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Thrombotic biomarkers for risk prediction of malignant disease recurrence in patients with early stage breast cancer

Cinzia Giaccherini,[1#] Marina Marchetti,[1#] Giovanna Masci,[2] Cristina Verzeroli,[1] Laura Russo,[1] Luigi Celio,[3] Roberta Sarmiento,[4] Sara Gamba,[1] Carmen J. Tartari,[1] Erika Diani,[1] Alfonso Vignoli,[1] Paolo Malighetti,[5] Daniele Spinelli,[5] Carlo Tondini,[6] Sandro Barni,[7] Francesco Giuliani,[8] Fausto Petrelli,[7] Andrea D'Alessio,[9] Giampietro Gasparini,[4] Filippo De Braud,[3] Armando Santoro,[2] Roberto Labianca[10] and Anna Falanga,[1,11] on behalf of the HYPERCAN Investigators**

[1]Immunohematology and Transfusion Medicine, Hospital Papa Giovanni XXIII, Bergamo; [2]Oncology Unit, IRCCS Humanitas Institute, Rozzano; [3]Oncology Unit, IRCCS National Cancer Institute, Milan; [4]Oncology Unit, Hospital San Filippo Neri, Rome; [5]Department of Management, Information and Production Engineering, University of Bergamo, Bergamo; [6]Oncology Unit, Hospital Papa Giovanni XXIII, Bergamo; [7]Oncology Unit, Hospital Treviglio-Caravaggio, Treviglio; [8]Oncology Unit, IRCCS Cancer Institute Giovanni Paolo II, Bari; [9]Medical Oncology and Internal Medicine, Policlinico San Marco, Zingonia-Bergamo; [10]Department Oncology Bergamo Province, Hospital Papa Giovanni XXIII, Bergamo and [11]University of Milan Bicocca, School of Medicine and Surgery, Milan, Italy

[#]CG and MM contributed equally as co-first authors.

***A full list of the Investigators of the HYPERCAN (HYPERcoagulation in CANcer) Study Group appears in the Appendix 1.*

ABSTRACT

In cancer patients, hypercoagulability is a common finding. It has been associated with an increased risk of venous thromboembolism, but also to tumor proliferation and progression. In this prospective study of a large cohort of breast cancer patients, we aimed to evaluate whether pre-chemotherapy abnormalities in hemostatic biomarkers levels: (i) are associated with breast cancer-specific clinico-pathological features; and (ii) can predict for disease recurrence. D-dimer, fibrinogen, prothrombin fragment 1+2, and FVIIa/antithrombin levels were measured in 701 early-stage resected breast cancer patients candidate to adjuvant chemotherapy and prospectively enrolled in the HYPERCAN study. Significant prognostic parameters for disease recurrence were identified by Cox regression multivariate analysis and used for generating a risk assessment model. Pre-chemotherapy D-dimer, fibrinogen, and prothrombin fragment 1+2 levels were significantly associated with tumor size and lymph node metastasis. After 3.4 years of follow up, 71 patients experienced a recurrence. Cox multivariate analysis identified prothrombin fragment 1+2, tumor size, and Luminal B HER2-negative or triple negative molecular subtypes as independent risk factors for disease recurrence. Based on these variables, we generated a risk assessment model that significantly differentiated patients at low- and high-risk of recurrence (cumulative incidence: 6.2 *vs.* 20.7%; Hazard Ratio=3.5; $P<0.001$). Our prospective clinical and laboratory data from the HYPERCAN study were crucial for generating a scoring model for assessing risk of disease recurrence in resected breast cancer patients, candidate to systemic chemotherapy. This finding stimulates future investigations addressing the role of plasma prothrombin fragment 1+2 in the management of breast cancer patients to provide the rationale for new therapeutic strategies. (The HYPERCAN study is registered at *clinicaltrials.gov identifier 02622815.*)

Correspondence:

ANNA FALANGA
annafalanga@yahoo.com

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Introduction

Cancer growth and dissemination is associated with the development of a subclinical hypercoagulable state in the host, detectable by coagulation laboratory tests.[1,2] The pathogenesis of blood coagulation activation in cancer is complex and multifactorial. Among other factors, the expression of tumor cell clot-promoting properties, and the inflammatory response of the normal host cells to the tumor play a central role in the cancer-associated prothrombotic diathesis by leading to the activation of the blood clotting system.[2,3] Importantly, tumor cells of different origin express different procoagulant profiles, and the clotting system can be triggered to various extents according to the type and stage of the malignant disease. [4] Furthermore, the patient's hypercoagulable state worsens with cancer progression and metastatic spread, which supports the concept of a tight relationship between tumor burden and hemostatic abnormalities.[5] All this contributes to an increased risk of thrombosis, and also affects the tumor biology by favoring tumor growth and metastasis.[6,7]

Over the last three decades, several studies have evaluated the role of clotting activation biomarkers in relation to cancer outcomes, including disease progression, response to chemotherapy, and mortality.[8-11] As recently reviewed,[12] however, most of these studies were retrospective in nature and not always specifically designed to address the impact of thrombotic biomarkers on cancer outcomes. In addition, recruitment was often mono-institutional, and included small heterogeneous cohorts of patients with different treatments. Therefore, despite the encouraging results reported, new data coming from large prospective cohorts are needed. In this respect, breast cancer patients with limited resected disease are an important setting to test whether abnormal levels of circulating thrombotic biomarkers may represent a novel non-invasive factor for better prediction of disease recurrence (DR) risk.

Breast cancer, the most common cancer in women, is a highly heterogeneous disease presenting with a broad range of clinical and molecular characteristics, as well as variability in clinical progression. In recent years, information campaigns and large-scale prevention screening programs have contributed to an increase in the rate of early diagnosis,[13] with a consequent improved rate of patients treated at an early stage of the disease.[14] For the treatment choice, patients are classified according to intrinsic biological subtypes within the breast cancer spectrum, using clinico-pathological criteria, i.e. the immunohistochemical definition of estrogen receptor (ER) and progesterone receptor (PR), the detection of overexpression and/or amplification of the human epidermal growth factor receptor 2 (HER2) oncogene, and Ki-67 labeling index. This classification allows for a more personalized approach to the medical treatments, with favorable results. However, in spite of this, almost 10-15% of these patients still experience local or distant recurrences in the first five years from diagnosis,[13,15,16] mainly in the high-risk category, characterized by worse prognostic factors in which the use of adjuvant systemic chemotherapy is justified.[17,18] In this category, the identification of patients at the highest risk of relapse is an important area in which to improve personalized treatments and, ultimately, cancer care.

