Differential influence of breast cancer stage, time from diagnosis and chemotherapy on plasma and cellular biomarkers of hypercoagulability

Mourad Chaari¹²³, Ines Ayadi⁴, Aurelie Rousseau², Patrick Vandreden⁵, Elmina Lefkou⁷, Fatoumata Sidibe², Hela Ketatni¹², Vassiliki Galea¹², Amir Khatachi¹², Racem Bouzguenda⁴, Mounir Frikha⁴, Lilia Ghorbal⁶, Jamel Daoud⁶, Choumous Kallel⁵, Martin Quinn⁵, Joseph Gligorov⁷, Jean Pierre Lotz⁷, Mohamed Hatmi⁸, Ismail Elalamy¹², Grigoris T Gerotziafas¹²

¹Service d’Hématologie Biologique Hôpital Tenon, Hôpitaux Universitaires Est Parisien, Assistance Publique Hôpitaux de Paris, France.
²ER2UPMC, Faculté de Médecine Pierre et Marie Curie, Université Paris VI, France.
³Laboratoire d'hématologie, Hôpital universitaire Habib Bourguiba, Sfax, Tunisia.
⁴Service de Carcinologie, Hôpital universitaire Habib Bourguiba, Sfax, Tunisia.
⁵Research and Development, Diagnostica Stago, Gennevilliers, France.
⁶Service de radiothérapie carcinologique, Hôpital universitaire Habib Bourguiba, Sfax.
⁷Service d’Oncologie Médicale et de Thérapie Cellulaire, Hôpitaux Universitaires de l’Est-Parisien, Institut Universitaire de Cancérologie, Université Pierre et Marie Curie APREC.
⁸Département Infection et Epidémiologie, Institut Pasteur, Paris, France.

Correspondence to

Grigoris T Gerotziafas, MD, PhD, Service d'Hématologie Biologique, Hôpital Tenon, 4, rue de la Chine, ER2UPMC 75020, Paris, Cedex 20, France.
tel: +33156016197; +33156018063  fax: +33156016044
e-mail : grigorios.gerotziafas@tnn.aphp.fr
Abstract

Introduction. In breast cancer patients VTE risk increases during chemotherapy and in advanced stage. Routine pharmacological thromboprophylaxis is not recommended but individualized risk assessment is encouraged. The incorporation of hypercoagulability biomarkers could increase the sensitivity of the risk assessment models (RAM) to identify patients at VTE risk. To this aim we investigated the impact of cancer-related characteristics on the biomarkers of hypercoagulability.

Methods. Thrombin generation (TG) assessed with the Thrombogramme-Thrombinoscope®, the levels of platelet derived microparticles (Pd-MP) assessed with flow cytometry, the procoagulant phospholid dependent clotting time (PPL-ct) measured with prothrombinase activity based clotting assay from Diagnostica Stago and D-Dimers (measured with the mini-Vidas assay from Biomerieux) were assessed in a cohort of 62 women with breast cancer and in 30 age matched healthy women. These biomarkers were analyzed in relation to the stage, the time elapsed since the diagnosis and the chemotherapy.

Results. Patients showed significantly higher TG, Pd-MP, D-Dimers levels and shortened PPL-ct compared to the control group. The PPL-ct was inversely correlated with the levels of Pd-MP, which were increased in 97% of patients. TG and D-Dimers were increased in 34% and 59% of patients respectively. TG increase was not associated with the stage of the disease. In patients on chemotherapy with <6 months since diagnosis TG was significantly higher as compared to those on chemotherapy who diagnosed in interval > 6 months (1627 ± 335 nM.min versus 1402 ± 327 nM.min, respectively; p<0.05). Patients with metastatic disease had significantly higher levels of Pd-MP and D-Dimers compared to those with non-metastatic disease. The presence of cardiovascular risk factors did not influence TG and Pd-MP levels but was associated with increase of D-Dimers.

Conclusion. The present study defined the most appropriate biomarkers for the diagnosis of blood borne hyperocagulability related to breast cancer. The levels and the procoagulant activity of Pd-MP are interconnected with the biological activity and the overall burden of cancer. TG reflects the procoagulant effect of chemotherapy in the initial period of cancer diagnosis. Thus the weighted incorporation of the biomarkers of cellular and plasma hypercoagulability in RAM for VTE might improve their predictive value.

Key-words: Breast cancer, venous thromboembolism, thrombin generation, microparticles, D-Dimers, risk assessment model
Introduction

The close association of cancer with hypercoagulability and the risk of thrombosis have been recognized since the 19th century [1-3]. The risk of venous thromboembolism (VTE) is about 7-fold higher in cancer patients compared to controls [4,5]. VTE significantly affects morbidity and is the second cause of mortality in hospitalized cancer patients [6-9]. Many aspects of the interplay between cancer and blood coagulation have been elucidated by experimental, clinical and epidemiological studies [10,11]. The histological type, the burden of cancer cells, the stage of the disease, the use of chemotherapy and the time since diagnosis are determinants of the VTE risk [12].

Breast cancer is the commonest malignancy in women and is considered to be associated with low VTE risk as compared to other malignancies. In women with newly diagnosed breast cancer the cumulative incidence of VTE is less than 1% [10, 12]. However VTE risk increases by 4- to 6-fold during chemotherapy as well as in advanced stage or metastatic disease [14]. Routine administration of thromboprophylaxis is not recommended in women with breast cancer undergoing adjuvant chemotherapy since there are no relevant clinical trials assessing the efficacy and safety of antithrombotic agents in this context [13]. However, expert consensus statements encourage an individualized approach for the identification of patients at risk of VTE who are eligible for pharmacological thromboprophylaxis [15]. To this aim, Korhana et al have developed and prospectively validated a risk assessment model that stratifies cancer patients to high, moderate or low risk for VTE prior to chemotherapy initiation [16].

