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This study was declared exempt by the Wake Forest School of Medicine Institutional Review Board.

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Thrombophilic status may predict prognosis in patients with metastatic BRAFV600-mutated melanoma who are receiving BRAF inhibitors

To the Editor: Coagulation and cancer are strictly linked. There is increasing evidence that the activation of the coagulation cascade correlates with prognosis in preclinical models.[1] Melanoma cells are able to change the thrombophilic status (TS) of endothelial cells and microenvironment,[2,3] and contribute to angiogenesis and metastatic progression through their proteolytic properties.[4] We investigated the TS and hemostatic variables in patients with BRAFV600-mutated melanoma (MPs) with the hypothesis that circulating TS could correlate with the extension of disease and predict clinical response and outcome in metastatic MPs who were receiving BRAF inhibitors (BRAFis). Two cohorts of MPs consecutively observed from November 2011 to August 2014 at the Division of Oncology, Hospital Papa Giovanni XXIII, in Bergamo, Italy were enrolled in the study. The main cohort included 43 MPs who were prospectively diagnosed with locally advanced or metastatic melanoma and who were treated with BRAFis. The second cohort included 37 MPs with stage I to III melanoma. Forty healthy subjects (20 men and 20 women) were included as reference group for coagulation biomarker studies. All investigations were approved by the local ethical committee (Comitato di Bioetica, Hospital Papa Giovanni XXIII, Bergamo), and all study subjects gave informed written consent to the study. The routine hematologic assays, coagulation biomarker studies, and immunohistochemical analyses used for assessment of these melanoma tissues can be viewed in Supplemental Table IS and Fig 1S (available online at http://www.jaad.org).

Levels of D-dimer in the group of MPs were significantly ($P < .01$) higher compared to healthy subjects. By using a logistic model, patients with higher D-dimer levels at enrollment into the study had a significantly lower probability to achieve an objective response (Table I).

Using multivariate analysis and after adjusting for age, sex, and tumor stage, high baseline levels of

Table I. Logistic model: objective response according to the circulating thrombophilic status in patients with BRAV600 melanoma receiving BRAF inhibitors

Variables	OR	95% CI	*P* value
ETP HEPES (for 500 increase)	1.547	0.791-3.025	.2025
Peak HEPES (for 50 increase)	1.991	0.982-4.037	.0562
ETP 1 pmol/L TF (for 500 increase)	0.891	0.373-2.127	.7954
Peak 1 pmol/L TF (for 50 increase)	0.860	0.610-1.211	.3868
ETP 5 pmol/L TF (for 500 increase)	0.821	0.356-1.894	.6437
Peak 5 pmol/L TF (for 50 increase)	0.738	0.471-1.155	.1838
Platelets (for 10 increase)	1.045	0.935-1.167	.4378
D-dimer (for 10 increase)	0.946	0.911-0.982	.0037
PPL (for 10 increase)	0.500	0.259-0.966	.0390

CI, Confidence interval; *ETP*, endogenous thrombin potential; *OR*, odds ratio; *PPL*, endogenous plasma phospholipids; *TF*, tissue factor.