In the present study, we tested the predictive role of circulating clotting biomarkers on DR risk in a large patient cohort with resected high-risk breast cancer scheduled to receive post-surgical adjuvant chemotherapy enrolled in the ongoing prospective observational Italian multicenter HYPERCAN ("HYPERcoagulation and CANcer") study.[19] Specifically, we investigated whether baseline levels of D-dimer, fibrinogen, prothrombin fragment 1+2 (F1+2), and FVIIa/antithrombin (FVIIa-AT) complex were associated with clinico-pathological characteristics of breast cancer, and whether these biomarkers might be effective in predicting DR in patients at an early stage of the disease.

Methods

Study design and study population

For the present study, we considered patients of both genders with a high-risk surgically treated breast cancer enrolled in the ongoing HYPERCAN study[19] (*Online Supplementary Appendix*). The study protocol was approved by the local ethics committee. A data extraction from the HYPERCAN database was performed in January 2018. Patients enrolled from March 2012 to December 2015 were considered for the study. Of the 1,042 patients with resected breast cancer enrolled during this period, 953 had an adequate follow-up time. Data on ER/PR and HER2 positivity were available in 788 patients; in this subgroup, biomarkers data were available for 701 patients (*Online Supplementary Figure S1*). Median time of follow up at the time of the present analysis is 3.4 years.

Patient classification

Patients were categorized in subtypes according to tumor expression of ER, PR, ki67, and HER2 according to the American Society of Clinical Oncology (ASCO) - College of American Pathologist (CAP) guideline.[20] Data were derived from clinical pathology reports. ER/PR positivity defined the luminal groups, which included: Luminal A [i.e. HER2-negative (neg) and low Ki67)]; Luminal B HER2-neg (i.e. HER2 negativity and high Ki67), and Luminal B HER2-positive (pos) (i.e. HER2-pos and any ki67). HER2-pos cancer subtype was defined by negativity for ER/PR and positivity for HER2, while triple negative (TN) cancer was defined by ER/PR and HER2 negativity.

Blood sampling and plasma preparation

Fasting peripheral venous blood was drawn at enrollment prior to start of systemic adjuvant chemotherapy using a 21-gauge needle into 6 mL vacutainer tubes containing 0.109 M citrate (9:1 vol/vol; Becton Dickinson) after discarding the first 2-3 mL of blood.[21] Within two hours from collection, plasma was isolated by two sequential centrifugations at 2,600 rpm for 15 minutes (min)[22] and stored at -80°C. All samples were anonymized and tested in blind at the Laboratory of Hemostasis and Thrombosis Center (Hospital Papa Giovanni XXIII, Bergamo, Italy).

Hypercoagulation biomarkers

Plasma levels of D-dimer (HemosIL D-dimerHS, Werfen Group) and fibrinogen (QFA thrombin, Werfen Group) were measured on an automated coagulometer analyzer (ACL TOP500, Werfen Group). F1+2 (Siemens Healthcare Diagnostics) and FVIIa-AT complex (Stago) were determined by commercial ELISA. Reference intervals for coagulation biomarkers were internally generated from a group of 200 apparently healthy controls (170 females; 30 males) with no chronic or acute diseases. Median age was 49 years (range, 35-64 years).

Outcome

The primary outcome of the present analysis was DR, defined as either loco-regional (limited to the ipsilateral breast or chest wall and/or axillary, infraclavicular, or supraclavicular lymph nodes) or distant metastasis.

Statistical analysis

Categorical data are summarized as frequencies and proportions, while for continuous variables, data are summarized as mean and standard deviation or median and 5th-95th percentile range, depending on their distribution. Differences between groups were tested by Pearson's χ^2 test, Student *t*-test or Mann-Whitney U-test. Survival functions were estimated using the Kaplan-Meier method, assuming as baseline time the beginning of chemotherapy, while survival analyses were performed using Cox's proportional hazard (PH) model. Statistical analysis was performed using SPSS v21.0 (IBM Corp).

Results

Characteristics of study population

Table 1 summarizes clinical and tumor histological characteristics of study patients (median age 52; range, 29-79 years). Breast-conserving resection was performed in 61% and mastectomy in 39%. HER-2 expression was positive in 203 and negative in 498 patient sample specimens, respectively. Most frequent molecular subtypes were Luminal B HER2-neg (33.4%) > Luminal A (22.7%) > Luminal B HER2-pos (20.3%) > TN (14.4%) > HER2-pos (8.6%) (4 missing data for ki67 =0.6%). According to histological subtype, the largest proportion was classified as invasive ductal carcinoma, diagnosed in 83% of patients. Lymph node involvement was found in 56% of patients. Based on primary tumor characteristics, ER/PR and HER2 status, age, and/or discretion of the treating physician, patients were candidates for systemic adjuvant chemotherapy. An anthracycline-based regimen was indicated for 35.1% of patients. The addition of taxane to an anthracycline regimen was considered in case of extensive disease burden or TN disease (50.8% of patients). A taxane-based regimen without anthracyclines was administrated to 9.3% of patients. In case of comorbidities or patient preference, intravenous CMF (i.e. cyclophosphamide, methotrexate and 5-fluorouracil) was given (4.8% of patients). All 203 HER-2 positive breast cancers received (in addition to chemotherapy) trastuzumab every 21 days for one year. Four hundred and seventy-five patients with ER/PR positivity (n=535) received endocrine therapy according to their premenopausal state (data missing in 48 patients).

