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TO THE EDITOR:

Co-occurrence of *DNMT3A*, *NPM1*, *FLT3* mutations identifies a subset of acute myeloid leukemia with adverse prognosis

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The impact of mutations in DNA methyltransferase 3 α (*DNMT3A*) at diagnosis as a prognostic marker in acute myeloid leukemia (AML) has been contradictory so far.[1-3] These discrepancies most likely arise from differences of therapeutic protocols used. Most important, few if any studies have evaluated the clinical importance of the 3-way co-occurrence of mutations affecting *DNMT3A*, nucleophosmin (*NPM1*), and fms-like tyrosine kinase 3 (*FLT3*) genes (in particular *FLT3* length mutations or *FLT3*–internal tandem duplication (ITD) for "internal tandem duplication") in patients treated outside of well-controlled clinical trials, a real-life setting that represents most low- and middle-income countries. Hence, we assessed the frequency and clinical impact of *DNMT3A* mutations and the co-occurrence of *DNMT3A*/*NPM1*/*FLT3*-ITD mutations on treatment outcomes of nonselected AML patients, followed from June 2003 to January 2019 at 5 Brazilian reference centers specialized on AML treatment.

Bone marrow samples from 507 consecutive patients with de novo AML (median age, 51 years; range, 18-94 years; 47% male) were obtained at diagnosis. Patients with acute promyelocytic leukemia, therapy-related AML, or with a previous history of myelodysplastic syndrome were excluded. Details for treatment protocols can be found in the supplemental Methods, available on the *Blood* Web site. The study adhered to the tenets of the Declaration of Helsinki and informed consents were obtained from all patients or their relatives. The local Research Ethics Board of each participating center approved the study.

The *DNMT3A* and *NPM1* mutations were analyzed by standard sequencing techniques. Details are described in supplemental Methods. Screening for *FLT3*-ITD mutations was performed by polymerase chain reaction according to the method of Kiyoi et al.[4] In parallel, we explored the *FLT3* allelic ratio in patients with the *FLT3*-ITD mutated status. *FLT3*-tyrosine kinase domain mutations were not evaluated in this study. Because most of the *DNMT3A* mutations in myeloid neoplasms occur at exon 23, with a significant enrichment for mutations at codon R882,[5,6] we evaluated the mutational and phenotypic profile of patients harboring *DNMT3A*-R882 and non-R882 mutations using The Cancer Genome Atlas database (TCGA) data set.[5] We observed that *NPM1* and *FLT3*-ITD mutations were significantly enriched in *DNMT3A*-R882 when compared with non-R882 mutations or *DNMT3A* wild type (supplemental Figure 1). Therefore, based on its biological and clinical significance[3,7-10] and our own experience, screening for *DNMT3A* mutations was restricted to the codon R882.

DNMT3A-R882 mutations were detected in 64 of 507 patients (13%), most of them identified as R882H (49 of 64; 76%), followed by R882C (12 of 64; 19%), and R882P (3 of 64; 5%). Samples without detectable *DNMT3A*-R882 mutations or carrying single-nucleotide polymorphisms are referenced here as "*DNMT3A* nonmutated." To decide which variables to include in the multivariate Cox proportional hazard model, we performed a backward elimination analysis using the Akaike Information Criteria (AIC) as fitness measure and getting the best-fitted model (supplemental Table 1). The basis (indispensable) variable used was cytogenetic risk stratification. Treatment-related variables were not included in the multivariate model due to the biased nature of a retrospective study.

