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# *DNMT3A/TET2/ASXL1* Mutations are an Age-independent Thrombotic Risk Factor in Polycythemia Vera Patients: An Observational Study

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## Abstract

**Background** Polycythemia vera (PV) patients are classified as high or low thrombotic risk based on age and prior history of thrombosis. Despite adherence to treatment recommendations, vascular events remain frequent, leading us to question whether thrombotic risk stratification could be improved. We previously reported an association between thrombotic events and mutations in DTA genes (*DNMT3A, TET2,* and *ASXL1*). The objective of this study was to confirm this observation in a larger series of PV patients.

**Methods** PV patients with a minimum follow-up of 3 years were recruited from 8 European centers. Medical history was searched for thrombotic event recorded at any time and next-generation sequencing carried out with a myeloid panel. Multivariable logistic regression evaluated the impact of variables on thrombotic risk. Kaplan–Meier thrombosis-free survival curves were compared by the log rank test. Associations in the total cohort were confirmed in a case–control study to exclude selection bias.

**Keywords**
- myeloproliferative neoplasm
- cardiovascular event
- next-generation sequencing
- prognosis
- CHIP

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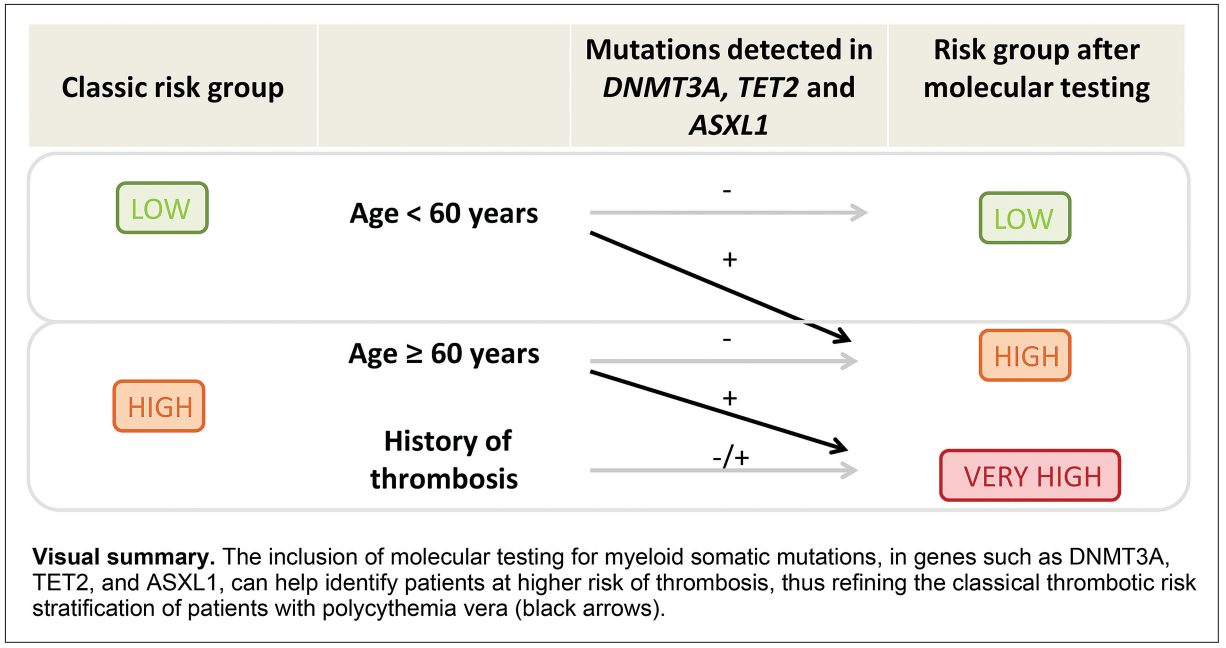


**Visual summary.** The inclusion of molecular testing for myeloid somatic mutations, in genes such as DNMT3A, TET2, and ASXL1, can help identify patients at higher risk of thrombosis, thus refining the classical thrombotic risk stratification of patients with polycythemia vera (black arrows).