Disease recurrence

The Kaplan-Meier curve shown in Figure 1 describes the cumulative incidence of DR during four years of follow up. The incidence of DR in the group of patients was 2% (95%CI: 1-3) at one year, 6% (95%CI: 4.3-7.8) at two years, 9.5% (95%CI: 7.2-11.9) at three years, and 11.2% (95%CI:8.5-14) at four years. Specifically, 630 out of 701 patients included into the analysis remained disease-free, while 71 relapsed. Demographics and clinical characteristics of patients who developed DR and of those who remained disease-free are shown in Table 1. The group of patients who relapsed comprised 68 females and three males, with a median age of 52 years (range, 34-78 years). Recurrences consisted of distant metastasis in 69% of cases, and loco-regional relapse in the remaining 31%. According to the molecular subtype, the majority of

Table 1. Characteristics of patients with resected breast cancer.

	All patients 701	No DR 630	DR 71	*P*
Gender				
Male	11 (1.6)	8 (1.3)	3 (4.2)	0.091
Female	690 (98.4)	622 (98.7)	68 (95.8)	
Age				
Median (5th-95th)	52 (37-73)	52 (36-72)	52 (37-76)	0.893
Stage				
IA	173 (24.7)	165 (26.2)	8 (11.3)	
IIA	225 (32.1)	209 (33.2)	16 (22.5)	
IIB	125 (17.8)	104 (16.5)	21 (29.6)	
IIIA	85 (12.1)	75 (11.9)	10 (14.1)	**0.037**[$]
IIIB	9 (1.3)	8 (1.3)	1 (1.4)	
IIIC	44 (6.3)	38 (6.0)	6 (8.4)	
Unknown	40 (5.7)	31 (4.9)	9 (12.7)	
Tumor size				
≤ 2 cm	80 (11.3)	76 (12.1)	4 (5.6)	
2-5 cm	245 (35.0)	227 (36.0)	18 (25.4)	**0.011**
≥ 5 cm	342 (48.8)	295 (46.8)	47 (66.2)	
Unknown	34 (4.9)	32 (5.1)	2 (2.8)	
N stage				
N0	287 (40.9)	268 (42.5)	19 (26.8)	
N1	268 (38.3)	236 (37.5)	32 (45.1)	
N2	77 (11.0)	70 (11.1)	7 (9.8)	**0.020**[+]
N3	48 (6.8)	40 (6.3)	8 (11.3)	
Unknown	21 (3.0)	16 (2.6)	5 (7.0)	
Histological type				
Ductal	580 (82.7)	526 (83.5)	54 (76.1)	
Lobular	56 (8.0)	49 (7.8)	7 (9.9)	
Mixed	4 (0.6)	3 (0.5)	1 (1.4)	0.193[&]
Others	44 (6.3)	38 (6.0)	6 (8.4)	
Unknown	17 (2.4)	14 (2.2)	3 (4.2)	
Grading				
G1	14 (2.0)	14 (2.2)	-	
G2	264 (37.7)	238 (37.8)	26 (36.6)	0.550[§]
G3	409 (58.3)	365 (57.9)	44 (62.0)	
Unknown	14 (2.0)	13 (2.1)	1 (1.4)	
Molecular subtype				
Luminal A	159 (22.7)	149 (23.7)	10 (14.1)	
Luminal B HER2-neg	234 (33.4)	201 (31.9)	33 (46.5)	
Luminal B HER2-pos	142 (20.3)	137 (21.7)	5 (7.0)	**<0.001**
HER2-pos	61 (8.6)	58 (9.2)	3 (4.2)	
Triple negative	101 (14.4)	82 (13.0)	19 (26.8)	
Unknown	4 (0.6)	3 (0.5)	1 (1.4)	
Surgery				**0.047**
Breast conserving	427 (61.0)	392 (62.2)	35 (49.3)	
Mastectomy	273 (38.9)	238 (37.8)	35 (49.3)	
Unknown	1 (0.1)	-	1 (1.4)	
ECOG-PS				
0	634 (90.4)	569 (90.3)	65 (91.6)	0.402
1	26 (3.7)	22 (3.5)	4 (5.6)	
Unknown	41 (5.9)	39 (6.2)	2 (2.8)	

Data are presented as number (percentage). *P* is statistical significance by Pearson's χ^2 test (or by Mann-Whitney test for age) for comparison between patients with disease recurrence (DR) and without relapse (no DR). [$]$\chi^2$ test performed on clustered groups (stage 1/2/3). [+]χ^2 test performed on clustered groups (N0 *vs.* N1, N2, N3). [&]χ^2 test performed on clustered groups (ductal *vs.* others). [§]χ^2 test performed on clustered groups (G1/G2 *vs.* G3). HER2: human epidermal growth factor receptor 2; ECOG-PS: Eastern Cooperative Oncology Group Performance Status; N: number; neg: negative; pos: positive.

relapses occurred in Luminal B HER2-neg (n=33) and in TN (n=19), while the remaining were in Luminal A (n=10), in Luminal B HER2-pos (n=3), and in HER2-pos (n=3) patients; for one patient, this information was not available. The risk of recurrence was increased 4-fold (P<0.01) in patients with Luminal B HER2-neg and TN subtypes (HR=4.2, 95%CI: 2.3-7.8) compared to the remaining patients, with a DR cumulative incidence at four years of 17.6% (95%CI: 12.9-22.4) and 4.9% (95%CI: 2.4-7.5), respectively. Finally, the group of patients with DR was characterized by a higher proportion of subjects with tumor size ≥ 5 cm (P=0.011), infiltrated axillary lymph nodes (P=0.020), mastectomy (P=0.047), and Luminal B HER2-neg and TN molecular subtypes.

Hypercoagulation biomarkers and hematologic parameters

Patients had significantly higher plasma levels (P<0.01) of D-dimer, fibrinogen, and F1+2, compared to internal reference values obtained from a control group of healthy subjects (Figure 2). To exclude any influence of surgery [median time to blood sampling: 43 days (5^{th}-95^{th} range: 26-72 days)] on coagulation, the levels of each hemostatic marker were correlated with the time from surgery. The results showed no statistically significant correlation between time from surgery and each of the biomarkers (*data not shown*), even after sex and gender correction.

At enrollment, 12 patients were on thromboprophylaxis with anti-platelet agents (i.e. aspirin) and four with anticoagulants. To exclude any influence of thromboprophylaxis on thrombotic biomarker levels, we compared patients on aspirin (n=12) with those who were not (n=689), and no significant differences were found. The comparison of biomarkers between the anticoagulated (n=4) *versus* non-anticoagulated (n= 697) subjects could not provide statistically reliable results due to the very small number of subjects on anticoagulants.

