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OUTCOMES OF GLOBAL COAGULATION ASSAYS IN PATIENTS WITH PHILADELPHIA-NEGATIVE MYELOPROLIFERATIVE NEOPLASMS WITH RESPECT TO GENETIC DETERMINANTS OF CLONAL PROGRESSION

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Phase 1. Evaluation of Platelet Fibrinogen Receptor in Myeloproliferative Neoplasms.

From: "Unexpected low expression of platelet fibrinogen receptor in patients with chronic myeloproliferative neoplasms: how does it change with aspirin?"

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1. Summary

This study was conducted to evaluate the expression of fibrinogen receptors on platelets of Philadelphia-negative chronic myeloproliferative neoplasms (MPNs) patients. We collected blood samples from 40 consecutive MPNs patients and healthy volunteers. We performed flow cytometry analysis of P-selectin expression, Integrin beta-3 and activation of GP IIb/IIIa and fibrinogen receptors exposure (PAC-1 binding). Surprisingly, we found a very low PAC-1 binding capacity in MPNs patients and the expression of PAC-1 was almost completely recovered with aspirin intake. We hypothesize that the hypercoagulable states observed in MPNs patient could depend on a primarily plasma-driven impairment of fibrin turnover and thrombin generation.

2. Background

Patients affected by Philadelphia-negative chronic myelopro- liferative neoplasms (MPNs) are considered at high risk of thrombo-haemorrhagic events, but the role of the platelet count in the assessment of the risk of vascular events is still controversial (Bucalossi *et al.*, 1996; Finazzi *et al.*, 1996).

A tight correlation was found between the platelet count and plasma sCD40L (Viallard *et al.*, 2002), which appears to be required for thrombus formation *in vivo* (André *et al.*, 2002). However, sCD40L is increased both in MPNs and reactive thrombocytosis

(Viallard *et al.*, 2002). Intravascular aggregates of platelets and leukocytes, mediated by P-selectin on the former and CD11b on the latter, have been observed (Cervantes *et al.*, 2009). The expression of CD11b is even more evident in patients with mutation of the *JAK2* gene Val617Phe (Coucelo *et al.*, 2014). Both of these processes should imply a perpetual — and measurable — platelet activation. Several studies on platelet function have been already proposed. Nevertheless, the mechanisms through which platelets are able to trigger vascular events are not yet adequately clarified. A refined method for the determination of platelet activation appears to be the use of platelet PAC-1 antibody, able to identify the expression of the fibrinogen receptor of platelet glycoprotein IIb/IIIa (Lu & Malinauskas, 2011). This expression is indeed unique in the process of platelet activation, and yet rarely analyzed. One of the few reports demonstrating platelet fibrinogen receptor expression in MPN patients documented a decrease in PAC-1 binding, despite a normal expression of GP IIb/IIIa. This finding was more marked in myelofibrosis (MF) than in essential thrombocythaemia (ET) or polycythaemia vera (PV) (Jensen *et al.*, 2000). Moreover, since the exposure of platelet fibrinogen receptor seems to be influenced by turbulence in blood flow, we estimated that its evaluation could provide important biological evidence to explain some clinical manifestations, such as microvascular disturbances.

3. Patients and Methods

After obtaining informed consent from patients and the approval of the local Ethics Committee, blood samples from 40 consecutive MPNs patients who never received cytoreductive agents were obtained. Clinical characteristics of patients are reported in Table I. In all, 28/40 patients were receiving a continuative antiplatelet prophylaxis with low-dose aspirin (ASA, 75–100 mg) at the time of collection, while 12/40 of them were not on such therapy.

Table I. Patients and characteristics.

MPNs	
No. patients	40
Gender	
Male	17 (42·5)
Female	23 (57·5)
Median age, years (range)	64 (32–86)
Pathology	
(A) Essential thrombocythaemia	22/40
Platelet count 10^9/l (mean ± SD)	691·18 ± 264·40
Haematocrit (mean ± SD)	41·00 ± 5·50
JAK2 allele burden (mean ± SD)	17·67 ± 15·46
CALR mut exon 9 – Type 2	2/22 (9%)
(B) Polycythaemia vera	13/40
Platelet count 10^9/l (mean ± SD)	415·08 ± 176·96
Haematocrit (mean ± SD)	48·93 ± 3·06
JAK2 allele burden (mean ± SD)	47·85 ± 34·18
CALR mut exon 9 – Type 2	0/13 (0%)
(C) Myelofibrosis	5/40
Platelet count 10^9/l (mean ± SD)	461·60 ± 32·18
Haematocrit (mean ± SD)	43·64 ± 7·73
JAK2 allele burden (mean ± SD)	12·63 ± 11·74
CALR mut exon 9 – Type 2	1/5 (20%)