**Results** Of the 136 patients recruited, 74 (56.1%) had a thrombotic event, with an incidence density of 2.83/100 person-years. In multivariable analysis, DTA mutation was a risk factor for thrombotic event, being predictive for shorter thrombosis-free survival in the whole cohort ($p = 0.007$), as well as in low-risk patients ($p = 0.039$) and older patients ($p = 0.009$), but not for patients with a prediagnostic event. A gender- and age-matched case–control study confirmed the increased risk of thrombotic event for PV patients with a DTA mutation.

**Conclusion** Our results support the use of molecular testing at diagnosis to help predict which PV patients are at higher risk of developing thrombosis.

## Background

The myeloproliferative neoplasms (MPN) are a group of diseases produced by a clonal expansion of an abnormal hematopoietic stem cell and characterized by a propensity for cardiovascular events. Polycythemia vera (PV) has the highest risk of cardiovascular events, affecting 34 to 39% of patients,[1] including those classified as low risk according to the current European LeukemiaNet recommendations.[2] Such events are often not only the cause for MPN diagnosis, but also occur frequently during follow-up despite strict adherence to the prophylactic recommendations set out in international guidelines.[3] Indeed, annual incidence rates of 3.6 events/100 person-years were reported in patients aged 70 years receiving hydroxyurea treatment.[4]

Carriers of clonal hematopoiesis of indeterminate potential (CHIP), a natural phenomenon associated with older age, are at risk of progression to hematological disease (~0.5–1% per year).[5,6] Moreover, patients with CHIP have a 40% higher mortality due to an increased cardiovascular predisposition, independently of classical risk factors such as diabetes, high cholesterol, etc.[7,8]

In the last few decades, the diagnosis, treatment, and follow-up of PV patients has changed dramatically as a result of our increased understanding of the underlying pathogenic mechanisms. However, the same risk stratification has continued in use for over 20 years.[9–11]

In a previous study we reported an association between the presence of mutations in DTA genes (*DNMT3A*, *TET2*, and *ASXL1*, the most frequently mutated genes in CHIP) and vascular events in patients with PV.[12] We aimed to confirm this observation in a larger series of PV patients from various European centers.

## Methods

### Patients

Patients aged ≥ 18 years with a confirmed diagnosis of PV according to the World Health Organization (WHO) criteria and a minimum follow-up of 3 years were recruited from seven hospitals in Spain and one in Poland. Patients with a diagnosis

 

prior to 2016 were reevaluated according to the revised WHO criteria.[13] All PV patients included in this study had either hemoglobin or hematocrit above the revised thresholds (>16.5g/dL or >49% males, >16 g/dL or >48% females, respectively) and a compatible bone marrow biopsy. Most patients also presented with a *JAK2* mutation (94%) and a subnormal serum erythropoietin level (minor criterion). Medical records were searched for thrombotic event recorded at any time in the medical history as well as information on cardiovascular risk factors (CVRF), including hypertension.

For the case–control study, two homogeneous groups of gender- and age-matched PV patients were formed. Patients with thrombotic events in their medical history (case) were identified from eligible patients. Then patients without thrombotic events (control) in their medical history were taken and gender- and age-matched with the cases. Cases that did not age-match with a control were removed from the case–control analysis.

### Next-Generation Sequencing

Next-generation sequencing (MiSeq, Illumina) was performed on 200 ng genomic DNA extracted from peripheral blood at diagnosis of 136 PV patients with the 30-gene panel Myeloid Solution (SOPHiA Genetics). Only variants with an allelic frequency (VAF) ≥2% and annotated as pathogenic or probably pathogenic were considered.[5]

### Statistical Analysis

Data normality was determined using the Shapiro–Wilk test. Differences between group means were compared using the Student's paired *t*-test of equal variance for unpaired samples. Incidence density (events/person-year) of thrombotic events was calculated by dividing the total number of thrombotic events by the total time of follow-up from diagnosis of all patients. Chi-squared univariable analyses were used to determine associations with thrombotic event and the level of significance calculated by the Fisher's exact test. Logistic regression multivariable analysis evaluated the impact of the independent variables on thrombotic risk and was only carried out for variables that reached statistical significance in the univariable analysis. Thrombosis-free survival (TFS) was measured from the time of diagnosis until the thrombotic event or date of last follow-up. Survival probabilities were estimated using the Kaplan–Meier method and the log rank test was used for statistical comparison. *p*-Values < 0.05 were considered statistically significant. Analyses were performed using the SPSS statistical software, version 22.0.