The clinical and baseline characteristics are summarized in Table 1. Overall, 302 of 507 patients (60%) achieved complete

Table 1. Clinical and baseline characteristics according to the *DNMT3A* mutational status and according to the *DNMT3A/NPM1/FLT3*-ITD mutations

Characteristics	All patients, no. (%)	*DNMT3A* mutated, no. (%)	*DNMT3A* nonmutated, no. (%)	*P**	Triple-mutated, no. (%)	Non–triple-mutated, no. (%)	*P**
Age, y				<.001†			.003†
18-40	157 (31)	6 (9.4)	151 (34.1)		3 (8.6)	154 (32.6)	
41-60	185 (36.5)	34 (53.1)	151 (34.1)		21 (60)	164 (34.7)	
60 and older	165 (32.5)	24 (37.5)	141 (31.8)		11 (31.4)	154 (32.6)	
Median (range)	50.6 (18, 93.8)	54.4 (27, 91)	49.2 (18, 93.8)	.003†	54.8 (27, 77.5)	49.9 (18, 93.8)	.08
Sex				.033†			.292
Female	269 (53.1)	42 (66.6)	227 (51.2)		22 (62.9)	247 (52.3)	
Male	238 (46.9)	22 (34.4)	216 (48.8)		13 (37.1)	225 (47.7)	
FAB subtype				.756			.928
M0	22 (4.7)	2 (3.4)	20 (4.9)		1 (3.1)	21 (4.8)	
M1	90 (19.3)	9 (15.3)	81 (19.9)		7 (21.9)	83 (19.1)	
M2	150 (32.1)	17 (28.8)	133 (32.6)		12 (37.5)	138 (31.7)	
M4	150 (32.1)	23 (39)	127 (31.1)		10 (31.3)	140 (32.2)	
M5	41 (8.8)	7 (11.9)	34 (8.3)		2	39 (9)	
M6	10 (2.1)	1 (1.7)	9 (2.2)			10 (2.3)	
M7	4 (0.9)		4 (1)			4 (0.9)	
Missing data	40	5	35		3	37	
Cytogenetic risk stratification‡				<.001†			.006†
Favorable	69 (19.5)		69 (22.6)			69 (21.1)	
Intermediate	227 (64.3)	43 (89.6)	184 (60.3)		24 (92.3)	203 (62.1)	
Adverse	57 (16.1)	5 (10.4)	52 (17)		2 (7.7)	55 (16.8)	
Missing data§	154	16	138			145	
***FLT3*-ITD**				<.001†			
Mutated	134 (26.4)	42 (65.6)	94 (20.8)				
Nonmutated	373 (73.1)	22 (34.4)	351 (79.2)				
NPM1				<.001†			
Mutated	145 (28.6)	44 (68.8)	103 (22.8)				
Nonmutated	362 (71.4)	20 (31.3)	342 (77.2)				
BM blasts, median (range), %	67 (20, 100)	74 (20, 96)	66 (10, 100)	.143	77 (21, 96)	66 (20, 100)	.117
Leukocyte count, median (range), $\times 10^9$/L	27.3 (0.28, 790)	61 (0.28, 435)	25.6 (0.6, 790)	.009†	67 (0.86, 435)	26 (0.28, 790)	.001†
Platelet count, median (range), $\times 10^9$/L	45 (3, 600)	59 (5, 404)	44 (3, 600)	.818	60 (10, 196)	45 (3, 600)	.763
Hg, median (range), g/dL	8.1 (3, 16.2)	8 (3.1, 11.6)	8.1 (3, 16.2)	.727	7.8 (3.9, 13.2)	8.1 (3, 16.2)	.534
LDH level, median (range), U/l	682.5 (116, 11 722)	775 (136, 5289)	662 (116, 11 722)	.188	767 (136, 2789)	672 (116, 11 722)	.607
Complete remission, %	60	53	60	.786	62	59	.725
OS, % (95% CI)	20 (16, 24)	9 (3, 18)	22 (17, 27)	.003†	3 (0-14)	22 (17, 26)	.011†
DFS, % (95% CI)	38 (31, 45)	19 (7, 34)	42 (34, 49)	<.001†	4 (0-19)	42 (35, 50)	<.001†
CIR, % (95% CI)	54 (48, 60)	72 (58, 86)	50 (42, 57)	<.001†	85 (71, 98)	50 (43, 56)	<.001†

Values are shown as number (percentage), unless otherwise specified in the row headings as median (range) or percentage (95% CI).