Flow cytometric analyses were performed using a FACS- Canto flow cytometer (Becton–Dickinson, Franklin Lakes, NJ, USA) and 50 000 events were recorded for each sample. Our aim was to verify the expression of platelet fibrinogen receptors (PFRs) in the two different groups of patients compared to healthy volunteers, using whole blood flow cytometry. In each experiment sodium citrate and heparin tubes were collected from the same patient (positive control of platelet activation). Within 10 min from blood sampling, 5 μl of whole blood from each tube was incubated for 20 min at room temperature in the dark with a saturated concentration of CD61 peridinin-chlorophyll proteins (PerCP), CD62P phycoerythrin (PE) and PAC-1 fluorescein isothiocyanate (FITC). Positive control was also incubated with PAC-1 in the presence of Arg–Gly–Asp–Ser (RGDS) in order to test the specific antibody binding. Samples were fixed with paraformaldehyde 1% for 30 min at 4°C in the dark and analyzed on a flow cytometer. Prism 8.0.1 (GraphPad, San Diego, CA, USA) was used for statistical analysis. To com- pare continuous response variables between two groups a Mann–Whitney *U*-test was performed. $P < 0{\cdot}05$ was

considered as statistically significant. To measure the linear correlation between two variables the Pearson correlation coefficient was used.

4. Results

Surprisingly, we were able to verify a very low binding of PAC-1 to platelets in patients with MPNs not receiving cytoreduction nor antiplatelet agents if compared to that observed in healthy subjects (35·3 ± 12·9 vs 65·3 ± 24·2 respectively, $P = 0{\cdot}008$). Conversely, the use of aspirin seems to restore the expression of platelet fibrinogen receptor, as PAC-1's binding capacity is comparable to that of healthy volunteers (56·7 ± 18·7) (Fig 1). No difference was found with respect to the *JAK2* Val617Phe mutation and its allele burden (data not shown). Interestingly, by focusing on the group of patients under antiplatelet prophylaxis and with no history of thrombosis, it was found that subjects with persistent microcirculatory disorders (MD) show a higher PAC-1 binding capacity if compared to the asymptomatic ones (67·7 ± 17·8 vs 51·7 ± 14·0 respectively, $P = 0{\cdot}030$) (Fig 2).

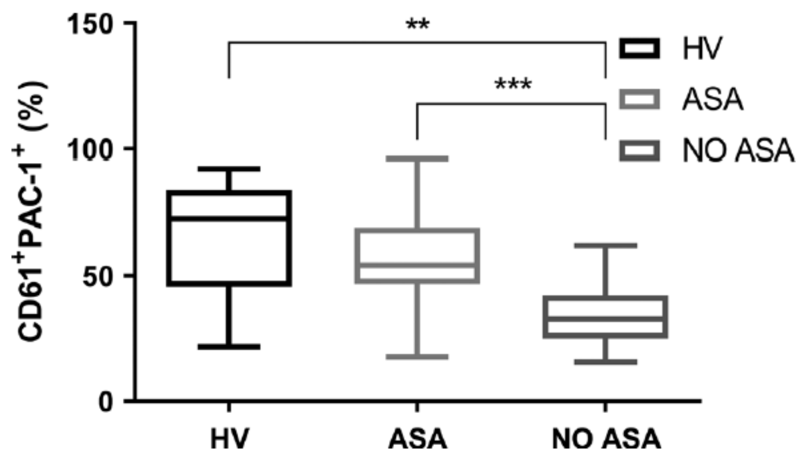

Fig 1. PAC-1 frequency in whole blood obtained from healthy volunteers (HV, $n = 10$) and myeloproliferative neoplasm (MPN) patients treated or not with low-dose aspirin (ASA, $n = 28$ and NO ASA, $n = 12$). $^{**}P = 0{\cdot}008$, $^{***}P < 0{\cdot}001$.