Hemochromocytometric tests showed most patients had low red blood cell count and hemoglobin levels as compared to the control reference range (Table 2).

Association of hypercoagulation biomarkers with tumor characteristics

Multivariate analyses were performed to search for any significant association between hypercoagulation biomarker levels, hematologic parameters and tumor characteristics. According to tumor-node-metastasis classification, tumor size was a significant determinant of D-dimer (β=0.134, P=0.001) and fibrinogen (β=0.110, P=0.006) levels, while lymph node involvement positivity was a significant predictor of D-dimer (β=0.216, P<0.001) and F1+2 (β=0.112, P=0.004) plasma levels. No significant associations were found between hypercoagulation biomarkers and tumor histological subtype or grading. Similarly, no significant associations were found between hematologic parameters and tumor characteristics.

Association between hypercoagulation biomarker abnormalities and disease recurrence

Plasma levels of hypercoagulation biomarkers and hematologic parameters according to DR occurrence are shown in Table 3. There was no statistical difference in levels of the D-dimer, FVIIa-AT and fibrinogen between patients who experienced a relapse as compared to patients who remained disease-free during follow up, while F1+2 levels were significantly higher in the group of relapsed patients (P<0.05). Interestingly, correlation analyses showed that pre-chemotherapy levels of fibrinogen were significantly and inversely associated with time to relapse (β = -0.317; P=0.012).

A receiver operating characteristic (ROC) curve analysis was performed to evaluate the contribution of F1+2 levels to predict DR at four years of follow up. The area under the curve was 0.595. Cut-off value was set at 202.5

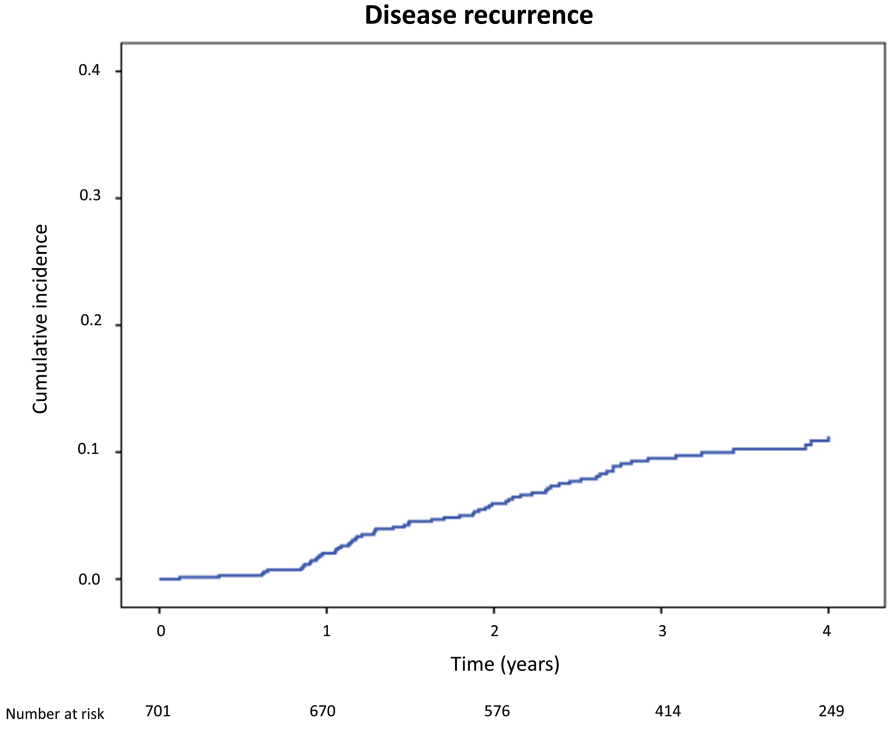

Figure 1. Cumulative incidence of disease recurrence in patients with resected breast cancer during four years follow up.

(pmol/L) (sensitivity=61%, specificity=53%). Kaplan-Meier analysis showed that in patients with F1+2 levels above the cut-off, cumulative incidence of DR was 14.7 % (95%CI:10.2-19.2) and was significantly (P<0.05) higher than in patients with F1+2 levels ≤ cut-off (8.7%; 95%CI: 5.4-12). A multivariate Cox regression analysis, stratified for age, was carried out testing for clinico-pathological covariates (Body Mass Index, smoking habit, infection, molecular subtype, tumor size, lymph node status, use of anticoagulant/anti-platelet agents, and type of surgery). After backward selection, only increased F1+2 value (HR 2; 95%CI: 1.1-3.6; P=0.019), tumor size ≥ 5cm (HR 2.6; 95%CI:1.4-4.6; P=0.001), and having Luminal B HER2-neg or TN molecular subtypes (HR 3.9; 95%CI: 2.1-7.5; P<0.001) were identified as independent risk factors for DR.

To exclude any influence of the pre-analytical phase on biomarkers levels, we performed the multivariate Cox regression analysis stratifying for recruiting centers. Results confirmed F1+2, tumor size and molecular subtypes as significant independent risk factors for DR.

The co-efficients of the multivariate analysis for each independent co-variate were used to generate a risk assessment score, as follows: F1+2 > 202.5 pmol/L = +1, tumor size ≥ 5cm = +1.3, Luminal B HER2-neg and TN molecular subtypes= +2. After score calculation, a ROC curve analysis was performed to evaluate the contribution of score value for DR (*Online Supplementary Figure S2*). The area under the curve was 0.72. The risk groups were created using a cut-off value of 2.65 points (sensitivity=66%, specificity=67%). Kaplan Meier curves according to risk groups are shown in Figure 3. Cumulative incidence of DR was 6.2% (IC 95%: 3.6-8.7) and 20.7% (IC 95%: 14.8-26.6), in the low-risk (score<3) and high-risk (score≥3) groups, respectively (HR 3.5; 95%CI: 2.1-6.0; P<0.001).

