako, Trappes, France), samples were performed in duplicate and analyzed using a NAVIOS cytometer (Beckman Coulter Immunotech, Miami, FL). MP number was calculated on the basis of the known number of Cytocount beads added to the sample. PMPs were identified as CD31$^+$/CD41$^+$/AV$^{+/-}$ events, and EMPs were identified as CD31$^{+/}$CD41$^-$/AV$^{+/-}$. Two MP-size regions (<0.5 μm and between 0.5 and 0.9 μm) were determined and

standardized daily with Megamix fluorescent beads (BioCytex, Marseille, France) containing two types of beads with a defined size (0.5 and 0.9 μm diameter, respectively).

Validation of circulating MP quantification by flow cytometry
The Bland and Altman graphs compared the AV$^+$MPs, MPs, and EMPs between tubes 1 and 2. These statistical graphs confirmed the good agreement and the reproducibility of our method of quantification and the beads of counting standardization technique used (data not shown).

Thrombogenic activity of MPs
Thrombogenic activity of MPs was evaluated by the functional coagulation test *STA Procoag-PPL* (Diagnostica Stago, Asnières sur Seine, France). It is a reproducible automated and marketed test that measures the coagulation time associated with phospholipids. The clotting time is dependent on the amounts of anionic phospholipid. A first reagent (freeze-dried citrated human plasma PPL-depleted) containing coagulation factors is added to 25 μl plasma including MPs and then centrifuged twice at 2,500 g for 15 minutes. It makes the test dependent on procoagulant phospholipids present in the tested sample. After incubation, a second reagent (factor X activated and Ca^{2+}) is added; it allows to trigger coagulation at the level of Xa factor, eliminating the interaction with the previous factors. The coagulation time was then measured. The shorter the clotting time,

the more important was the procoagulant activity of phospholipids (normal values: 72 ± 5.6 seconds).

To confirm that the variation of STA PPL values was associated with the presence of MPs, we carried out the following experiment. The plasmas of five patients with melanoma were pooled in a tube. This process was performed five times to obtain five tubes. The content of each tube was then separated in two identical volumes and distributed in two identical tubes for the five samples (S1–S5 and S1′–S5′). In tubes (S1–S5), PMPs were counted by flow cytometry, and a dosage of STA PPL was made. For the other tubes (S1′–S5′), an ultracentrifugation was carried out at 20,000 g during 90 minutes to obtain MP-free plasma. Then, an STA PPL and a PMP dosage by flow cytometry were performed for each plasma obtained.

Statistical analysis

The descriptive analysis of qualitative and ordinal variables includes the number and frequency of each modality. The quantitative variables include the mean, s.d., median, and extreme values. Comparisons between different stages of melanoma and between patients and healthy volunteers' usual tests involved the nonparametric Wilcoxon or Kruskal–Wallis test for semiquantitative or quantitative non-Gaussian variables. $P < 0.005$ was considered statistically significant after applying the Bonferroni correction because of the number of tests performed. Coefficients of correlation (spearman) were calculated between clotting time and MP levels in patients, and between tumor thickness and clotting time or MP levels. Statistics were performed by the Clinical Investigation Centre of the University Hospital of Besançon using SAS software for Windows 9.3 (Stastistica 6.1, Statsoft, Maisons-Alfort, France).

CONFLICT OF INTEREST

The authors state no conflict of interest.

Author contributions

CL, FP, FA, FG-O, and ES designed the research study. CL, FP, and TL performed experiments, analyzed data, prepared the figures and tables, and drafted the manuscript under the supervision of FG-O, SB, GM, and ES. CL, FP, and FA wrote the paper. CL, FP, FG-O, ES, and PH reviewed the paper.

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