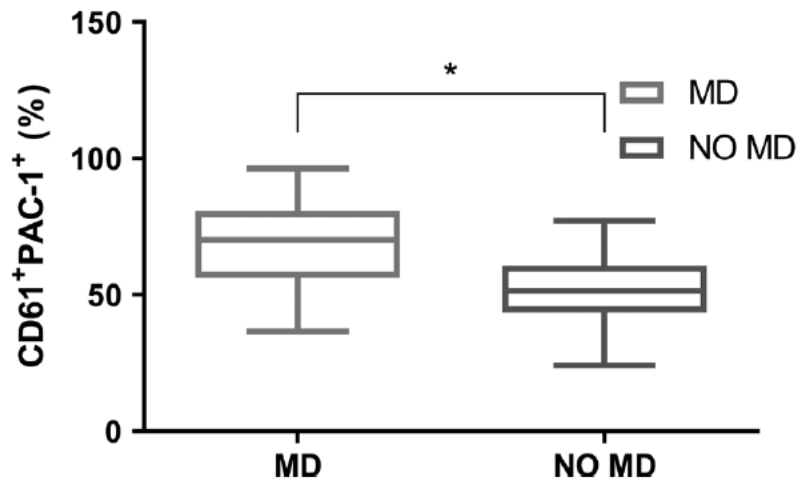

Fig 2. PAC-1 frequency in whole blood obtained from MPN patients stratified according to the presence of microcirculatory disorders (MD, n = 13 and NO MD, n = 9). *$P < 0{\cdot}05$.

5. Discussion

The expression of fibrinogen receptors has already been previously evaluated in patients with microcirculation disorders similar to those observed in MPNs (Raynaud's phenomenon). Interestingly, in the paper by Polidoro et al. (2012), particularly high values of PAC-1 expression were reported, but with an inverse relation to the circulating levels of thromboxane B2. The authors hypothesized a possible downregulation of GP IIb/IIIa as a response to chronic exposure to thromboxanes. In MPNs, platelet counts are usually high, as well as the rate of vascular events. Moreover, since MPNs are characterized by high turbulence of blood flow — and consequently by an increase in endothelial shear stress — the PAC-1-binding capacity was expect likely to be high, by analogy to Raynaud's phenomenon. As a matter of fact, there is already evidence of how the binding between fibrinogen and GP IIb/IIIa is important in shear stress-induced platelet aggregation (SIPA). Inhibitors of thromboxane synthesis seem able to counteract SIPA (Ikeda et al., 1988; Floyd & Ferro, 1988). Surprisingly, we observed that in untreated MPNs large amounts of platelets are resting in a conformation that is unable to bind fibrinogen, as demonstrated by the low PAC-1 expression in cytofluorometry. This lack of activity can be reversed by administering ASA, which is also known for its fibrinolytic and hypoprothrombinaemic effects. We hypothesize that the hypercoagulable states observed in these patients could depend on a primarily plasma-driven impairment of fibrin turnover and thrombin generation. A study by Moore et al. (2013) found an impaired fibrinogen binding in ET

patients, despite normal levels of GP IIb/IIIa receptors. In the same study, interest- ingly, the authors describe enhanced Protease-Activated Receptor-1 (PAR1)-mediated expression of GP IIb/IIIa after thrombopoietin stimulation, followed by the disappearance of fibrinogen binding sites. Starting from these observations, we can speculate that an increased generation of thrombin in MPN patients could secondarily lead to PAR1 activation, determining both a major conversion of fibrinogen into fibrin and the disappearance of fibrinogen binding sites on platelets with a reduced PAC-1 expression. As is well known, PAR receptors are expressed in platelets, endothelium, and smooth muscle, contributing to both normal and pathological haemostasis (Leger et al., 2006). PAR1 activation could thus be a key element for the pathogenesis of thrombosis in MPNs. The results of our experiment are also similar to those observed in patients with pre-infarct angina, where a reduced expression of PAC-1 is described (Scalone et al., 2013), and to what occurs months after an acute ST segment elevation myocardial infarction, with a progressive increase in the expression of the platelet fibrinogen receptor despite dual antiplatelet therapy (Scalone et al., 2011). Indeed, in acute cardiovascular diseases the role of plasma procoagulants such as tissue factor was considered fundamental (Steffel et al., 2006). Finally, PAC-1 could at the same time be a good marker of aspirin resistance in patients experiencing microcirculatory disorders. Further investigations are continuing in our group.

Phase 2. Design of a model of interplay between fibrosis and coagulation in myeloproliferative neoplasms.