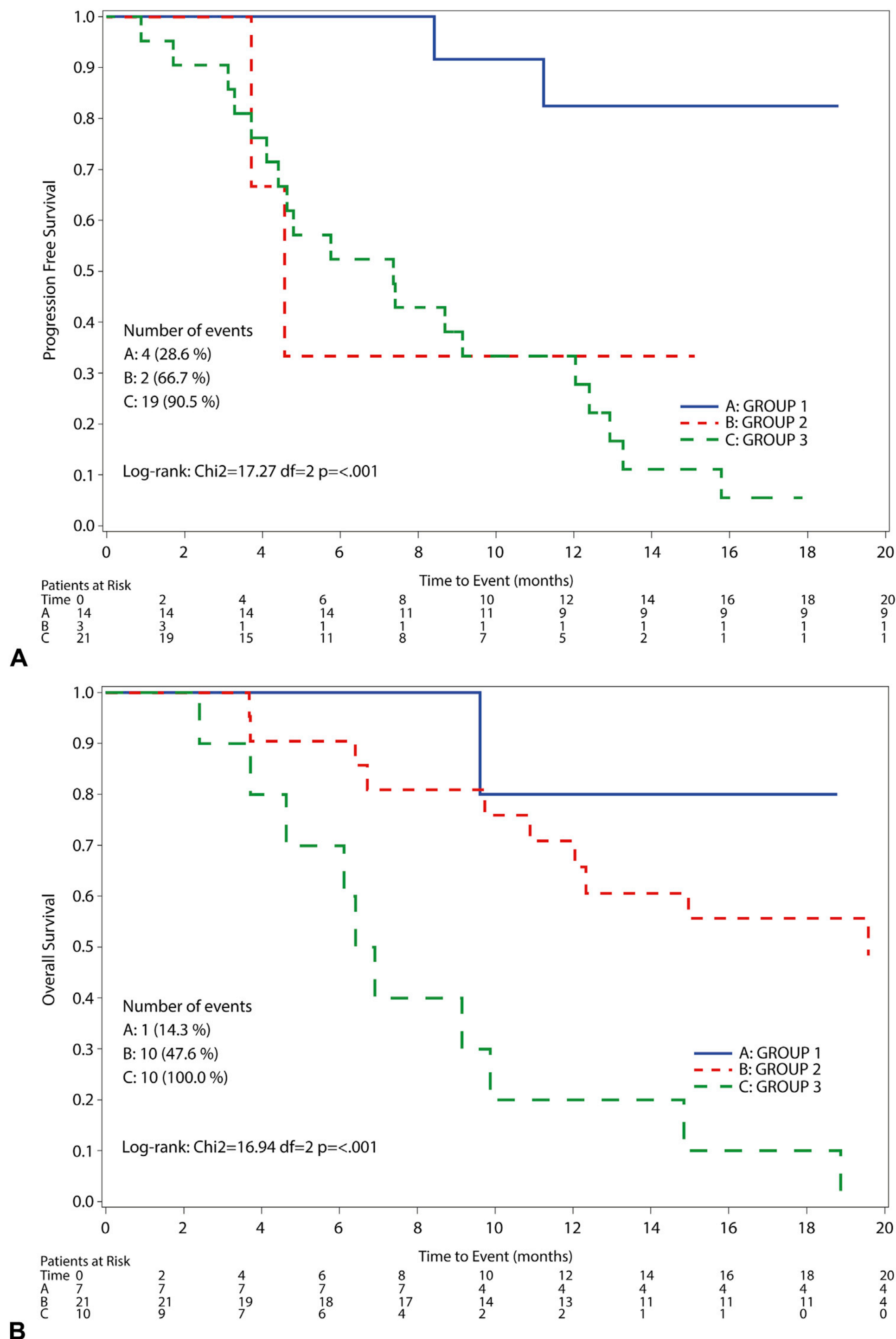

Fig 1. Kaplan–Meier curves for D-dimer levels. **A**, Progression-free survival curves. Group 1: D-dimer at baseline <161.5 and D-dimer <349 at subsequent visit. Group 2: D-dimer at baseline <161.5 and D-dimer ≥ 349 at subsequent visit. Group 3: D-dimer at baseline ≥ 161.5. **B**, Overall survival curves. Group 1: D-dimer at baseline <433.5 and D-dimer <149.5 at subsequent visit. Group 2: D-dimer at baseline <433.5 and D-dimer ≥ 149.5 at subsequent visit. Group 3: D-dimer at baseline ≥ 433.5.

D-dimer were associated with a statistically significantly poorer progression-free survival (PFS) and overall survival (OS; hazard ratio [HR] for 10-ng/mL increase = 1.01 [95% confidence interval {CI}, 1.00-1.02]; $P = .005$ for PFS and HR for 10-ng/mL increase = 1.01 [95% CI, 1.01-1.02], $P = .001$ for OS).

Based on the recursive partitioning algorithm, the best predictive marker of PFS was a D-dimer baseline level at the 161.5-ng/mL cutoff. Among subjects with D-dimer levels <161.5 ng/mL, the model split the subjects into groups depending on D-dimer measured at a subsequent visit (ie, 1 month after starting the BRAFi; cutoff: 349 ng/mL). According to this algorithm, a poorer PFS was found in patients with low baseline D-dimer levels and high D-dimer levels at a subsequent visit (HR = 9.23 [95% CI, 1.28-66.66]; $P = .0276$) and in patients with high D-dimer levels at baseline (HR = 12.18 [95% CI, 2.8-53.09]; $P = .0009$) when compared to patients with low D-dimer levels both at baseline and at a subsequent visit (Fig 1).

In addition, patients with high D-dimer levels at baseline had a poorer OS (HR = 13.40 [95% CI, 1.69-106.79]; $P = .0142$) when compared to patients with low D-dimer levels at baseline and at a subsequent visit (1 month after starting the BRAFi; Fig 1). Finally, tissue TS correlated with circulating TS (Supplemental Table IS and Fig 1). Therefore, among the investigated coagulation biomarkers, only D-dimer showed a statistically significant association with PFS and OS. Should our results be validated in an independent cohort, D-dimer should be incorporated as a stratification biomarker in future clinical trials.