Discussion

In this large, prospective cohort study of 701 patients with early stage, surgically resected, high-risk breast cancer, enrolled in the HYPERCAN study, we characterized the baseline hypercoagulability status before starting systemic chemotherapy and investigated the capacity of plasma thrombotic biomarkers to predict DR. This represents the first large prospective study specifically designed to assess hemostatic activation and its association with recurrence risk in breast cancer patients.

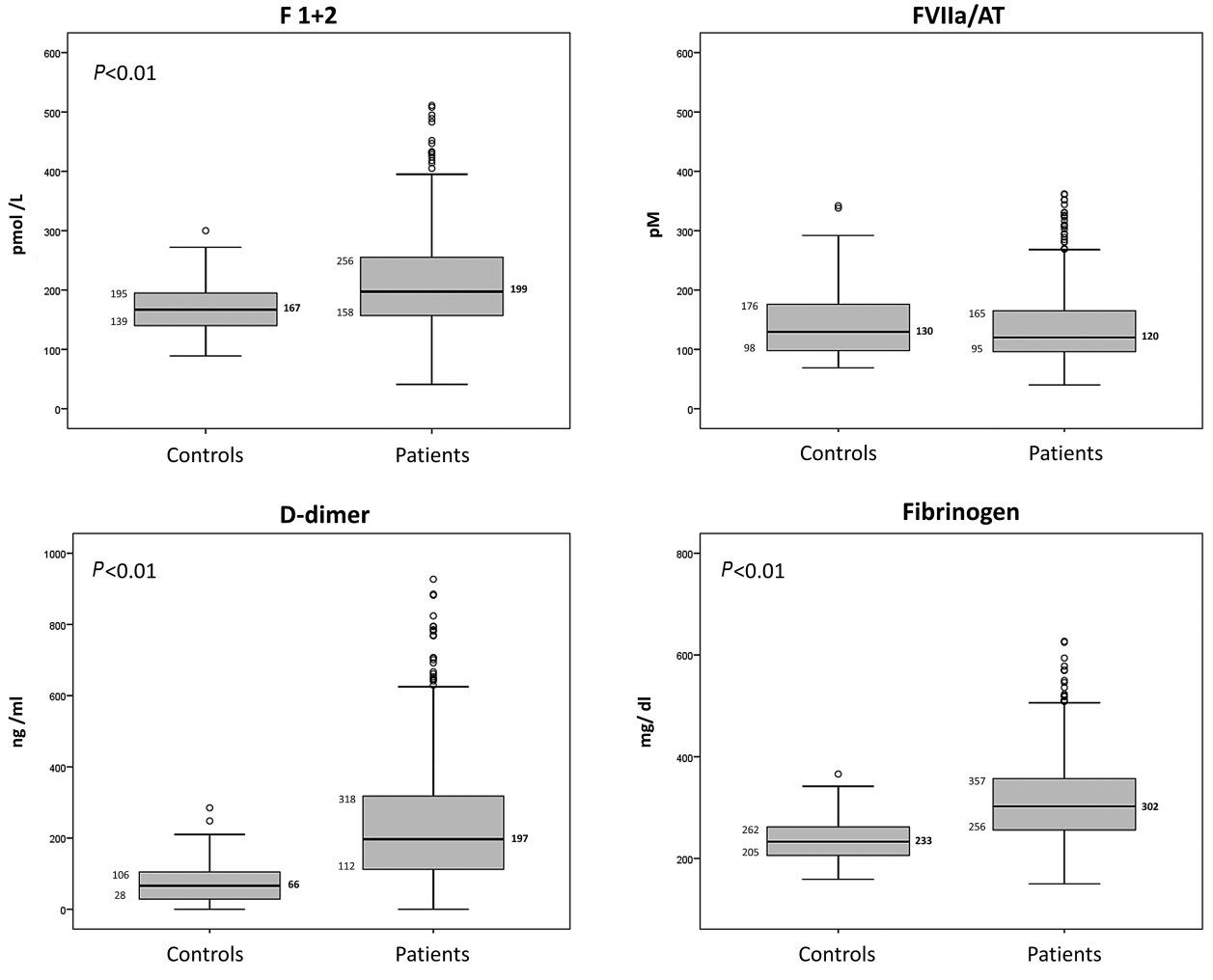

Figure 2. Distribution of F1+2, FVIIa-AT complex, D-dimer and fibrinogen values in patients compared to controls. The 25th, 50th and 75th percentile values are indicated for each biomarker and group. *P*-value calculated by Mann-Whitney test. AT: antithrombin; F1+2: prothrombin fragment 1+2.

In particular, we chose to study the role of fibrinogen, FVIIa-AT, F1+2, and D-dimer. Fibrinogen, a glycoprotein synthesized by the liver, is enzymatically converted to fibrin by thrombin during the coagulation process. In normal conditions, it circulates in plasma at high concentrations, and its levels increase in inflammatory states as part of the acute-phase response. Circulating levels of FVIIa-AT complexes reflect the degree of exposure of cellular tissue factor to blood; increased FVIIa-AT levels have been reported in a number of prothrombotic conditions, including solid and hematologic malignancies.[23] F1+2 is a peptide released during the proteolytic activation of prothrombin into active thrombin, and represents a parameter of *in vivo* thrombin formation, while D-dimer is the primary degradation product of cross-linked fibrin, representing an index of both coagulation and fibrinolysis activation. It has been suggested that fibrinogen is involved in several stages of cancer progression.[24] *In vivo*, in breast cancer patients, high fibrinogen levels have been associated with poorer overall survival[25,26] and poor response to therapy,[27] while increase in D-dimer levels has been related to growth rate, tumor burden, progression rate, and survival.[12,28]

In the present prospective cohort of patients with resected tumors and candidates to adjuvant therapy, we found the occurrence of a moderate, but significant, hypercoagulable state, as indicated by the increased plasma levels of fibrinogen, F1+2, and D-dimer. The lack of correlation between thrombotic biomarker levels and the time from surgery suggests involvement of a tumor-related mechanism in the hypercoagulability observed in these patients. Indeed, levels of thrombotic biomarkers, and particularly D-dimer and fibrinogen, positively and significantly correlated with tumor size and lymph node metastasis. The association with tumor characteristics might suggest an imprinting of the primary tumor on the coagulation system after resection, and might also represent the activity of residual occult circulating tumor cells on blood coagulation. In fact, it is well known that breast cancer cells can activate blood coagulation through several mechanisms,[4,29,30] and *in vivo* studies in breast cancer patients show significant correlations between increased D-dimer levels with circulating tumor cells and number of metastasis,[31] as well as with lymphovascular invasion, clinical stage, and lymph node involvement.[32]

To understand the relevance of our observation in rela-

Table 2. Hematologic parameters in the study subjects.