## Results

A total of 136 adult PV patients were recruited with an average age of 61.1 years (52.9% were aged 60 or above) and 51.5% were males (►**Supplementary Table S1**, available in the online version). Of these, 74 patients had a thrombotic event recorded at any time in their medical history (56.1%) and 58 had no thrombotic event (43.9%, information on vascular events was not available from the medical history of 4 patients).

Seventy-seven patients (77/136, 56.1%) presented ≥1 additional mutation (i.e., pathogenic nondriver mutation). The most frequently mutated genes were *TET2* 27.9%, *ASXL1* 11.8%, and *DNMT3A* 11% (termed "DTA"; ►**Fig. 1**). As expected, patients with DTA mutations were older than those without DTA mutations (65.4 vs. 57.8 years, $p = 0.002$; Student's *t*-test). Patients with a larger *JAK2*-mutated clone size (≥50% VAF) were not more likely to harbor a DTA mutation ($p = 0.858$).

With a median follow-up of 9.4 years, the incidence density of vascular events was 2.83 per 100 person-years. Patients classically stratified as high risk had shorter TFS as expected (►**Fig. 2A**, $p = 0.034$, median TFS 209 months for low risk vs. 136 months for high risk).

Thirty-seven events (52.9%) were prediagnostic (including the cause for diagnosis) and 44 (62.9%) were postdiagnostic; 11 patients had both a pre- and postdiagnostic event. Of the postdiagnostic events with information available ($n = 37$), 25.7% were venous and 50.0% arterial. The most frequent events were cerebral (30.9%), acute myocardial infarction (25.5%), and portal thrombosis (7.3%).

Comparing the group of PV patients with a thrombotic event at any time in their medical history ($n = 74$) to those without ($n = 58$), there was no significant difference in patient age or the VAF of *JAK2* mutation, but the group with an event had a higher leukocyte count at diagnosis and more additional mutations (mean 1.1 vs. 0.7, $p = 0.004$) and DTA mutations (0.53 vs. 0.31, $p = 0.001$; ►**Supplementary Table S2**, available in the online version). Patients with a prediagnostic event were more likely to have a postdiagnostic event ($p = 0.036$) and also more likely to have a DTA mutation ($p = 0.038$).

In univariable analysis, thrombotic event was not associated with age ≥ 60 years or VAF of *JAK2* mutation but was associated with the presence of any additional mutation (odds ratio [OR]: 3.4, $p = 0.001$) and with any DTA mutation (OR: 2.5, $p = 0.014$). Statistical significance was lost when DTA mutations were not included (non-DTA additional mutation, ►**Supplementary Table S3**, available in the online version). The association with any "adverse mutation" (*SRSF2, IDH2,* or *ASXL1,* as defined by Tefferi et al[14] based on impact on overall survival) was marginal ($p = 0.064$).

Notably, DTA mutation was predictive of postdiagnostic TFS ($p = 0.015$, ►**Fig. 2B**, median TFS 231 months without DTA mutation vs. 111 months with DTA mutation). Moreover, DTA mutation remained predictive of postdiagnostic TFR when selecting the low-risk patients (<60 years of age and no prior event, $p = 0.039$, ►**Fig. 3A**), patients aged ≥60 years ($p = 0.009$, ►**Fig. 3B**), and the high-risk group with no prior events ($p = 0.016$, ►**Fig. 3C**). The presence of DTA mutation was not predictive for the postdiagnostic TFS of patients who had a prediagnostic event ($p = 0.534$).