Thrombosis is a multifactorial disease occurring when the Virchow’s triade (blood hypercoagulability, vessel wall lesion and alteration of blood flow) is fulfilled. However, current risk assessment models for VTE in cancer patients are restricted to some clinical risk factors and are missing the evaluation of blood borne hypercoagulability factors, although this is one of the basic components of Virchow’s triad. The expression of tissue factor (TF) by cancer cells as well as the formation of procoagulant microparticles derived from activated platelets, are pivotal events leading to enhanced thrombin generation in patients with cancer [reviewed in 17-20]. TF-induced activation of blood coagulation in cancer patients leads to sustained thrombin generation and fibrin formation [21]. The D-Dimers are
degradation products of cross-linked fibrin, indicating either enhanced fibrin formation or activation of the fibrinolytic system, or increased levels of fibrinogen and likely reflect the biological activity of cancer cells [22]. Increased concentration of D-Dimers in plasma has been observed in patients with breast, prostate or bowel cancer [23].

It has been reported that incorporation of biomarkers of cellular or plasma hypercoagulability increases the sensitivity of the risk assessment models to identify cancer patients at risk for VTE [24]. The aim of the present study was to investigate the potential relation between cancer-related characteristics and the biomarkers of plasma and cellular hypercoagulability. The capacity of thrombin generation in patients’ plasma, the concentration of procoagulant platelet-derived microparticles expressing phosphatidylinerin (Pd-MP/PS+) in plasma, the procoagulant phospholipid (PPL) dependent clotting time and D-Dimers were assessed in a cohort of women suffering from breast cancer. These biomarkers of plasma and cellular hypercoagulability were analyzed in relation to the stage of the disease, the time elapsed since diagnosis and the administration of chemotherapy.

**Methods**

*Cancer patients*

Out-patients with histologically proven breast cancer were enrolled in the study from January to June 2012. Patients were considered under chemotherapy if they had received a chemotherapy cycle 21 days earlier. The exclusion criteria were: age less than 18 years, recent (<6 months) documented episode of VTE (deep venous thrombosis and/or pulmonary embolism) or acute coronary syndrome, confirmed pregnancy, major psychiatric disorders, life expectancy less than 3 months, active anticoagulant treatment, recent (<3 months) hospitalization for acute medical illness or major surgery, recent surgery (<2 months).

*Classification of the patients.* Patients were classified according to the tumor, node, metastases (TNM) system of stratification: Local stage was defined by the absence of axillary nodes and distant metastasis (TxN0M0). Regional stage was defined by the presence of axillary node(s) and the absence of distant metastasis (TxN+M0). The metastasis stage was defined by the presence of one or more distant metastases (TxNxM+) [25]. Patients were also stratified according to the presence or not of at least one cardiovascular risk factor.
**Control group**

The control group consisted of 30 age-matched healthy women who did not have breast cancer and who were not taking any medication for at least one month before blood sampling. Healthy volunteers had normal prothrombin time (PT) and activated partial thromboplastin time (aPTT) and had no personal history of thrombotic or hemorrhagic episodes. The values obtained in this population, comparable in age to the breast cancer patients, were used to establish reference intervals for the assays. All patients and healthy individuals gave written informed consent for participation in the study.

**Blood samples**

Blood samples were obtained by traumatic puncture of the antecubital vein, using a 20-gauge needle, and placed into siliconized vacutainer tubes containing 0.129 mol/L trisodium citrate (from Becton and Dickinson France) as anticoagulant, in a ratio of nine parts of blood to one part of citrate. Platelet poor plasma (PPP) was obtained after double centrifugation of citrated whole blood for 20 minutes at 2000g. Platelet-free plasma was prepared immediately after blood sampling using a 2-step centrifugation procedure: initially at 1500g for 15 minutes at 20°C to prepare platelet rich plasma and then at 13000g for 2 minutes at 20°C to prepare PFP. Samples were aliquoted and frozen at -80°C until assayed. All measurements were done in thawed plasma samples. All PPP samples were from vein punctures performed for routine evaluation of blood coagulation tests. Blood anticoagulated with EDTA was used for the determination of complete blood count. The study was performed in accordance with the principles embodied in the Declaration of Helsinki after approval of the institutional ethics committee.

**Thrombin generation in plasma**

Thrombin generation in PPP was assessed using the Calibrated Automated Thrombogram assay (CAT®, Diagnostica Stago, France) as described by Hemker et al [26]. Briefly 80 µl of PPP was added to 20 µl of PPP-reagent 5 pM (Thrombinscope b.v., Maastricht, Netherlands), that is a mixture of TF (5 pM final concentration in plasma) and phospholipids (4 µM final concentration in plasma). Each patient’s plasma was studied in duplicate. In a third well, PPP
reagent 5 pM was replaced with the same volume of Thrombin Calibrator\textsuperscript{*} (Thrombinscope bv, Maastricht, Netherlands) to correct thrombin generation curves for substrate consumption and the inner filter fluorescence effects. Thrombin generation was triggered with a 20 µl solution containing CaCl\textsubscript{2} (16.7 mM final concentration) and the fluorogenic substrate Z-Gly-Gly-Arg-AMC (417 pM final concentration). Fluorescence was measured using a Fluoroscan Ascent\textsuperscript{*} fluorometer (ThermoLabsystems, Helsinki, Finland). Acquisition of thrombin generation parameters was performed using the appropriate software (Calibrated Automated Thrombogram\textsuperscript{*} bv, Maastricht, Netherlands). Among thrombogram parameters we analyzed the endogenous thrombin potential (ETP) that reflects the integral thrombin activity, the Peak concentration of thrombin and the mean rate index (MRI), which reflects the rate of the propagation phase of thrombin generation [calculated by the formula MRI=Peak/(ttPeak – lag-time)].