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Distance of travel to phototherapy is associated with early nonadherence: A retrospective cohort study

To the Editor: Phototherapy is an effective management option for many dermatologic diseases.[1,2] Although adherence to a phototherapy regimen is essential for success, few studies have evaluated factors impacting adherence among phototherapy patients.[3] Our retrospective cohort study aimed to identify patient-related factors associated with early non-adherence to phototherapy, in which patients discontinue phototherapy for reasons unrelated to treatment efficacy.

A database of phototherapy patients at Brigham and Women's Hospital (BWH) was generated through a query of a registry of patients with medical documentation at the BWH Phototherapy Center from November 2009 to February 2015. The registry contains information on the total number of phototherapy treatments, treatment indication, reasons for declining treatment, distance to hospital, insurance, and copay, all of which were included in the analysis. All patients in the database had been consented and counseled regarding phototherapy at BWH during an office visit. This study was approved by the Partners Institutional Review Board.

Each patient record generated by the query was individually reviewed. Patients who consented to treatment after November 1, 2014 were excluded to

Supplemental materials

The white blood cell differential count, hematocrit, hemoglobin, red blood cell count, and platelet count were determined by a CELL-DYN Emerald hematology analyzer (Abbott Diagnostics, Lake Forest, IL).

Peripheral venous blood was collected by a team of experienced nurses from fasting patients in the morning (9-11 AM). For patients in the main cohort, blood samples were collected at the time of enrollment and after 2 and 4 weeks of treatment with BRAF inhibitors (BRAFis). For patients in the control cohort, blood samples were collected only once, at the time of enrollment.

After the application of a light tourniquet, venous blood was collected using a 21-gauge needle into 6-mL tubes containing 3.2% citrate (0.109 mol/L, 1:9 vol/vol; BD Vacutainer, Becton, Dickinson and Co, Franklin Lakes, NJ), after discarding the first 2 to 3 mL of blood. Anticoagulant was mixed during blood collection with gentle inversion of the tube. The tubes of blood were transported carefully to avoid unnecessary agitation. Blood samples were kept at room temperature (20-24°C) and processed within 2 hours of collection. Platelet-poor plasma was obtained by centrifugation at 2600 *g* for 15 minutes at 24°C, and platelet-free plasma (PFP) by further centrifugation of platelet-poor plasma at 2600 *g* for 15 minutes at 24°C, avoiding application of the centrifuge brake. Aliquots of PFP were snap-frozen in liquid nitrogen and stored at −80°C until use.

The duration of storage of plasma samples before testing ranged from 4 to 6 months. Aliquots of 1 mL of frozen samples of PFP were thawed for 10 minutes at 37°C before use or until completely thawed. The processing of blood samples and the assays for coagulation study (ie, thrombin generation potential, D-dimer and endogenous plasma phospholipids [PPL] assay) were performed at the Laboratory of the Hemostasis and Thrombosis Unit, Division of Immunohematology and Transfusion Medicine, Hospital Papa Giovanni XXIII, Bergamo.

Thrombin generation potential of plasma was measured by the calibrated automated thrombogram method (Thrombinoscope BV, Maastricht, The Netherlands) using reagents from Diagnostica Stago (Parsippany, NJ).[E1] Briefly, 80 μL of PFP were incubated for 10 minutes at 37°C with 20 μL of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (ie, in the absence of exogenous tissue factor [TF]), 1 pmol/L TF, or 5 pmol/L TF. Coagulation was started by the addition of $CaCl_2$ and a fluorogenic thrombin substrate (Z-Gly-Gly-Arg-AMC, Diagnostica Stago). Thrombin generation was measured using a Fluoroskan Ascent reader (Thermo Labsystems OY, Helsinki, Finland), and the following parameters were calculated using software (Thrombinoscope BV): peak height; the maximum concentration of thrombin generated, expressed in nmol/L of thrombin; and the endogenous thrombin potential, expressed in nmol/L thrombin*min. Thrombin generation assay was performed in batch with inclusion of internal controls (2 levels) for validation of the runs.

The STA-Procoag-PPL (Diagnostica Stago) assay was used to evaluate the influence of PPL on the activation of the coagulation cascade.[E2] The PPL assay measures clotting time, in the presence of factor Xa and $CaCl_2$, of a system in which all the factors are present in physiologic levels (supplied by procoagulant-PPL-depleted plasma), except the PPL, which are supplied by the plasma sample being tested. The assay was performed on an ACL TOP500 coagulometer (Instrumentation Laboratory, Lexington, MA). Results are expressed as coagulation time in seconds: the shorter the coagulation time, the higher the PPL activity. PPL assay was performed in batch with inclusion of positive and normal control samples, provided by the kit, for validation of the runs.