From: "Platelets Contribution to Thrombin Generation in Philadelphia-Negative Myeloproliferative Neoplasms: The 'Circulating Wound' Model"

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1. Abstract

Current cytoreductive and antithrombotic strategies in MPNs are mostly based on cell counts and on patient's demographic and clinical history. Despite the numerous studies conducted on platelet function and on the role of plasma factors, an accurate and reliable method to dynamically quantify the hypercoagulability states of these conditions is not yet part of clinical practice. Starting from our experience, and after having sifted through the literature, we propose an in-depth narrative report on the contribution of the clonal platelets of MPNs—rich in tissue factor (TF)—in promoting a perpetual procoagulant mechanism. The whole process results in an unbalanced generation of thrombin and is self-maintained by Protease Activated Receptors (PARs). We chose to define this model as a "circulating wound", as it indisputably links the coagulation, inflammation, and fibrotic progression of the disease, in analogy with what happens in some solid tumours. The platelet contribution to thrombin generation results in triggering a vicious circle supported by the PARs/TGF-beta axis. PAR antagonists could therefore be a good option for target therapy, both to contain the risk of vascular events and to slow the progression of the disease towards end-stage forms. Both the new and old strategies, however, will require tools capable of measuring procoagulant or prohaemorrhagic states in a more extensive and dynamic way to favour a less empirical management of MPNs and their potential clinical complications.

2. Introduction

Philadelphia-negative Myeloproliferative Neoplasms (MPNs) are diseases characterised by a high risk of thrombo-haemorrhagic events. In the past years, the focus has been on the mechanistic model of clonal proliferation, often attributing the states of hypercoagulability to the alteration of cell counts. First-line therapeutic strategies, almost always based on cytoreduction, aim at "numerical" rather than functional objectives. Thrombosis in MPNs can occur at any platelet count, therefore it is questionable regarding what the correct way is to monitor the efficacy of therapeutic strategies. Although the importance of cellular interactions has been demonstrated, in particular between white blood cells, platelets, and the vascular endothelium in JAK2-mutated patients, there are numerous experiences on the alteration of platelet function in MPN, which is partly secondary to a state of hypercoagulability, primarily plasma-driven, and has increased thrombin generation (TG) as its central element.

3. Platelets in MPNs: Role and Controversies

The role of platelets in the pathogenesis of vascular events in MPNs remains controversial [1,2]. Several studies did not observe a correlation between platelet count and thrombotic risk [3,4]. A recent review points out the correlation between the presence of driver mutations and platelet activation, underlining how this may actually depend on the consequences of the activation of myeloproliferative pathways on endothelial and systemic inflammation. It is also reiterated that cell counts have a substantially dubious role both on thrombotic risk (only in ET, leukocytosis appears to be a risk factor) and haemorrhagic risk (the majority of acquired von Willebrand diseases have a platelet count below 1000×10^9 /L [5]. On the contrary, Michiels et al. described a platelet-mediated microvascular thrombotic syndrome, documenting thrombotic processes by a reduced platelet survival and by an increase in beta-thromboglobulin, platelet factor 4, and thrombomodulin levels in Polycythemia Vera (PV) and Essential Thrombocythemia (ET) patients [6]. A recent review published by Marin Oyarzùn and Heller accurately describes the platelet contribution to procoagulant states [7]. In ET and MF, a higher platelet activation rate has been observed [8,9] probably because of both intrinsic and extrinsic factors [7]. Among intrinsic platelet characteristics, we can discern membrane abnormalities [10] and JAK2-signalling hyperactivation. Endothelium can be also JAK2-mutated, releasing increased

quantities of P-selectin and the von Willebrand Factor (vWF), and expressing higher levels of adhesion molecules and receptors, enhancing platelet activation [11]. The role of megakaryocytes in platelets activity has also been investigated, observing alterations in gene expression and the transcriptome [12,13]. One of the most recent and interesting findings is an altered expression of β-1,4-galactosyltransferase1 (B4GALT1) in megakaryocytes from MPN, which can induce the genesis of platelets with aberrant galactosylation [14]. Consequently, TPO synthesis by hepatocytes is promoted regardless of the circulating platelet mass. The expression of B4GALT1 can be modulated by JAK1/2 inhibitors, but the gene product should be considered a potential target for therapy.

Additionally, among extrinsic factors involved in platelet activation, we can discern the higher interaction with the endothelium and leukocytes, as well as the influence of miRNA-signalling and thrombin generation [7,15]. In fact, there is evidence of how driver mutations (and their allelic burden) may be responsible for a state of hypercoagulability induced both by a greater probability of interaction between platelets, leukocytes, and the endothelium, and by a more massive release of plasma factors [16] [15]. Among these is the tissue factor (TF), which is an element whose function directly affects the generation of thrombin and whose circulating levels are particularly high in MPNs [17]. Often in clinical practice, it is thought to use hydroxyurea (and cytoreduction in general) with the aim of controlling cell counts, but probably the greatest advantage this strategy offers lies in the reduction of P-selectin-mediated TF expression in polymorphonuclear leukocytes [18]. The periodic assessment of cell counts may be a method too coarse to understand whether the patient is at risk for a cardiovascular event. Functional tests, such as global coagulation assays, which we will discuss later, would perhaps be more useful. In fact, these tests provide a reliable measure of the thrombogenic potential of thrombocytosis.