**Microparticle labelling and flow cytometry analysis**

Platelet-derived microparticles were measured in platelet free plasma using a flow cytometry assay as described by Robert et al [27]. Briefly, for Pd-MP/PS\textsuperscript{+} labelling, 30 µL of fresh PFP was incubated with 10 µL of a solution of phycoerythrin (PE) bound monoclonal antibody against platelet glycoprotein IIb (CD41). For the detection of phosphatidylserine expression by Pd-MP the plasma samples were additionally spiked with 10 µL of fluorescein isothiocyanate (FITC) labelled recombinant human annexin V. Anti-CD41 monoclonal antibody was purchased from BioCytex (Marseille, France). Human annexin V-FITC kit was obtained from AbCys (Paris, France). Concentration-matched isotype antibodies (IgG\textsubscript{1}–PE, 15 ng/µL, clone 2DNP-2H11, from BioCytex) or Annexin V-FITC with phosphate-buffered saline without calcium were used as controls. Analyses were performed on Cytomics FC500 flow cytometer (Beckman-Coulter, Villepinte, France). To limit background noise from dust and crystals, the instrument was operated using a 0.22 µm filtered sheath fluid (Isoflow\textsuperscript{TM}; Beckman-Coulter, France). The software packages CXP ACQUISITION\textsuperscript{*} and CXP ANALYSIS\textsuperscript{*} (Beckman-Coulter, France) were used for data acquisition and analysis, respectively. Standardization of the Pd-MP protocol was done using a blend of mono-disperse fluorescent beads (Megamix, BioCytex Marseille, France) of three diameters (0.5, 0.9 and 3 µm). Forward scatter and side scatter parameters were plotted on logarithmic scales to best cover a wide size range. Pd-MP were defined as single positive CD41\textsuperscript{+} events. CD41 positivity was
displayed on single parameter histograms. Pd-MP/PS$^+$ were defined as dual-positive phosphatidylserine PS$^+$/CD41 events as displayed on dual-color fluorescence plots after staining with annexin V-FITC and CD41-PE. In each studied sample 30 μl of counting beads with an established concentration close to 1000 beads/μl (Flow Count™Fluorosphores Beckman-Coulter) were added in order to express counts as absolute numbers of microparticles per microliter of PFP. All plasma samples were assessed for Pd-MP within one week after blood collection and after one cycle of freezing/thawing. Application of the same experimental conditions reduced the impact of the eventual error introduced by the freezing/thawing on Pd-MP concentration.

**Assessment of procoagulant phospholipid dependent activity in plasma**

Procoagulant phospholipid-dependent clotting time (PPL) was measured in thawed PPP using the factor Xa - based coagulation assay (PPL clotting time) STA®Procoag-PPL, (DiagnosticaStago, Asnières, France) in which shortened clotting times are associated with increased levels of procoagulant phospholipids. The PPL clotting time was performed according to the manufacturer's instructions on a STA®-R analyser.

**D-Dimers**

The concentration of D-Dimers in platelet poor plasma was determined using the enzyme linked fluorescent assay on a mini VIDAS system (bio-Merieux, Paris, France). The assay employs a quantitative sandwich enzyme immunoassay technique combining a bound anti-D-Dimer monoclonal immunoglobulin with an unbound enzyme labeled anti-D-dimer monoclonal immunoglobulin. Results are reported in ng/mL of fibrinogen equivalent units. According to manufacturer’s instructions, D-Dimers concentrations equal or lower than 500 ng/ml were considered as normal.

**Routine biochemical and hematological assessment**

Blood samples were also obtained for the assessment of transaminase levels (ASAT and ALAT), CRP, urea and creatinine. Routine hemogram parameters as well as prothrombin time (expressed as percentage of prothrombin) and activated partial thromboplastin time (expressed as ratio of patients/control values), were also analyzed. Routine hematological
and biochemical measurements were performed with standardized assays existing in the central hematological and biochemical hospital laboratory.

**Statistical analysis**
The parametrically distributed data were expressed as mean ± standard deviation (sd). The results of D-dimer levels were expressed as the median [(Q1-Q4), interquartile range]. Comparisons between normally distributed continuous variables were performed using Student’s t-test. The correlation between the studied parameters was examined by the Spearman correlation coefficient. When appropriate, the upper and lower normal limits (UNL and LNL respectively) for each parameter of the studied biomarkers of hypercoagulability were defined as mean ± 2 sd of the values obtained by assessing thrombogram and Pd-MP in the group of healthy volunteers. Thrombin generation was considered as increased when at least one of the studied parameters (ETP, Peak or MRI) showed a value higher than the UNL. Two-sided p-value <0.05 was considered significant. Statistical analysis was performed using SPSS 20.0 (SPSS Inc., Chicago, IL).
Results

**Patients characteristics**

A total of 62 women with breast cancer were included in the study. There was no statistical difference in the mean age of the cancer patients (52 ± 11) compared to the control group (55 ± 10 years), p>0.05. Basic hematological parameters in cancer patients were within the normal range and not significantly different compared to the control group. The body mass index was also not significantly different between the two groups.