BM, bone marrow; CIR, cumulative incidence of relapse; DFS, disease-free survival; FAB, French-American-British; Hg, hemoglobin; LDH, lactate dehydrogenase; OS, overall survival

*Missing values were excluded for the calculation of *P* values.

†Indicates statistically significant differences.

‡The cytogenetic risk groups were defined according to Medical Research Council criteria.[24]

§Material not available or no metaphases detected.

remission (CR), of whom 37 of 64 (53%) and 265 of 443 (60%) were assigned to the *DNMT3A*-mutated and *DNMT3A*-nonmutated groups, respectively ($P = .786$). The median follow-up among survivals was 39 months (95% confidence interval [CI], 26-53 months). Patients with *DNMT3A* mutations had significantly lower 5-year overall survival (OS) (9%; 95% CI, 3% to 18%) compared with those without *DNMT3A* mutations (22%; 95% CI, 17% to 27%) ($P = .0035$) (Figure 1A). The best-fitted multivariate Cox proportional hazards model for OS was age (>60 years old), leukocyte counts ($>50 \times 10^9$/L), *DNMT3A* status, and cytogenetic risk stratification. This model showed that *DNMT3A* mutational status was independently associated with poor OS (hazard ratio [HR], 1.4; 95% CI, 1.01-2.1; $P = .04$) (Figure 1G). Of the 302 patients who achieved CR, 133 patients (44%) relapsed. Considering nonrelapse death as a competing cause of failure, the 5-year cumulative incidence of relapse (CIR) rate was 54% (95% CI, 48% to 60%). CIR rates for patients assigned to the *DNMT3A* mutated and nonmutated groups were 72% (95% CI, 58% to 86%) and 50% (95% CI, 42% to 57%), respectively ($P < .0001$; Figure 1B). Patients with *DNMT3A* mutations had a significantly lower disease-free survival (DFS) rate (19%; 95% CI, 7% to 34%) in comparison with patients without *DNMT3A* mutations (42%; 95% CI, 34% to 49%) ($P < .0001$; Figure 1C).

Considering its clinical[11] and biological[9,10,12] relevance and the strikingly high frequency of 3-way co-occurrence mutations in AML,[5] we evaluated the clinical relevance of the co-occurrence *DNMT3A/NPM1/FLT3*-ITD in a real-life setting. The frequency of triple-mutated patients in our cohort (35 of 507; 7%) was very similar to other studies.[5,11] Table 1 summarized the main baseline and clinical characteristics. Triple-mutated patients had significantly lower OS (4%; 95% CI, 2% to 15% vs 21%, 95% CI, 17% to 26%; $P = .011$), higher CIR rate (85%; 95% CI, 71% to 98% vs 50%, 95% CI, 43% to 56%; $P < .0001$), and lower DFS rates (5%; 95% CI, 1% to 20% vs 42%, 95% CI, 34% to 49%; $P < .0001$) compared with non–triple-mutated patients (Figure 1D-F). In multivariate analysis, the lowest AIC for DFS was achieved when *FLT3*-ITD and *NPM1* status, triple-mutant AML, and cytogenetic risk stratification were included. In this model, triple-mutant AML presented a higher relapse risk (HR, 2.49, 95% CI, 1.3-5.5; $P = .02$) (Figure 1H). The co-occurrence of *DNMT3A/NPM1/FLT3*-ITD mutations had no impact on CR achievement ($P = .725$).