	Patients	Reference range	Out of range
White blood cells (x10^9/L)	6.8 (2.0)	(4.2-9.4)	4% (<4.2) 9% (>9.4)
Red blood cells (x10^{12}/L)	4.47 (0.51)	(4.7-5.82)	76% (<4.7) 0.5% (>5.82)
Hemoglobin (g/dL)	13.1 (1.3)	(14-17)	75% (<14)
Platelets (x10^9/L)	267 (80)	(150-400)	5% (>400)

Data of patients are shown as mean (standard deviation). Reference ranges are internally defined.

Table 3. Plasma levels of coagulation biomarkers according to disease recurrence.

	No-DR	DR	*P*
D-dimer (ng/mL)	196 (49-647)	224 (30-653)	0.270
FVIIa-AT (pM)	120 (70-269)	110 (62-335)	0.382
F 1+2 (pmol/L)	197 (117-385)	219 (114-628)	0.024
Fibrinogen (mg/dL)	298 (211-489)	321 (209-455)	0.909

Data are shown as median and range (5th-95th percentiles). *P*-value calculated by Mann-Whitney test. AT: antithrombin; F 1+2: prothrombin fragment 1+2; DR: disease recurrence.

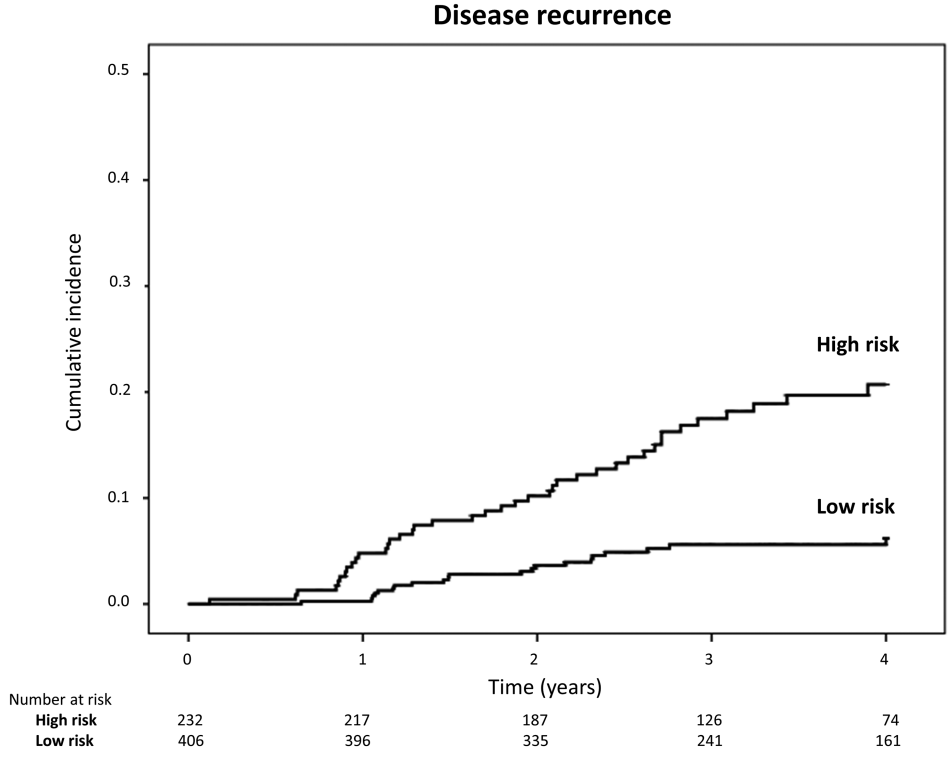

Figure 3. Kaplan-Meier analysis of disease recurrence cumulative incidence in patients according to risk-groups derived from the score (low-risk<3, high-risk≥3).

tion to the primary outcome of the study, we analyzed the patient thrombotic biomarkers according to DR. Relapses occurred in 71 patients, with distant metastasis in 69% and loco-regional metastasis in 31% of cases, respectively, providing a 10.8% cumulative incidence of DR after four years of follow up. As expected, most patients with DR belong to the Luminal B HER2-neg (46.5%) and TN (26.8%) subtypes. As regards thrombotic biomarkers, patients who subsequently experienced DR showed significantly (P<0.05) higher circulating levels of F1+2, compared to disease-free subjects. Remarkably, high fibrinogen levels were significantly associated with shorter time to DR, also after age and gender correction by multivariate analysis. Recurrence can be considered to be the result of cancer cells that persist in the host after treatments and start to proliferate and disseminate after years of quiescence. Activation of the clotting system might represent the first sign of tumor cell proliferation, and increased levels of thrombotic biomarkers can be an echo of occult tumor cell action on the hemostatic system. This is probably what is happening in our population, as detected by hypercoagulability biomarkers. That blood clotting activation might be an overt sign of an occult cancer is supported by clinical studies in cancer-free subjects, showing that hypercoagulability is a risk factor for subsequent death from cancer.[33,34] Analyses of hemostatic biomarkers in 19,303 male participants from three English cohorts followed for up to 30 years identified elevated circulating levels of fibrinogen and F1+2 as predictors of risk of smoking-related cancers.[35] In the setting of breast cancer, in a Scandinavian case-control study, high D-dimer and low antithrombin significantly predicted for breast cancer diagnosis,[36] while fibrinogen, in combination with cancer antigen 15-3 and platelet distribution width, distinguished breast cancer from benign breast disease in non-conclusive mammography patients.[37]

Furthermore, we were able to combine a clotting biomarker with previously established breast cancer prognostic factors in order to improve the individual prediction recurrence in this high-risk population. In fact, we found that higher F1+2 values, TN and Luminal B HER2-neg molecular subtypes, and tumor size were independent risk factors for DR. Therefore, F1+2 might be very helpful to identify those subjects who deserve special attention and those who can instead avoid the side effects of sustained chemotherapy. Finally, the significant association between fibrinogen levels and time to DR suggests the potential utility of this marker in those subjects identified at greater risk of DR as a parameter indicative of time to DR.