Patients were stratified according to the stage of the disease as follows: 13 had a local stage, 29 had a regional stage and 20 had metastatic disease. Age, BMI and basic hematological parameters were not significantly different among these subgroups, as well as between each subgroup and the control group. Thirty six patients were having chemotherapy, 5 patients were having chemotherapy and hormone therapy, 11 patients were on hormone treatment and 3 were having radiotherapy. The 7 remaining patients were not receiving any anticancer treatment but were treated with zoledronic acid. In the subgroup with metastatic stage disease, 95% had bone metastasis and 40% also had liver or lung metastases.

Patients were also stratified according to the time elapsed since the diagnosis of cancer: <6 months (n=27), 6-12 months (n=10), 12-36 months (n=13), >36 months (n=12). Invasive ductal carcinoma of the breast was diagnosed in 90% of patients. Curative surgery was performed in 82% of the patients included in the study. All surgical procedures were completed at least 2 months before enrolment. In 42 out of 62 patients (67%) at least one cardiovascular risk factor was present. Demographic and clinical characteristics of the studied groups are summarized in Table 1.

**Thrombin generation in breast cancer patients**

Thrombin generation was significantly increased in breast cancer patients as compared to the control group (Table 2). The MRI was significantly higher in the group of patients as compared to the control group (159 ± 47 nM/min versus 109 ± 33 nM/min respectively; p<0.001). The thrombin peak was also higher in cancer patients as compared to the control group (341 ± 65 nM versus 288 ± 48 nM, respectively; p=0.001). The ETP was not
significantly different between the cancer group and the control group (1531 ± 337 nM.min versus 1498 ±225 nM.min).

The distribution of the individual values of thrombogram parameters in cancer patients and controls is shown in Figure 1. Representative thrombograms of patients with increased and normal thrombin generation profile are depicted in Figure 2. The MRI was higher than the UNL in 21 patients (34%). The Peak was higher than the UNL in 18 patients (29%). The ETP was higher than the UNL in 8 patients (13%). Sixteen patients (26%) had two thrombogram parameters (MRI and Peak) higher than the UNL. One patient had thrombin generation inferior than the LNL.

**Influence of stage, time and chemotherapy on thrombin generation**

The increase in thrombin generation was not associated with the stage of the disease (Table 2). The analysis of patients on active chemotherapy (n=41) also showed that the stage of the disease did not significantly alter thrombin generation (Table 3). In addition, thrombin generation was not influenced by the time elapsed since the diagnosis of the breast cancer (Table 4). Thrombin generation was not significantly different between the subgroups of patients who were diagnosed with cancer within 6 months before inclusion as compared to those who were diagnosed with cancer in an interval longer than 6 months.

Analysis of the group of patients on active chemotherapy (n=41) showed that the ETP was significantly higher in patients < 6 months since diagnosis compared to those >6 months (1627 ± 335 nM.min versus 1402 ± 327 nM.min, respectively; p<0.05) (Table 4, Figure 3).

Analysis of thrombogram parameters showed that thrombin generation was not significantly affected by the time since the diagnosis, at each stage of the disease (Table 5).

**Procoagulant platelet-derived microparticles in breast cancer patients**

In the control group the concentration of Pd-MP and Pd-MP/PS⁺ was 756 ± 429/μl and 695 ± 361/μL respectively. In the breast cancer group Pd-MP and Pd-MP/PS⁺ were both significantly increased (p<0.001) compared to the control group (Table 2). The concentration of Pd-MP and Pd-MP/PS⁺ was higher than the UNL in 97% and 93% of patients respectively. Accordingly, the PPL clotting time was significantly shorter in patients as compared to the control group (43.5 ± 10 sec versus 72.8 ± 9.9; p=0.03). The PPL clotting time was
significantly correlated with both Pd-MP and Pd-MP/PS⁺ ($r^2=0.7; \ p<0.0001$). In 51 patients (82%) the PPL clotting time was shorter than the LNL of the assay.

Influence of stage, time and chemotherapy on platelet-derived microparticles

There was no significant difference in Pd-MP or Pd-MP/PS⁺ between the subgroups of patients with local or regional stage. Patients with metastatic disease had significantly higher levels of Pd-MP and Pd-MP/PS⁺ compared to those with non-metastatic disease (Table 2). The concentration of Pd-MP and Pd-MP/PS⁺ was not influenced by the time since the diagnosis of the breast cancer (Table 4). The stratification of each subgroup according to the administration of chemotherapy did not show any significant difference between the subgroups (Table 5). Similarly, the concentration of Pd-MP and Pd-MP/PS⁺ was not significantly different between the subgroups of patients with regional stage of the disease to whom cancer was diagnosed within less or more than 6 months before the inclusion in the study. The PPL clotting time, similarly to Pd-MP, was not influenced by chemotherapy and time since diagnosis but it was shorter in patients with metastatic disease.