To validate our findings, we took advantage of 2 publicly available AML data sets (TCGA[5] and Gene Expression Omnibus,[13] GEO; www.ncbi.nlm.nih.gov/geo, accession number: GSE6891). For survival analysis, only de novo AML patients submitted to intensive therapy were included. *DNMT3A*-R882 mutations were reported in 22 of 142 (15%) and 64 of 479 patients (13%) included in the TCGA and GEO cohorts, respectively. *DNMT3A* mutations were significantly associated with poor OS (HR, 1.65; 95% CI, 1.1-2.7; $P = .046$), but not with DFS (HR, 1.64; 95% CI, 0.95-2.83; $P = .072$) in TCGA patients. In contrast, *DNMT3A* mutational status had no impact on OS (HR, 1.22; 95% CI, 0.95-1.58; $P = .115$), but was significantly associated with poor DFS for patients included in the GEO cohort (HR, 1.47; 95% CI, 1.1-2; $P = .015$). The co-occurrence of *DNMT3A/NPM1/FLT3*-ITD mutations was significantly associated with poor DFS (HR, 1.3, 95% CI, 1.1-1.68; $P = .038$), but not with OS (HR, 1.16, 95% CI, 0.92-1.48; $P = .19$) in TCGA patients. Similar results were obtained for GEO patients: DFS (HR, 2.02; 95% CI, 1.3-3.17; $P = .002$) and OS (HR, 1.5; 95% CI, 1.01-2.24; $P = .48$).

In summary, we demonstrated that *DNMT3A* mutations might be useful for AML outcome prediction, although results remain conflicting. Several groups speculate that patient-related features and differences in treatment protocols could explain the contradictory results.[1-3,7] In fact, the functional genomic landscape of AML suggest that the response to drugs is specific to combinatorial mutational events.[14] Therefore, the impact of *DNMT3A* mutations in clinical decision-making remains disputable. Importantly, we restricted the screening for *DNMT3A* mutations to codon R882, which probably explains our lower rate of *DNMT3A* mutations in comparison with other studies.[1,2] Although, one may argue that restricting our analysis to the codon R882 may limit our study, it is important to notice that current literature supports the idea that only *DNMT3A*-R882 mutations contribute to prognostication[3,7,15] and pathophysiology of AML.[9,10,16] For instance, non-R882 mutations found on the DNMT3A enzyme affecting different domains showed few biochemical consequences.[17] Furthermore, *DNMT3A*-R882 mutations (but not non-R882 mutations) may have an impact on clonal hematopoiesis.[18-20] Finally, a remarkable difference in DNA methylation signatures between samples with *DNMT3A*-R882 and non-R882 mutations has been reported,[8] suggesting that these mutations should not be pooled together. It is possible that mutations in different *DNMT3A* domains lead to different neomorphic functions, resulting in pathogenetic variabilities. Nevertheless, whether *DNMT3A* non-R882 mutations harbor biological or clinical importance in AML requires further studies.

Yet, the co-occurrence *DNMT3A/NPM1/FLT3*-ITD mutations is more representative regarding the biology of the disease and may constitute a more robust strategy for outcome prediction in AML. Reasons for such robustness remain to be elucidated, although functional studies have identified a link between the co-occurrence of *DNMT3A/NPM1/FLT3*-ITD mutations and AML resistance to anthracycline based-chemotherapy.[9] In agreement, triple-mutated AML showed a unique differentiation response to the FLT3 inhibitor AC220[21] and increased sensitivity to the Food and Drug Administration-approved drug ibrutinib.[14] More recently, transcriptomic and immunophenotypic data describe triple-mutant blasts to be associated with high leukemia stem cell frequency, and synergistic upregulation of specific leukemia stem cell regulator.[12] Finally, specific DNA methylation signatures were characterized in triple-mutated patients.[22] We speculate that these findings may help us to better understand the poor prognosis of this specific AML subtype.

This is one of the first studies to describe the prognostic importance of *DNMT3A* mutations and the co-occurrence of *DNMT3A/NPM1/FLT3*-ITD mutations involving consecutive nonselected patients treated outside well-controlled clinical trials. As such, any conclusion drawn from this "real-world" data should be interpreted with caution. In contrast to previous studies that draw their conclusions based on a uniformly treated patient population,[11] our study is confronted with many variables (including drug unavailability, risk-adapted treatment, comorbidities and time from diagnosis to treatment initiation) that cannot be fully controlled. Nevertheless, we are firm believers that clinical data obtained from real-world studies, if properly