Other authors have reported different results as regards the F1+2 prognostic role in breast cancer. Specifically, in a recently published study including 235 patients with stage I-IIA breast cancer, F1+2 was not predictive for disease-free survival.[11] This discrepancy may be due to different characteristics of that study: a single center study, involving a smaller cohort of patients with stage I and IIa breast cancer (whereas our study enrolls patients up to stage IIIC). In addition, in that study, the disease-free survival was evaluated in a subgroup of 62 patients and blood samples for biomarker testing were obtained before surgery, while our samples were collected in tumor-resected condition before starting chemotherapy.

One limitation of our study is that some data on factors which can have an impact on coagulation cascade, including treatments with corticosteroids, oral contraceptives, and hormone replacement therapy, were not collected at the time of blood sampling. In any case, the hemostatic assays performed in this study directly represent the *in vivo* activity of the coagulation system, which already reflects all possible factors. In addition, at the time of this analysis, data on hormone therapy and radiotherapy were not available for all patients, and therefore were not included in the analysis. An evaluation of the contribution of these data on prognosis in these patients should be a subject of future study.

Several patients relapsed after a relatively long period of time and, therefore, the question may arise as to whether the time elapsed between blood collection and cancer relapse might be a source of uncontrolled confounding bias. This is a common limitation of prognostic biomarkers which aim to predict the cancer patient outcomes in order to decide on a best treatment option strategy. Validated genetic and biochemical prognostic biomarkers in early breast cancer provide a risk of DR for an event that may occur up to ten years later. In this study, we aimed to identify new prognostic biomarkers to help the selection of patients at highest risk of relapse at presentation, before planning the antitumor strategy. In this sense, we are consistent with the aim of the study, as our results identify a prognostic score based on the F1+2 applicable at presentation.

In conclusion, our prospective study is the first to demonstrate the utility of F1+2 as a potential circulating independent predictive biomarker for DR in a large cohort of patients with high-risk early breast cancer. Having reached this goal, we are now planning to validate the results in an independent cohort of patients. Our findings stimulate future investigations into the utility of longitudinal measurement of plasma F1+2 in the surveillance of women following surgery for primary breast cancer and to provide the rationale for new therapeutic strategies.

Appendix 1

The members of the HYPERCAN Study Group (all in Italy) are as follows: Investigators (by center) - Immunohematology and Transfusion Medicine, Hospital Papa Giovanni XXIII, Bergamo: Falanga A., Brevi S., Caldara G., Diani E., Gamba S., Giaccherini C., Marchetti M., Russo L., Schieppati F., Tartari C.J., Verzeroli C., Vignoli A.; Oncology Unit, IRCCS Humanitas Institute, Rozzano: Santoro A., Masci G.; Oncology Unit, IRCCS National Cancer Institute, Milan: De Braud F., Celio L., Nichetti F., Martinetti A.; Oncology Unit, Hospital Papa Giovanni XXIII, Bergamo: Tondini C.; Oncology Unit, Hospital San Filippo Neri, Rome: Gasparini G., Sarmiento R., Gennaro E, Meoni G; Oncology Unit, Hospital San Giovanni, Rome: Minelli M.; Oncology Unit, Hospital Treviglio-Caravaggio, Treviglio: Barni S., Petrelli F., Ghilardi M.; Dept. of Management, Information and Production Engineering, University of Bergamo: Malighetti P., Spinelli D.; Dept. Oncology Bergamo Province, Hospital Papa Giovanni XXIII, Bergamo: Labianca R.; IRCCS Cancer Institute Giovanni Paolo II, Bari: Giuliani F.; Medical Oncology and Internal Medicine, Policlinico San Marco, Zingonia-Bergamo: D'Alessio A., Cecchini S.