DTA mutations were significantly associated with arterial events (OR: 4.6, $p < 0.001$) and marginally with venous events (OR: 2.7, $p = 0.06$). Although no association was found between thrombotic event and individual DTA genes (*DNMT3A* $p = 0.299$, *TET2* $p = 0.169$, *ASXL1* $p = 0.281$), the risk was almost doubled for *TET2* mutations (66.7% of

 

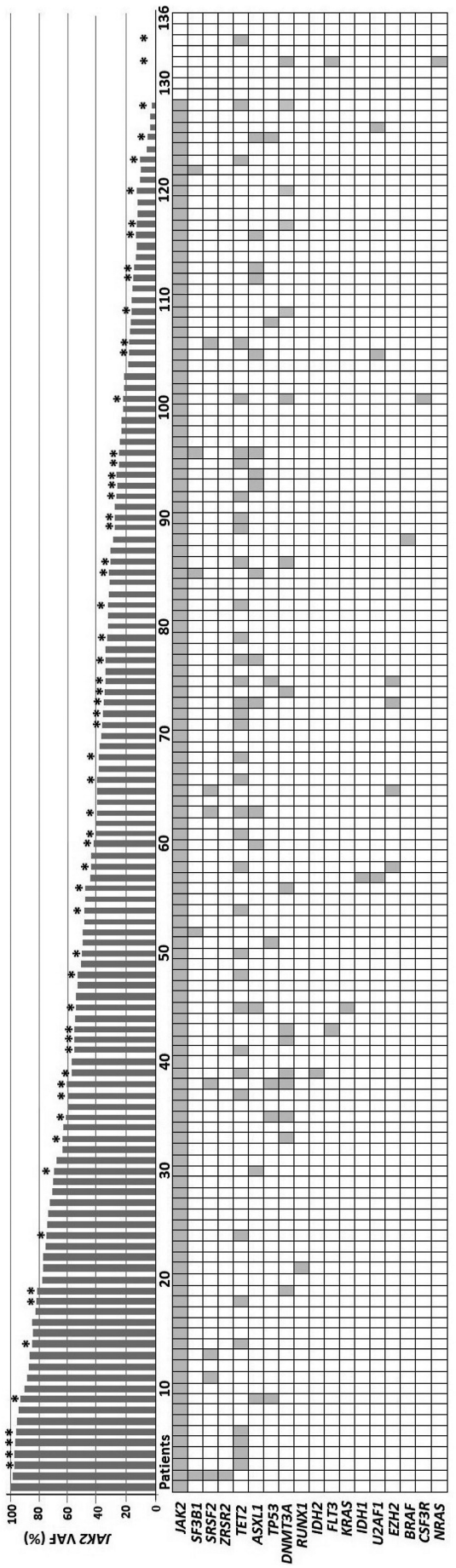


**Fig. 1** Mutations detected in the series of 136 polycythemia vera patients. The variant allele frequency (VAF) of *JAK2* is represented in the top panel. Asterisks (*) mean that a DTA (*DNMT3A*, *TET2*, or *ASXL1*) mutation was detected. Variants detected in genes covered by the targeted myeloid panel (listed on the left) are represented in the bottom panel. Gray square: a pathogenic (or probably pathogenic) variant was detected in the gene; white square: no variant was detected.

patients with *TET2* mutation had an event vs. 33.3% no event). When considered in groups of two genes, the association with thrombotic event was significant for *TET2* with *DNMT3A* ($p = 0.037$), and *TET2* with *ASXL1* ($p = 0.005$), but not for *DNMT3A* with *ASXL1* ($p = 0.123$), suggesting that the significant association with thrombotic events was mainly contributed by mutations in *TET2*.

Data on CVRF and hypertension were available for 115 patients. Seventy-three of 115 patients (63.5%) were hypertensive. The DTA and CVRF variables are closely related (OR: 6.8, $p = 0.009$), in particular the *TET2* mutation with hypertension ($p = 0.025$). Nevertheless, DTA mutation and additional mutation remained as risk factors for a thrombotic event in hypertensive patients (OR: 4.4, $p = 0.052$; OR: 5.8, $p = 0.026$, respectively; Fisher's exact test), with additional mutations losing statistical significance when the DTA genes were removed from the analysis ($p = 1.0$). The association between thrombotic event and CVRF or hypertension was confirmed in multivariable analysis (OR: 3.8, $p = 0.030$; OR: 3.85, $p = 0.002$, respectively), whereas the association with DTA mutation was marginal (OR: 2.1, $p = 0.075$; ►**Supplementary Table S4**, available in the online version).