4. From Platelet Fibrinogen Receptors to Thrombin Generation: the "Circulating Wound" Model

The platelet fibrinogen receptor (PFR) is exposed only after a conformational change in glycoprotein (GP) IIb/IIIa, which is the most abundant integrin on the platelet surface. This process is exquisitely attributable to platelet activation, particularly in conditions of shear stress [19]. MPNs are generally characterised by hyper-viscosity phenomena, therefore the flow cytometric evaluation of the expression of the fibrinogen receptor should represent a

refined method for the evaluation of platelet activation. As we will see in this section, the experimental data lead us to other considerations.

The active conformation of the CD41/CD61 complex (glycoprotein IIb/IIIa) is recognised by the PAC-1 antibody. In details, after vessel injury, CD41 (platelet glycoprotein IIb) interacts with CD61 (platelet glycoprotein IIIa) to form a functional receptor, exposing the fibrinogen-binding site and promoting platelet aggregation [20,21]. In MPNs, intrinsic cellular abnormalities in the GPIIb/IIIa complex activation have been described [22] and JAK2-mutated ET patients presented an altered functionality of the PI3 kinase/Rap1, with a reduced activation of the GPIIb/IIIa receptor [23]. Moreover, in ET, a decreased concentration of glycoprotein IIb and IIIa was observed, with a reduced sialysation and a consequent low fibrinogen binding [24–27].

A recent study by Marín Oyarzùn et al. focused on the role of platelets in immunity, inflammation, and thrombosis, showing an enhanced toll-like receptor 2-mediated translocation of granule membrane proteins with preserved GPIIb/IIIa activation [28]. A decrease in PAC-1-binding was also documented by Jensen et al., despite a normal expression of GPIIb/IIIa [22]. Finally, our recent work confirmed a reduced activation of GPIIb/IIIa in MPN patients, with a recovery of the PAC-1-binding capacity after acetylsalicylic acid intake [29]. The most interesting aspect of our experience was the recovery of PFR expression—to levels very close to that of healthy subjects—in patients under prophylaxis with low doses of acetylsalicylic acid. Paradoxically, in the context of diseases characterised by a state of hypercoagulability, we observed the presence of platelets unable to bind fibrinogen. The most credible explanation of this phenomenon, however, distances itself from an alleged “thrombasthenia”.

In fact, the experimental observations obtained by Moore in 2013 showed an altered binding of fibrinogen to platelets in patients with ET [23]. Although GPIIb/IIIa was normally expressed under baseline conditions, the authors observed a significant increase in its protease-activated Receptor-1 (PAR-1)-mediated exposure after stimulation with thrombopoietin. A rapid disappearance of the fibrinogen binding sites on the platelets followed soon after. We therefore speculated that a more marked thrombin generation in MPNs could be at the basis of an increase in PAR-1 activity, for instance, to determine a continuous conversion of functional fibrinogen into fibrin, as well as concerning the reduction of PFR. Acetylsalicylic acid (ASA), a drug also known for its fibrinolytic and

hypoprothrombinemic properties, would thus be able to intervene on plasma factors and restore the ability to bind fibrinogen to platelets [30].

Protease-activated receptors (PARs) are G protein-coupled receptors responsible for protease-signalling and for regulating cellular processes, survival, and apoptosis. They are expressed in platelets and the endothelium, with a role in haemostasis regulation [31,32]. Genetic polymorphisms of PAR-1, PAR2, and PAR-4 have been recently described [33] and they seem able to act on the gene-coding sequences and their degree of expression, or may regulate the downstream signalling. In particular for PAR-4, single nucleotide polymorphisms could influence the downstream response of PARs, even though their specific effects on platelet activation still need to be clarified [34].