D-Dimer levels in breast cancer patients

The concentration of D-Dimers was significantly increased in cancer patients (1250 ± 1773 ng/ml, range: 220 – 8520 ng/ml) compared to the control group (230 ± 50 ng/ml; $p<0.05$ (Table 2). The concentration of D-Dimers tended to increase in advanced stages of the disease. However no significant difference was observed between the subgroups of patients with local and regional stage (605 ± 499 ng/ml versus 1123 ± 1429 ng/ml; $p>0.05$). The concentration of D-Dimers in patients with metastatic stage (1853 ± 2497 ng/ml) was significantly higher as compared to that in patients with local stage ($p=0.04$). The concentration of D-Dimers in patients with regional stage was not significantly different as compared to patients with metastatic stage (Figure 4). The analysis of the data from the subgroup of the patients on chemotherapy showed a similar trend of elevation of D-Dimers in parallel with the stage of the cancer.

In patients with localized disease receiving chemotherapy, the median concentration of D-Dimers was significantly lower (410 ng/mL, range 220-1230 ng/mL) compared to patients having chemotherapy for metastatic disease (1920 ng/mL, range, 242-6547 ng/mL,
p=0.088). The time since diagnosis of cancer did not show any significant influence on D-Dimer levels in the subgroup of patients having chemotherapy.

In 37 patients (59%) the concentration of D-Dimers in plasma was higher than the upper normal cut-off levels of 500 ng/ml. Both increased thrombin generation and D-Dimer level were observed in 11 patients (18%).

**Cardiovascular risk factors and markers of cellular and plasma hypercoagulability in breast cancer patients**

Thrombin generation, PPL clotting time and the concentration of Pd-MP, PdMP/PS\(^+\) and the D-Dimers were not significantly different between the subgroup of patients presenting with at least one risk factor of cardiovascular disease compared to those who did not have any cardiovascular risk factor (Table 6). The analysis of the impact of cardiovascular risk factors on the biomarkers of plasma and cellular hypercoagulability at each stage of cancer showed no significant difference for thrombin generation, Pd-MP, Pd-MP/PS\(^+\) and PPL clotting time. When only patients with regional stage were considered, the concentration of the D-Dimers was significantly higher in patients with cardiovascular risk factors (Table 6).

**Correlation of cellular and plasma markers of hypercoagulability with routine hematological and biochemical parameters.**

Age and BMI of patients did not correlate with any of the studied biomarkers of hypercoagulability. Among thrombogram parameters the Peak and the ETP were significantly correlated with the CRP (r=0.3; p=0.028 and 0.019 respectively). The peak was also correlated with the ASAT levels (r=0.3; 0= 0.03).

The concentration of D-Dimers was inversely correlated with Hb (r=-0.52; p<0.0005) and positively correlated with the concentration of transaminases. In addition, alkaline phosphatase was correlated with the concentration of D-Dimers (r=0.38; p<0.005). The levels of D-Dimers did not correlate with creatinine, urea and CRP.

The concentration of Pd-MP and Pd-MP/PS\(^+\) was inversely correlated with Hb (r=-0.3; p=0.01) and positively correlated with the platelet count (r=0.3; p=0.02).

All the other hematological and biochemical parameters did not correlate with thrombin generation parameters and Pd-MP or PPL clotting time. None of thrombin generation parameters was correlated with the concentration of D-Dimers or Pd-MP or with aPTT or PT.
Discussion

The present study demonstrates that in patients with breast cancer the hypercoagulable state is characterized by marked increase of procoagulant Pd-MP, enhanced thrombin generation and increased degradation of fibrin. Cancer-related variables have a differential impact on the biomarkers of plasma and cellular hypercoagulability. Almost all studied patients showed high levels of procoagulant Pd-MP and short procoagulant phospholipid-dependent clotting times in plasma. Thus, in patients with breast cancer platelet activation leading to the release of microparticles expressing phosphatidylserine is a dominant characteristic of the blood hypercoagulability. This finding is in accordance with previous studies which showed that breast cancer patients treated with chemotherapy or receiving adjuvant endocrine therapy have increased numbers of mainly Pd-MP and a high microparticle-dependent thrombin generation [28].

Our study shows that the increase of Pd-MP is related to the underlying cancer rather than specific anticancer treatment. Indeed, our study has shown that the stage of the disease has a significant influence on the concentration of the procoagulant Pd-MP and the PPL clotting time: patients with metastatic disease had significantly higher concentrations of Pd-MP and shorter PPL clotting time compared to those with localized stage. Interestingly, chemotherapy did not induce any significant change in either microparticle concentration or the PPL clotting time. These findings are in accordance with previous studies [29-32] and support the hypothesis that Pd-MP concentration and the PPL clotting time are biomarkers that reflect the close association and interaction between the burden of cancer cells and platelets. Whether the release of procoagulant microparticles by platelets stems from the direct interaction of platelets with breast cancer cells or is the consequence of an inflammatory reaction triggered by cancer merits further investigation. The concept that platelet activation is a dominant phenomenon in cancer is supported by several recent studies conducted in patients with other types of cancer [33-36] and may have therapeutic implications in the management of cellular derived hypercoagulability.