To confirm these observations, two homogeneous groups of 47 gender- and age-matched PV patients were formed with (case, $n = 47$) and without thrombotic events (control, $n = 47$) in their medical history to exclude selection bias.

In the case–control study, both CVRF and hypertension lost significance in multivariable analysis while DTA mutation was confirmed as a risk factor for thrombotic event (OR: 2.9, $p = 0.027$; ►**Supplementary Table S4**, available in the online version), observing a higher number of additional mutations in the group of cases (cases 1.0 vs. controls 0.68, $p = 0.026$). Importantly, the association between thrombotic event and DTA mutation remained in the group of PV patients aged <60 years ($n = 44$; OR: 6.67, $p = 0.033$; ►**Supplementary Table S5**, available in the online version) from the case–control study and was predictive for TFS in younger patients ($p = 0.024$, ►**Supplementary Fig. S1**, available in the online version). When all patients in the cohort were dichotomized according to age at diagnosis, the association between thrombosis and DTA mutation was of borderline significance in both age groups (≥60 years OR: 2.44, $p = 0.064$; <60 years OR: 2.89, $p = 0.065$). Moreover, when data were dichotomized according to the VAF of mutated *JAK2*, the association between DTA mutations and thrombosis was significant for the smaller *JAK2*-mutated clones (VAF < 50%: OR: 3.34, confidence interval [CI]: 1.11–13.67, $p = 0.034$) and borderline for the larger *JAK2*-mutated clones (VAF ≥ 50%: OR: 3.50, CI: 0.82–14.91, $p = 0.091$).

 

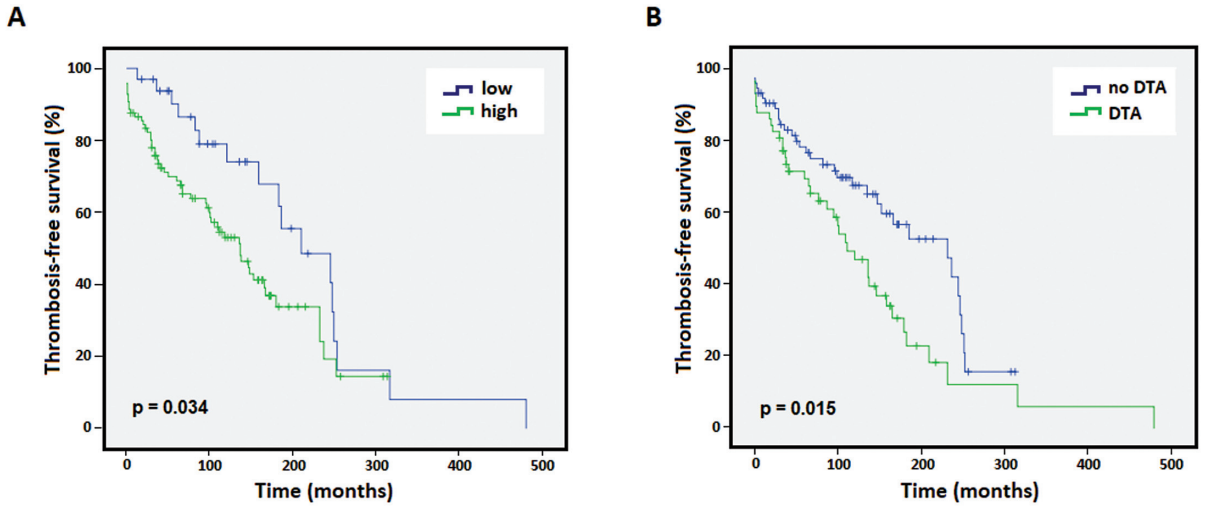


**Fig. 2** Thrombosis-free survival for the total cohort of polycythemia vera patients. **(A)** Kaplan–Meier curve with data stratified as high or low risk according to conventional thrombotic risk algorithms and **(B)** Kaplan–Meier curve with data stratified according to presence or absence of any DTA (*DNMT3A*, *TET2*, or *ASXL1*) mutation. Time calculated from date of diagnosis to date of thrombotic event or date of last follow-up. Significance determined using the log rank test.