D-dimer levels were evaluated by the HemosIL D-dimer HS, an automated latex enhanced immunoassay for the quantitative determination of D-dimer levels, using ACL TOP500 coagulometer (both manufactured by Instrumentation Laboratory). Results were reported in ng/mL.

Metastatic and primary tumor samples were retrieved from matrix metalloproteinase-19 and -14, respectively. For the study, 4 μm-thick sections were cut from formalin-fixed, paraffin-embedded tissue samples and immunostained according to standard procedures. Briefly, antigen retrieval was performed by immersing the slides in a thermostat bath containing Epitope Retrieval Solution EDTA (pH 9.0; Dako Milan, Italy) for 20 minutes at 98°C, followed by cooling for 20 minutes at room temperature. After blocking with UltraV Block (Thermo Scientific, Milan, Italy), sections were incubated at 4°C overnight with anti-TF (FL-295; 1:50 Santa Cruz Biotechnology Inc, Dallas, TX) and antithrombin receptor (ATAP2; protease-activated receptor-1, 1:25, Santa Cruz Biotechnology Inc) antibodies. Signal was detected by using UltraVision Quanto Detection System AP, and the bound antibody was visualized using a permanent Fast Red as chromogen (Thermo Scientific). Nuclei were counterstained with Mayer hematoxylin. A negative control was performed by substituting the primary antibody with a nonimmune serum at the same concentration.

Stained tumor sections were initially evaluated by a dermatopathologist (D. M.) and then independently reviewed by a second pathologist (C. S.), both blinded to clinical outcome. Discrepancies in interpretation or scoring (<5% of cases) were resolved by consensus conference at a double-headed microscope. Staining intensity was scored as follows: 0 (no staining), 1+ (weak), 2+ (moderate), or 3+ (strong). Samples were categorized as a continuous variable and correlated with circulating TS using the Spearman correlation coefficient. Cytoplasmic cellular staining had to unequivocally exceed background to be considered positive.

Odd ratio was defined as the proportion of patients with complete or partial response. Progression-free survival (PFS) was defined as the time from starting BRAFi treatment to the first appearance of progressive disease or death from any cause. Patients known to be alive and without progressive disease at the time of analysis were censored at their last available disease assessment. PFS and OR were based on the European Organization for Research and Treatment of Cancer's Response Evaluation Criteria in Solid Tumors guidelines (v 1.1). Overall survival (OS) was defined as the time from starting BRAFi to the date of death from any cause or the date of the last follow-up. The thrombophilic status of metastatic BRAFV600-mutated melanoma and patients with limited disease radically resected were compared by means of Kruskal-Wallis test. Correlation between TF and protease-activated receptor-1 immunohistochemical overexpression and circulating thrombophilic status was described using Spearman correlation coefficient.

A logistic regression model was used to assess the effect of the thrombophilic status on OR rate. Time-to-event data were described by the Kaplan-Meier curves. Cox proportional hazard models were used for univariate and multivariate analysis—adjusted for sex, age, and tumor stage—to estimate and test the thrombophilic status for its association with OS and PFS. Results were expressed for logistic models as odds ratios and for Cox models as hazard ratios and their relative 95% confidence intervals.

To identify a subset of patients with different prognosis defined by the thrombophilic status at baseline and 30 days after baseline, a recursive partitioning analysis was used both for response and survival. Once the classification tree was defined, a Cox proportional hazard regression model was used to estimate the hazard of progression/death associated with risk groups defined using cut-points suggested by the recursive partitioning.