In about one third of patients thrombin generation was higher than the upper normal limit. Platelet-derived microparticles manifested significant procoagulant activity as documented by the almost linear, inverse correlation between the concentration of both Pd-
MP and Pd-MP/PS⁺ with the PPL clotting time. The thrombogram-thrombinoscope assay in platelet poor plasma was performed using 5 pm of TF and saturating concentration of procoagulant phospholipids. Preliminary experiments from our group showed that in these experimental conditions, the thrombogram assay is not sensitive to the procoagulant activity of microparticles present in the plasma samples (data not shown). For this reason neither the concentration of Pd-MP nor the PPL clotting time were correlated with thrombin generation. Consequently, the two settings of tests describe different components of hypercoagulability; the cellular and the plasma one. The increase in thrombin generation was marked in patients with recently diagnosed cancer who were receiving chemotherapy. This implicates chemotherapy in triggering of plasma hypercoagulability. Chemotherapy induced hypercoagulability is one of the mechanisms which increase the risk of VTE in cancer patients [37-42]. The increase of thrombin generation was not universal to all patients but in about one third of them. The Vienna Cancer and Thrombosis Study, which prospectively evaluated the capacity of biomarkers of hypercoagulability to detect the risk of VTE in cancer patients showed that high thrombin generation is an independent risk factor for VTE [43]. Interestingly the presence of cardiovascular risk factors was not associated with any significant impact on either thrombin generation or the concentration of Pd-MP and the PPL clotting time. This finding further supports the concept that the increase of Pd-MP concentration and the enhancement of thrombin generation are related to cancer characteristics. Interestingly the poor correlation between Pd-MP and the studied biochemical parameters of inflammation, renal and liver function further enhances the assumption that platelet related hypercoagulability is closely associate with cancer. Thrombin generation showed a weak correlation with CRP and liver function revealing that plasma hypercoagulability might stem from interactions of cancer cells with plasma components as well as from the presence of an inflammatory state.

Breast cancer patients also showed enhanced fibrin degradation documented by the significant increase in D-Dimers concentration in cancer patients’ plasma as compared to healthy age matched individuals. In 59% of patients D-Dimers concentration was higher than the upper normal limit. The D-Dimers concentration in patients with metastatic disease was higher as compared to that in patients with localized cancer. This is in accordance with previous studies which demonstrated that augmentation of D-Dimers might be in part a reflection of ongoing fibrinogen metabolism within the actively remodeled tumor stroma.
and are related to the clinically measured growth rate of breast cancer [44]. The concentration of D-Dimers did not correlate with Pd-MP or thrombin generation and only 18% of patients showed combined increase of D-Dimers and thrombin generation. Noteworthy the presence of cardiovascular risk factors was linked with a significant increase of the concentration of D-Dimers revealing that the D-Dimers is a less specific biomarker for cancer induced hypercoagulability.

The evidence presented in this study demonstrates for the first time that in addition to hypercoagulability related to increased release of Pd-MP – which is an almost constitutional phenomenon in breast cancer patients – enhanced thrombin generation is only present in about one third of them. The combined use of biomarkers of cellular and plasma hypercoagulability allows identification of a sub-population of about 30% breast cancer patients who present marked hypercoagulability, characterized by increased thrombin generation and platelet activation.

Conclusions

The present study has defined the most appropriate biomarkers for the diagnosis of blood borne hypercoagulability related to breast cancer. The concentration and the procoagulant activity of Pd-MP are interconnected with the biological activity and the overall burden of cancer cells. Assessment of thrombin generation reflects the procoagulant effect of anticancer treatment administration within 6 months after the diagnosis of the disease. Thus the weighted incorporation of the biomarkers of cellular and plasma hypercoagulability in risk assessment models for VTE might improve their predictive value.
List of abbreviations

VTE: venous thromboembolism
TF: tissue factor
PPL: procoagulant phospholipid dependent clotting time
TNM: tumor, node, metastases
PT: prothrombin time
aPTT: activated partial thromboplastin time
PPP: platelet poor plasma
ETP: endogenous thrombin potential
MRI: mean rate index
Pd-MP: platelet derived microparticles
UNL: upper normal limit
LNL: lower normal limit
Pd-MP/PS*: platelet derived microparticles expressing phosphatidylserin

Conflict of Interest

The authors do not have any conflict of interest for this study
Authors’ contributions

MC has made substantial contributions to study design and organization, acquisition, analysis and interpretation of data, has been involved in drafting the manuscript
IA had substantial contribution to the recruitment of patients
AR carried out the assessment of thrombin generation and procoagulant phospholipid clotting time and had substantial contribution in the acquisition and the analysis of the respective data
PV has made substantial contributions to interpretation of data, has been involved in drafting the manuscript
EL has made substantial contribution in drafting the manuscript and revising it critically for important intellectual content
FS had substantial contribution to the recruitment of patients
HK carried out the assessment of D-Dimers and had substantial contribution in the acquisition and the analysis of the respective data
VG participated in the statistical analysis and had substantial contribution in the interpretation of the data
AK carried out the flow cytometry assay and had substantial contribution in the acquisition and the analysis of the respective data
RB had substantial contribution to the recruitment of patients
MF had substantial contribution to the recruitment of patients
LG had substantial contribution to the recruitment of patients
JD had substantial contribution to the recruitment of patients
CK had substantial contribution to the recruitment of patients
MQ performed the statistical analysis and has been involved in the data base construction
JG has made substantial contributions to conception, design, interpretation of data
JPL has made substantial contributions to conception, design, interpretation of data
MH has made substantial contribution to interpretation of data
IE has made substantial contribution to design and interpretation of data
GG has made substantial contributions to conception and design of the study, analysis and interpretation of data, has been involved in drafting the manuscript has given final approval of the version to be published, agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved

Acknowledgement. The authors acknowledge Professor Gordon Wishard for the critical reading of the manuscript. The authors are also grateful to Marie-Paule Roman and Severin Bouffard for the excellent technical assistance.
References