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References

1. Falanga A, Schieppati F, Russo D. Cancer Tissue Procoagulant Mechanisms and the Hypercoagulable State of Patients with Cancer. Semin Thromb Hemost. 2015;41(7):756-764.
2. Falanga A, Marchetti M, Vignoli A. Coagulation and cancer: biological and clinical aspects. J Thromb Haemost. 2013;11(2):223-233.
3. Falanga A, Russo L, Verzeroli C. Mechanisms of thrombosis in cancer. Thromb Res. 2013;131 Suppl 1:S59-62.
4. Marchetti M, Diani E, ten Cate H, Falanga A. Characterization of the thrombin generation potential of leukemic and solid tumor cells by calibrated automated thrombography. Haematologica. 2012;97(8):1173-1180.
5. Rickles FR, Falanga A. Activation of clotting factors in cancer. Cancer Treat Res. 2009;148:31-41.
6. Falanga A, Panova-Noeva M, Russo L. Procoagulant mechanisms in tumour cells. Best Pract Res Clin Haematol. 2009;22(1):49-60.
7. Adams GN, Rosenfeldt L, Frederick M, et al. Colon Cancer Growth and Dissemination Relies upon Thrombin, Stromal PAR-1, and Fibrinogen. Cancer Res. 2015;75(19):4235-4243.
8. Guo R, Yang J, Liu X, Wu J, Chen Y. Increased von Willebrand factor over decreased ADAMTS-13 activity is associated with poor prognosis in patients with advanced non-small-cell lung cancer. J Clin Lab Anal. 2018;32(1).
9. Falanga A, Marchetti M, Massi D, et al. Thrombophilic status may predict prognosis in patients with metastatic BRAFV600-mutated melanoma who are receiving BRAF inhibitors. J Am Acad Dermatol. 2016;74(6):1254-1256.e4.
10. Ma C, Lu B, Diao C, et al. Preoperative neutrophil-lymphocyte ratio and fibrinogen level in patients distinguish between muscle-invasive bladder cancer and non-muscle-invasive bladder cancer. Onco Targets Ther. 2016;9:4917-4922.
11. Mandoj C, Pizzuti L, Sergi D, et al. Observational study of coagulation activation in early breast cancer: development of a prognostic model based on data from the real world setting. J Transl Med. 2018;16(1):129.
12. Falanga A, Marchetti M. Hemostatic biomarkers in cancer progression. Thromb Res. 2018;164 Suppl 1:S54-S61.
13. Howlader N, Noone AM, Krapcho M et al. SEER cancer statistics review, 1975–2013. Bethesda, MD: National Cancer Institute. Available at http://seer.cancer.gov/csr/1975_2013/. 2016.
14. American cancer society. Breast Cancer Facts & Figures 2017-2018. Available at https://www.cancer.org/research/cancer-facts-statistics/breast-cancer-facts-figures.html.
15. Kennecke H, Yerushalmi R, Woods R, et al. Metastatic behavior of breast cancer subtypes. J Clin Oncol. 2010;28(20):3271-3277.
16. Colleoni M, Sun Z, Price KN, et al. Annual Hazard Rates of Recurrence for Breast Cancer During 24 Years of Follow-Up: Results From the International Breast Cancer Study Group Trials I to V. J Clin Oncol. 2016;34(9):927-935.
17. Gnant M, Harbeck N, Thomssen C. St. Gallen/Vienna 2017: A Brief Summary of the Consensus Discussion about Escalation and De-Escalation of Primary Breast Cancer Treatment. Breast Care (Basel). 2017;12(2):102-107.
18. National Comprehensive Cancer Network NCCN Clinical Practice Guidelines in Oncology: Breast Cancer. Fort Washington, PA: NCCN; 2017. Ver. 3.2017. Available at: https://www.nccn.org/professionals/physician_gls/pdf/breast.pdf. 2017.
19. Falanga A, Santoro A, Labianca R, et al. Hypercoagulation screening as an innovative tool for risk assessment, early diagnosis and prognosis in cancer: the HYPERCAN study. Thromb Res. 2016;140 Suppl 1:S55-59.
20. Wolff AC, Hammond ME, Hicks DG, et al. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. J Clin Oncol. 2013;31(31):3997-4013.
21. D'Alessio A, Marchetti M, Tartari CJ, et al. Long Term Low Molecular Weight Heparin Anticoagulant Therapy Modulates Thrombin Generation and D-dimer in Patients with Cancer and Venous Thromboembolism. Cancer Invest. 2017;35(7):490-499.
22. Marchetti M, Tartari CJ, Russo L, et al. Phospholipid-dependent procoagulant activity is highly expressed by circulating microparticles in patients with essential thrombocythemia. Am J Hematol. 2014;89(1):68-73.
23. Spiezia L, Campello E, Valle FD, Woodhams B, Simioni P. Factor VIIa-antithrombin complex: a possible new biomarker for activated coagulation. Clin Chem Lab Med. 2017;55(4):484-488.
24. Palumbo JS, Kombrinck KW, Drew AF, et al. Fibrinogen is an important determinant of the metastatic potential of circulating tumor cells. Blood. 2000;96(10):3302-3309.
25. Wen J, Yang Y, Ye F, et al. The preoperative plasma fibrinogen level is an independent prognostic factor for overall survival of breast cancer patients who underwent surgical treatment. Breast. 2015;24(6):745-750.
26. Krenn-Pilko S, Langsenlehner U, Stojakovic T, et al. An elevated preoperative plasma fibrinogen level is associated with poor disease-specific and overall survival in breast cancer patients. Breast. 2015;24(5):667-672.
27. Liu YL, Lu Q, Liang JW, et al. High plasma fibrinogen is correlated with poor response to trastuzumab treatment in HER2 positive breast cancer. Medicine. 2015;94(5):e481.
28. Dirix LY, Salgado R, Weytjens R, et al. Plasma fibrin D-dimer levels correlate with tumour volume, progression rate and survival in patients with metastatic breast cancer. Br J Cancer. 2002;86(3):389-395.
29. Rousseau A, Van Dreden P, Mbemba E, et al. Cancer cells BXPC3 and MCF7 differentially reverse the inhibition of thrombin generation by apixaban, fondaparinux and enoxaparin. Thromb Res. 2015;136(6):1273-1279.
30. Rousseau A, Larsen AK, Van Dreden P, et al. Differential contribution of tissue factor and Factor XII to thrombin generation triggered by breast and pancreatic cancer cells. Int J Oncol. 2017;51(6):1747-1756.
31. Mego M, Zuo Z, Gao H, et al. Circulating tumour cells are linked to plasma D-dimer levels in patients with metastatic breast cancer. Thromb Haemost. 2015;113(3):593-598.
32. Pradhan S, Hassani I, Seeto WJ, Lipke EA. PEG-fibrinogen hydrogels for three-dimensional breast cancer cell culture. J Biomed Mater Res A. 2017;105(1):236-252.
33. Folsom AR, Delaney JA, Lutsey PL, et al. Associations of factor VIIIc, D-dimer, and plasmin-antiplasmin with incident cardiovascular disease and all-cause mortality. Am J Hematol. 2009;84(6):349-353.
34. Miller GJ, Bauer KA, Howarth DJ, et al. Increased incidence of neoplasia of the digestive tract in men with persistent activation of the coagulant pathway. J Thromb Haemost. 2004;2(12):2107-2114.
35. Silvestris N, Scartozzi M, Graziano G, et al. Basal and bevacizumab-based therapy-induced changes of lactate dehydrogenases and fibrinogen levels and clinical outcome of previously untreated metastatic colorectal cancer patients: a multicentric retrospective analysis. Expert Opin Biol Ther. 2015;15(2):155-162.
36. Tinholt M, Viken MK, Dahm AE, et al. Increased coagulation activity and genetic polymorphisms in the F5, F10 and EPCR genes are associated with breast cancer: a case-control study. BMC Cancer. 2014;14:845.
37. Fu S, Yun ZY, Cui MM, et al. Cancer antigen 15-3, platelet distribution width, and fibrinogen in combination to distinguish breast cancer from benign breast disease in non-conclusive mammography patients. Oncotarget. 2017;8(40):67829-67836.