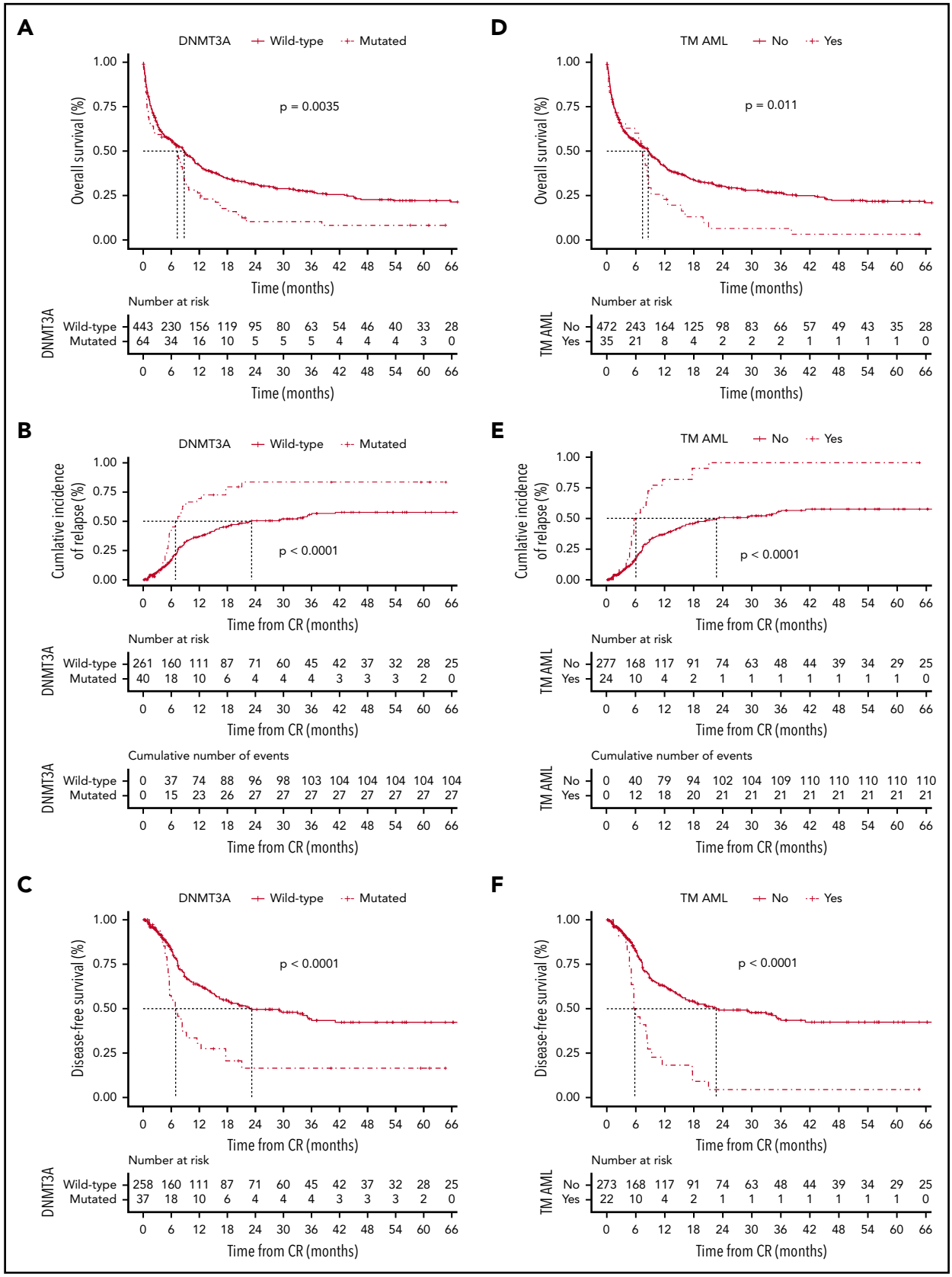

Figure 1. Probability according to *DNMT3A* mutations. The probability of overall survival (A), cumulative incidence of relapse (B), and disease-free survival (C) in patients with AML according to *DNMT3A* mutations. Overall survival (D), cumulative incidence of relapse (E), and disease-free survival (F) in triple-mutated (TM-AML) patients. Survival curves were estimated using the Kaplan-Meier method, and the log-rank test was used for comparison. Cumulative incidence curves for nonrelapse death and relapse with or without death were constructed to reflect time to relapse and time to nonrelapse death as competing risks. Time to relapse and time to nonrelapse death were measured from the date of complete remission. Multivariate Cox model for overall survival (G) and disease-free survival (H).

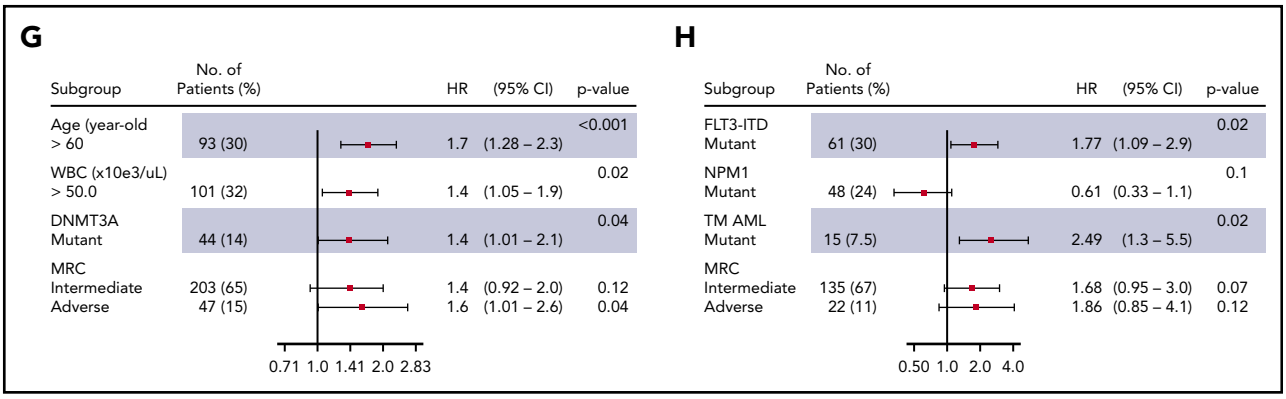

Figure 1. (Continued).

registered and with guaranteed accessibility, can provide representative evidence from routine practice about the clinical outcomes of patients, without the classical selection criteria of clinical trials.[23] Most importantly, these findings can serve as a more reliable basis for extrapolation of data to understudied populations.

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Authorship

Contribution: M.F.B. performed experiments, collected, analyzed, and interpreted data, and drafted the manuscript; D.R.S. and A.R.L.-A. performed the statistical analyses, interpreted the data, and drafted the manuscript; A.S.L., M.-R.P.-B., D.R.S., J.L.C.-S., D.A.P.-M., I.W., P.L.F.-N., L.Q., A.C., M.M.O., M.M.L., R.A.d.A., P.d.M.C., B.K.D., I.B., V.R., E.M.R., F.T., S.T.S., E.I.B., and M.A.B. obtained patient samples, updated the clinical data, collected, analyzed, and interpreted data, and drafted the manuscript; M.F.B. and A.R.L.-A. conceived and designed the study and reviewed the manuscript; and A.R.L.-A. gave final approval of the version to be submitted.

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Footnote

The online version of this article contains a data supplement.