**Figure 1.** Distribution of individual values of thrombin generation rate (frame A), Peak of thrombin (frame B) and ETP (frame C) in the control group (open cycles) and in the group of patients (dark cycles). The distinct line shows the Upper Normal Limit (UNL).
Figure 2. Representative thrombograms from a healthy individual (a) and three patients with high thrombin generation (c,d,e).
Figure 3. Impact of the interval since the diagnosis (shorter or longer than 6 months) on the ETP in patients on chemotherapy. Distinct lines show the corresponding mean values for each group.
Figure 4. Impact of the stage of breast cancer on the concentration of D-Dimers. The distinct lines show the corresponding mean values for each group.
<table>
<thead>
<tr>
<th>Age (year)</th>
<th>Control group (n=30)</th>
<th>All patients (n=62)</th>
<th>Localized Stage (n=13)</th>
<th>Regional Stage (n=29)</th>
<th>Metastatic Stage (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>55 ± 10</td>
<td>52 ± 11</td>
<td>53 ± 10</td>
<td>54 ± 11</td>
<td>49 ± 13</td>
<td></td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>27 ± 6</td>
<td>28 ± 4.7</td>
<td>29.6 ± 4.7</td>
<td>27.6 ± 4.2</td>
<td>27.7 ± 5.6</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>11.6±2</td>
<td>11.5 ± 1.5</td>
<td>11.2 ± 1.1</td>
<td>11.8 ± 1.4</td>
<td>11.3 ± 1.8</td>
</tr>
<tr>
<td>Leukocytes (10⁹/L)</td>
<td>7.3 ± 1.8</td>
<td>7.1 ± 4.8</td>
<td>7.2 ± 2.4</td>
<td>7.1 ± 2.8</td>
<td>7.1 ± 5</td>
</tr>
<tr>
<td>Platelets (10⁹/L)</td>
<td>270±62</td>
<td>242 ± 96</td>
<td>262 ± 65</td>
<td>264 ± 98</td>
<td>198 ± 98</td>
</tr>
<tr>
<td>PT (% of prothrombin)</td>
<td>100±8</td>
<td>92±8.3</td>
<td>94±6</td>
<td>95±7</td>
<td>89±11</td>
</tr>
<tr>
<td>aPTT ratio</td>
<td>1±0.2</td>
<td>0.9±0.06</td>
<td>0.9±0.05</td>
<td>0.9±0.06</td>
<td>0.9±0.08</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>3.9±0.6</td>
<td>4.4±1.2</td>
<td>4±0.8</td>
<td>4.7±1.4</td>
<td>4.4±1.1</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>-</td>
<td>14.5±50</td>
<td>32±99</td>
<td>7±20</td>
<td>10±15</td>
</tr>
<tr>
<td>ALAT (IU/L)</td>
<td>-</td>
<td>25±13</td>
<td>30±16</td>
<td>24±10</td>
<td>25±15</td>
</tr>
<tr>
<td>ASAT (IU/L)</td>
<td>-</td>
<td>36±23</td>
<td>31±18</td>
<td>34±17</td>
<td>43±32</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>-</td>
<td>65±17</td>
<td>67±23</td>
<td>67±17</td>
<td>62±13</td>
</tr>
<tr>
<td>Urea (mg/dL)</td>
<td>-</td>
<td>5±2</td>
<td>5±1.6</td>
<td>5.5±2.7</td>
<td>5±2.2</td>
</tr>
<tr>
<td>Time since diagnosis (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-6 months</td>
<td>27</td>
<td>8</td>
<td>15</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>6-12 months</td>
<td>10</td>
<td>2</td>
<td>7</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>12-36 months</td>
<td>-</td>
<td>13</td>
<td>3</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>&gt; 36 months</td>
<td>12</td>
<td>0</td>
<td>2</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Cardiovascular risk factors (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-Hypertension</td>
<td>no</td>
<td>14</td>
<td>3</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>-Varicose veine</td>
<td>no</td>
<td>8</td>
<td>1</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>-Hyperlipidemia</td>
<td>no</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>-Diabetes</td>
<td>no</td>
<td>6</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.** Demographic data, clinical characteristics and routine hematological and biochemical parameters of breast cancer patients and controls.
<table>
<thead>
<tr>
<th></th>
<th>Control group (n=30)</th>
<th>Breast cancer group (n=62)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All patients (n=62)</td>
<td>Local Stage (n=13)</td>
</tr>
<tr>
<td>MRI (nM/ min)</td>
<td>109±33</td>
<td>159±47**</td>
</tr>
<tr>
<td>Peak (nM)</td>
<td>288±48</td>
<td>341±65*</td>
</tr>
<tr>
<td>ETP (nM.min)</td>
<td>1498±225</td>
<td>1531±337</td>
</tr>
<tr>
<td>Pd-MP $^-$(/μL)</td>
<td>756±429</td>
<td>10015±8223$^6$</td>
</tr>
<tr>
<td>Pd-MP/PS $^+$ (/μL)</td>
<td>695±361</td>
<td>9698±7931$^6$</td>
</tr>
<tr>
<td>PPL (sec)</td>
<td>72.8 ± 9.9</td>
<td>43.5± 10.3</td>
</tr>
<tr>
<td>D-Dimers (ng/ml)</td>
<td>230±50</td>
<td>1250 ± 1773</td>
</tr>
</tbody>
</table>