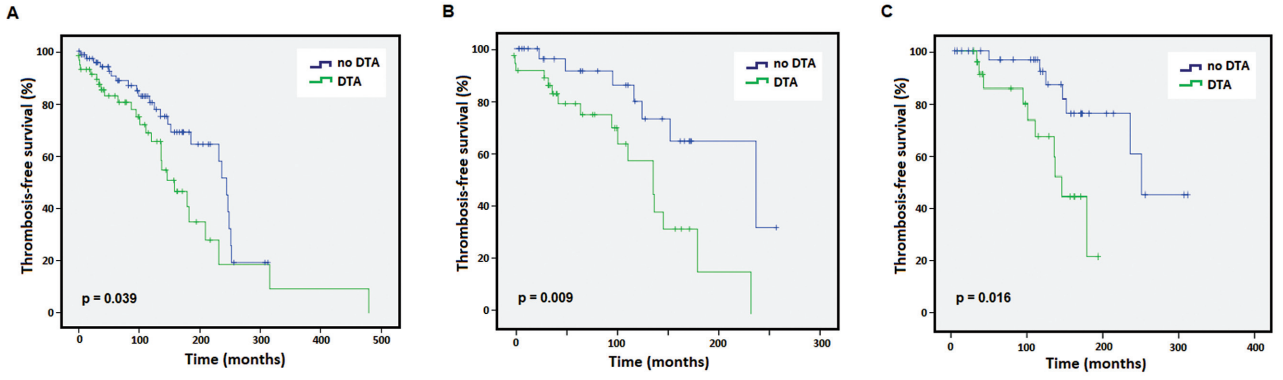


**Fig. 3** Thrombosis-free survival of **(A)** low-risk patients (<60 years of age and no prior event), **(B)** patients aged ≥60 years, and **(C)** the high-risk group with no prior events. Kaplan–Meier curves of thrombosis-free survival for subgroups of polycythemia vera patients stratified according to presence or absence of any DTA (*DNMT3A*, *TET2*, or *ASXL1*) mutation. Time calculated from date of diagnosis to date of thrombotic event or date of last follow-up. Significance determined using the log rank test.

## Discussion

In this observational multicenter study, we confirmed our previous observation that the presence of additional myeloid mutations at diagnosis, particularly DTA mutations, is a risk factor for thrombotic event in PV patients.[12] Although patients with DTA mutations were on average 10 years older than the patients without DTA mutations, we do not believe that the increased risk of thrombosis associated with DTA mutation is simply an age effect but rather an age-independent additional risk factor.

Our results show that the presence of DTA mutations predicts for TFS in older patients but also in patients aged <60 years and the low-risk group. As such, the determination of DTA mutation status at diagnosis for patients with no prior thrombotic history would be clinically useful since if would change the risk category of "low-risk" patients and further risk stratifying older patients at increased risk of later developing a thrombotic event. The association with thrombotic risk was lost when the DTA genes were considered individually, probably due to low frequencies. This association was age independent and, despite the intimate relationship between DTA mutations and CVRF, was an additional risk factor for hypertensive patients.[15]

The majority of events were arterial and the association between arterial thrombosis with DTA mutations was significant while only marginal significance was found between DTA mutation and venous thrombosis. The most frequent site of thrombosis in our cohort was cerebral, with a high incidence of ischemic stroke, in accordance with the recent observation of an age-independent association between DTA mutation and a history of stroke.[16] Some 64% of patients had

 

hypertension, and while hypertension itself is associated with a significantly higher rate of arterial (but not venous) events,[15] we observed a close association between presence of a *TET2* mutation and hypertension.