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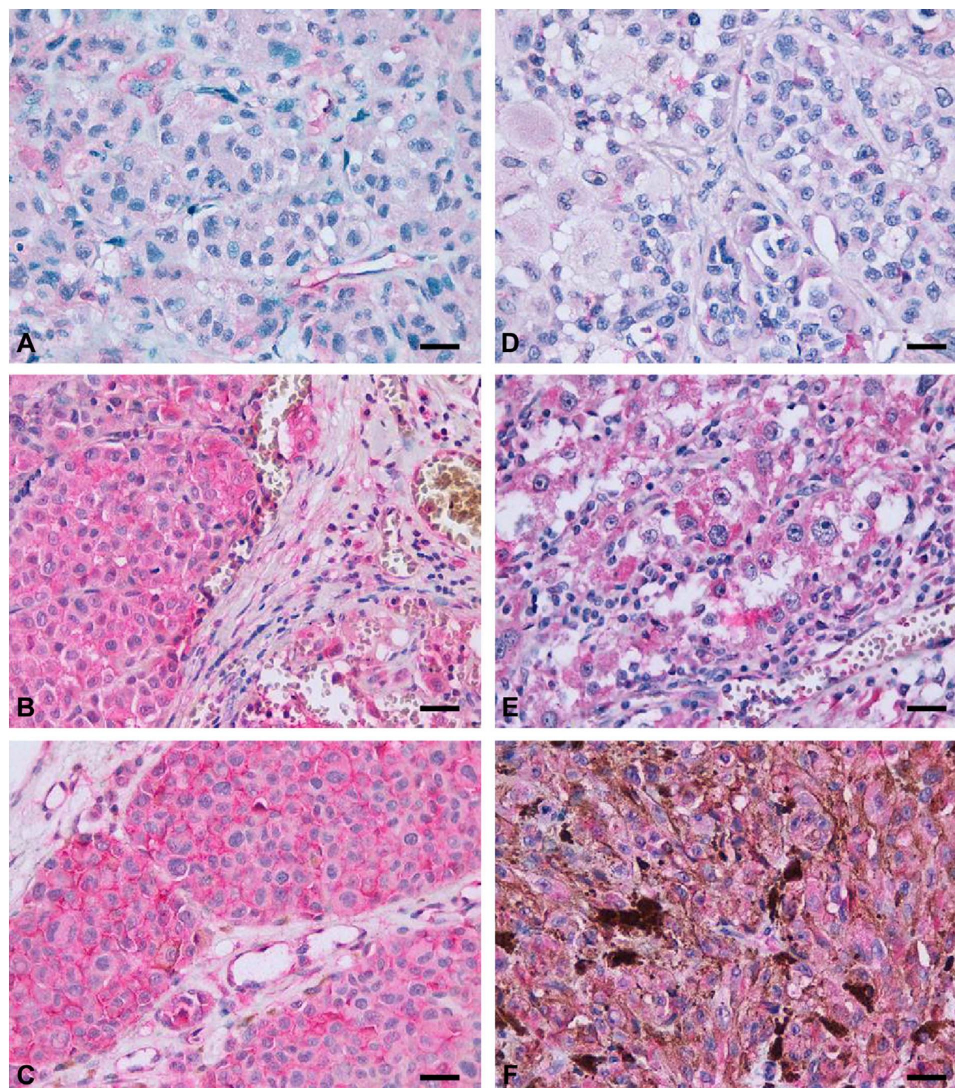

Supplemental Fig 1S. Protease-activated receptor (PAR)-1 and tissue factor (TF) immunohistochemical expression in melanoma tissues. **A**, Weak PAR-1 staining in melanoma cells. **B**, Q14 PAR-1 is strongly and diffusely expressed in melanoma cells. Note that endothelial cells decorating blood vessels express PAR-1 protein. **C**, Example of PAR-1 staining in melanoma Q15 tissues. Both cytoplasmic and membranous positivity is observed in tumor cells; scattered stromal fibroblasts and macrophages are also positive. **D**,Weak and focal TF immunostaining in scattered melanoma cells. **E**, Aggregates of melanoma cells with strong TF cytoplasmic positivity. TF is also expressed in intratumoral flattened vascular endothelial cells. **F**, Strong TF cytoplasmic immunostain (*red*) in highly pigmented melanoma cells, partly colocalizes with melanin pigment (*brown*). Note the presence of abundant extracellular granular melanin depositions within the tumor. Scale bar = 50 μm. (**A** to **F**, Original magnification: ×40.)

Supplemental Table IS. Correlation between immunohistochemical tissue factor/protease-activated receptor-1 expression and circulating thrombophilic status at baseline

	Correlation with IHC TF		Correlation with IHC PAR-1	
	Spearman correlation coefficient	*P* value	Spearman correlation coefficient	*P* value
T0_ hepes_etp	0.11	.590	0.26	.185
T0_ hepes_peak	0.06	.761	0.02	.925
T0_TF1_etp	0.22	.261	0.48	.010
T0_TF1_peak	-0.03	.867	0.06	.758
T0_TF5_etp	0.42	.025	0.47	.012
T0_TF5_peak	0.34	.074	0.22	.256
T0_plt_K_uL	-0.03	.882	0.13	.507
D-dimer_T0	0.15	.445	0.39	.041
ppl_T0	0.03	.894	0.27	.157

etp, Endogenous thrombin potential; *IHC*, immunohistochemical; *PAR*, protease-activated receptor; *ppl*, endogenous plasma phospholipids; *plt*, platelet; *T0*, time of enrollment; *TF*, tissue factor.