**Table 2.** Thrombogram parameters in patients and controls. Values are depicted as mean±sd.

* p=0.001 versus controls, ** p<0.001 versus controls, $^6$ p<0.01 versus controls, $^6^6$ p<0.05 versus controls, $^6^6^6$ p<0.05 versus metastatic stage.
<table>
<thead>
<tr>
<th></th>
<th>Local stage (n=8)</th>
<th>Regional stage (n=23)</th>
<th>Metastatic stage (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRI (nM/ min)</td>
<td>160±54</td>
<td>154±53</td>
<td>154±58</td>
</tr>
<tr>
<td>Peak (nM)</td>
<td>345±62</td>
<td>348±78</td>
<td>328±82</td>
</tr>
<tr>
<td>ETP (nM.min)</td>
<td>1502±264</td>
<td>1557±381</td>
<td>1416±326</td>
</tr>
<tr>
<td>Pd-MP (/μL)</td>
<td>10802±8618</td>
<td>7459±6743</td>
<td>11714±7173</td>
</tr>
<tr>
<td>Pd-MP/PS+ (/μL)</td>
<td>10478±8273</td>
<td>7209±6463</td>
<td>11403±7132</td>
</tr>
<tr>
<td>PPL (sec)</td>
<td>45±12</td>
<td>43±11</td>
<td>44±11</td>
</tr>
</tbody>
</table>

**Table 3.** Thrombogram parameters and Pd-MP levels in patients on active chemotherapy according to the stage of breast cancer. Values are described with mean±sd.
Table 4. Thrombogram parameters and Pd-MP levels in all patients and in patients on chemotherapy according to the time since the diagnosis (less than 6 months or more). Values are mean±sd. *p<0,05 versus patients on chemotherapy and diagnosis >6 months.
<table>
<thead>
<tr>
<th></th>
<th>Local stage (n=13)</th>
<th>Regional stage (n=29)</th>
<th>Metastatic stage (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-6 months (n=8)</td>
<td>&gt;6 months (n=5)</td>
<td>0-6 months (n=15)</td>
</tr>
<tr>
<td>MRI (mM/min)</td>
<td>177±35</td>
<td>164±64</td>
<td>154±71</td>
</tr>
<tr>
<td>Peak (nM)</td>
<td>376±61</td>
<td>357±102</td>
<td>347±114*</td>
</tr>
<tr>
<td>ETP (mM.min)</td>
<td>1620±250</td>
<td>1635±471</td>
<td>1596±420*</td>
</tr>
<tr>
<td>Pd-MP (/μL)</td>
<td>12236±8617*</td>
<td>4786±2515</td>
<td>8909±5190</td>
</tr>
<tr>
<td>Pd-MP/PS* (/μL)</td>
<td>11876±8272*</td>
<td>4699±2483</td>
<td>8531±4939</td>
</tr>
<tr>
<td>PPL -ct (sec)</td>
<td>43±9</td>
<td>48±11</td>
<td>42±7</td>
</tr>
</tbody>
</table>

Table 5. Thrombogram parameters and Pd-MP according to the stage and the time since the diagnosis (less than 6 months or more). Values are described with mean±sd. *p<0,05 versus regional stage and diagnosis >6 months. ** p<0,05 versus local stage and diagnosis >6 months.
<table>
<thead>
<tr>
<th></th>
<th>Total population of patients</th>
<th>Local stage (n=13)</th>
<th>Regional stage (n=29)</th>
<th>Metastatic stage (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CV RF (n=21)</td>
<td>No CV RF (n=41)</td>
<td>CV RF (n=5)</td>
<td>No CV RF (n=8)</td>
</tr>
<tr>
<td>MRI (mM/min)</td>
<td>134±51</td>
<td>167±51</td>
<td>190±45</td>
<td>144±64</td>
</tr>
<tr>
<td>Peak (nM)</td>
<td>317±89</td>
<td>357±72</td>
<td>336±101</td>
<td>389±52</td>
</tr>
<tr>
<td>ETP (mM.min)</td>
<td>1490±346</td>
<td>1552±335</td>
<td>1571±475</td>
<td>1660±238</td>
</tr>
<tr>
<td>Pd-MP (/μL)</td>
<td>8133±6480</td>
<td>10955±8893</td>
<td>6413±6848</td>
<td>11218±8082</td>
</tr>
<tr>
<td>Pd-MP/PS⁺ (/μL)</td>
<td>7896±6328</td>
<td>10599±8552</td>
<td>6326±6817</td>
<td>10858±7681</td>
</tr>
<tr>
<td>PPL (sec)</td>
<td>42±10</td>
<td>44±11</td>
<td>46±12</td>
<td>44±10</td>
</tr>
</tbody>
</table>


CV RF : Cardiovascular risk factors
Pd-MP : Platelet derived microparticles
Pd-MP/PS⁺ : Platelet derived microparticles expressing phosphatidylserin
PPL : Procoagulant phospholipid dépendent clotting time
MRI : Mean rate index of the propagation phase
ETP : Endogenous thrombin generation

*p=0.05 versus No CV RF
Figure 3

The scatter plot shows the distribution of ETP (nM.min) for two groups: 0–6 months and >6 months. The p-value for the difference between the two groups is 0.036.
Figure 4

The figure illustrates the distribution of D-Dimers (ng/ml) across different cancer stages: local stage, regional stage, and metastatic stage. The data points are color-coded, with different shades for each stage. The p-value indicated is 0.04, suggesting a statistically significant difference between the stages.