Since the cardiovascular risk imparted by mutations in CHIP genes has been convincingly demonstrated in healthy individuals,[6] its effect in PV patients is not surprising given the additional thrombotic risk imparted by the *JAK2* V617F mutation.[17] Nevertheless, not all studies have observed an association between DTA mutations and thrombosis in PV patients.[18]

As expected, a previous thrombotic event, i.e., at or prior to diagnosis, was a strong predictor for having another postdiagnostic thrombotic event. DTA mutation was not predictive for postdiagnostic TFS in the group of patients with a prior event, although this group did have significantly more DTA mutations.

One limitation of our study was the high proportion of PV patients with a postdiagnostic thrombotic event (44 events, 2.83 per 100 person-years), although the incidence of our cohort was in accordance with previous studies.[4] Two groups of a similar number of PV patients were initially included: with and without a thrombotic event recorded in their medical history. However, in a number of cases, records were found of thrombotic events upon closer scrutiny of their medical history, some of which were reported in unusual sites, such as the retina. For this reason, a case–control analysis of two equal groups of sex- and age-matched patients was also performed to exclude potential selection bias. Finally, treatment status for CVRF, such as hypertension and dyslipidemia, was not available and so we could not evaluate the association with thrombosis.

It is intriguing that some patients present with a thrombotic event leading to their PV diagnosis while others are diagnosed due to blood count abnormalities. It thus appears that a group of PV patients are prone to thrombotic events, including both "low-risk" patients and high-risk PV patients receiving prophylaxis, consequently increasing their comorbidities and reducing their life expectancy. Perhaps there are fundamental differences between these two groups of patients that result in one group being more prone to thrombosis. One such difference could be the presence of DTA mutations, or the order of mutation acquisition, associated with differences in MPN phenotype.[19] However, to demonstrate this, serial sampling and/or phylogenetic methods would be required.[20]

## Conclusion

Our results support the use of molecular testing at diagnosis to help predict which PV patients are at higher risk of developing thrombosis.[12,14,21] Such patients with additional myeloid mutations, particularly in the DTA genes, could perhaps benefit from a more aggressive therapy although randomized controlled trials would be required to confirm optimal prophylaxis management.

In conclusion, patients with a DTA mutation at diagnosis (including both younger and older patients, as well as those with classical risk factors) should be considered to be at higher risk of thrombosis. We question whether the current risk stratification and recommendations of cytoreduction and antiplatelet therapy are effective enough. The incorporation of biomarkers into current algorithms to predict thrombotic events is an unmet need that needs to be addressed.

### What is known about this topic?

- Patients with PV have a high risk of thrombosis, even those classified as low risk, and despite strict adherence to treatment recommendations.
- In a previous study we reported an association between thrombotic risk and the presence of mutations in DTA genes (*DNMT3A, TET2,* and *ASXL1)*, the most frequently mutated genes in clonal hematopoiesis of indeterminate potential, or CHIP, a natural phenomenon associated with older age and which conveys a higher cardiovascular risk.

### What does this paper add?

- Our study confirms that the presence of DTA mutations is an age-independent risk factor for a thrombotic event and predicts for shorter TFS in PV patients.
- Our results support the use of molecular testing at diagnosis to help predict which PV patients (including younger patients and those with classical risk factors) are at higher risk of developing thrombosis.

#### Ethics Approval Statement

This retrospective noninterventional study was approved by our Institutional Review Board (Comité Ético de Investigación Clínica, ref. 2019-230-1) on March 28, 2019 and conducted in accordance with the Declaration of Helsinki.

All patient data were dissociated and anonymized; informed consent was not required due to the retrospective nature of the study and because the results did not affect the clinical management of patients.

#### Data Availability Statement

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

#### Authors' Contribution

A.S.D. contributed data, curated data, analyzed the data, and wrote the paper; R.S. coordinated the study, curated data, analyzed the data, performed statistical analysis, and wrote the paper; Y.F. performed next-generation sequencing; M.S., A.A.L., F.F.M., M.P.E., G.C.T., M.L.F., B.T.V., and B.C. contributed data, J.F.L.R. and N.F.S. curated data; J.M.G.M. performed statistical analysis; M.T.G.C. designed the research study; C.B.S. designed the research study, performed statistical analysis and wrote the paper.

All authors read and approved the final version of the manuscript.

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Conflict of Interest
None declared.

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