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TO THE EDITOR:

Ticagrelor causes false-negative functional tests for heparin-induced thrombocytopenia

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Ticagrelor, a reversible inhibitor of the adenosine 5′-diphosphate (ADP) receptor $P2Y_{12}$, is recommended as a first-line $P2Y_{12}$ receptor antagonist after coronary interventions or acute coronary syndrome.[1,2] In contrast to the irreversible inhibitors aspirin, clopidogrel, and prasugrel, ticagrelor binds to the ADP receptor reversibly, and the drug is present at high concentrations in plasma (227-770 ng/mL).[3]

Heparin-induced thrombocytopenia (HIT) is a serious adverse reaction that is caused by platelet-activating anti–platelet factor 4 (PF4)/heparin antibodies and leads to an increased risk for thrombosis[4]. The most widely used system for clinical diagnosis is the 4Ts score (0-8 points).[5] The diagnosis is confirmed by laboratory tests. Antigen tests for anti-PF4/heparin antibodies[6] have a high sensitivity[7]; however, only a subset of anti-PF4/heparin antibodies that activate platelets is clinically relevant. In some patient populations, such as patients undergoing cardiac surgery, this accounts for <25% of anti-PF4/heparin antibodies detected by antigen tests.[8] Clinically relevant anti-PF4/heparin antibodies typically activate washed platelets of

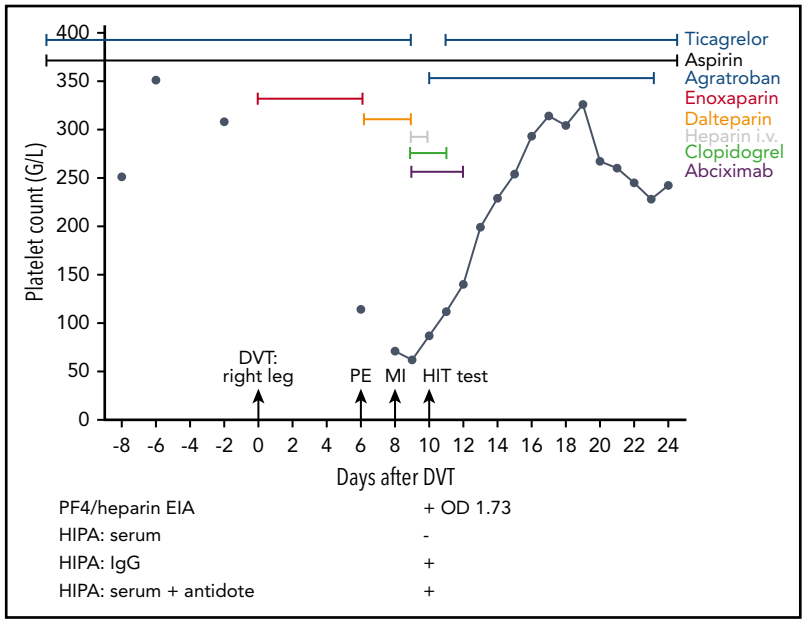

Figure 1. At day −20, the 59-year-old female patient had an ST-segment elevation myocardial infarction and cardiac arrest. She required cardiopulmonary resuscitation during which a bolus of unfractionated heparin was given, followed by placement of 3 drug-eluting stents in the right coronary artery. Resuscitation was complicated by liver laceration requiring surgery. No thrombosis prophylaxis was given because of the anticipated risk of bleeding after liver injury. Therapeutic-dose low molecular weight heparin was started at day 0 upon diagnosis of a deep vein thrombosis (DVT) of the right leg (enoxaparin; 1 mg/kg body weight twice a day subcutaneously). Severe pulmonary embolism (PE) occurred at day 6, and the platelet count had decreased to 114 per microliter. Anticoagulation was switched to dalteparin (5000 aFXaU twice a day subcutaneously). Three days later, the patient was transferred to the hospital of 1 of the authors (C.P.) with a further myocardial infarction (MI) due to in-stent thrombosis of the right coronary artery. Heparin-induced thrombocytopenia was suspected; the 4T score was 7. All heparins were stopped, and anticoagulation was switched to argatroban. In addition, abciximab and clopidogrel were given. The patient was discharged at day 25 and has remained stable. "HIT test" denotes the time point at which a blood sample was taken for HIT diagnosis; test results are shown below the graph. The PF4/heparin EIA was strongly positive (OD 1.73), but the HIPA was negative.