The thrombin promotes platelet activation by the cleavage of the NH2-terminal domain of PAR-1 and PAR-4. Activated PAR-1 stimulates RhoA activation though ERK1/2 kinases [32,35,36], inhibits the accumulation of cAMP, activates phospholipase C, and promotes Ca2+ mobilisation [37,38]. Moreover, PAR-1 activation influences endothelial barrier permeability due to the stimulation of the MAPKs cascade [39]. PAR-4 exerts several functions similar to PAR-1 [40,41], as it presents a more massive calcium signal [42] and is involved in cellular blebbing due to RhoA and B-arrestin activation [43]. Finally, it promotes platelet activation through G protein-coupled receptor kinase 5 (GRK5) [44].

The PAR4 structure shows several differences from the other PARs, its extracellular amino-terminus and intracellular carboxy terminus has little sequence similarity to the corresponding regions of other PARs [45]. PAR-4 does not show a high affinity thrombin-binding domain, as found in the other thrombin receptors [46].

Several studies described a role of PARs in cancer development, in particular in the setting of pancreatic cancer, where PAR-1 appears to be crucial for disease progression, for promoting an immunosuppressive microenvironment, and for conferring chemoresistance [47–51]. PAR-1 germline polymorphisms are linked to the prognosis of the tumour [52]. On the contrary, we still know little about the pathogenetic role that PARs play in MPNs. However, if we start from the consideration that both pancreatic cancer and MPNs result in TGF-beta-mediated fibrosis in their advanced stage [53,54], we could be determined in designing the next experiments. In a 2018 paper published by Ungefroren et al. in this Journal, the signalling crosstalk between the TGF-beta/ALK5 and PAR-2/PAR-1 pathways was accurately described [55]. In summary, in different models of disease ranging from

cancer to simple wound healing, the activation of PARs generates platelet activation and the release of TGF-beta, which in turn regulates PAR-1 and PAR-2 at a traditional level, generating a vicious circle that is self-maintaining and pushes towards both fibrosis and tumour growth.

In conclusion, MPNs would therefore be a model that—starting from a TF-rich clonal platelet—feeds a "**circulating wound**".

5. Thrombin Generation and Platelet Dysfunctions

Thrombin generation is a finely regulated process. After injury, the damaged endothelium exposes the tissue factor (TF), activating the coagulation cascade with thrombin production. Thrombin is responsible for the conversion of fibrinogen into fibrin, for the activation of several factors of the coagulation cascade (such as V, VIII, and XI), and for platelet recruitment and activation [56]. Platelets are activated through the cleavage of glycoprotein V and through the stimulation of PAR-1 and PAR-4 receptors [57]. The formed plug is then protected by the inhibition of ADAMTS13 activity [58]. Activated platelets present a procoagulant potential and can expose phosphatidylserine as a substrate for the conversion of prothrombin into thrombin [59]. MPNs showed the downregulation of several genes involved in thrombin generation [13], with a direct correlation between JAK2(V617F) allele burden and thrombin generation potential [60]. ET presented a higher production of platelet-induced thrombin in JAK2-mutated patients [61] and a recent study, performed in JAK2-mutated primary myelofibrotic mice models, documented a reduction in thrombus and size formation [62]. MF patients presented, indeed, a reduced endogenous thrombin potential, significantly correlated with platelet count [63].

Higher levels of platelet-released procoagulant microparticles, increasing thrombin generation in PV and ET patients, have also been detected [64,65], leading to a thrombomodulin resistance and probably contributing to the hypercoagulable state of patients [64]. For PV and ET patients, a reduced thrombin potential, if compared to healthy controls, has been described, also showing the occurrence of an acquired activated protein C resistance [66]. Thrombin generation has also been proposed as a potential biomarker of thrombotic risk in MPN [67].

Another aspect of considerable interest is the thrombin-mediated generation of intraceullary reactive oxygen species (ROS), which also require the activation of PAR-4 [68]. ROS overexpression is known to be linked to a proliferative advantage of mutated JAK2 clones and associated with an increased incidence of thrombotic events [69]. This is another excellent example of the interconnection between the hypercoagulability and inflammation in MPNs.

6. How to Evaluate Coagulation Parameters: Global Coagulation Assays

Patients with myeloproliferative neoplasms (MPN), even within the course of thrombosis, show little or no abnormalities of conventional coagulation tests. Conventional coagulation tests (CCTs) are not able to assay interactions between clotting factors, blood cell elements, and the vascular endothelium, thus they cannot predict and/or guide therapy in acute haemorrhages and are unable to predict thrombotic risk [70].

Global haemostatic assays (i.e., thrombin generation in platelet-rich plasma and thromboelastometry or thromboelastography in whole blood) have been evaluated for their role in detecting signs of procoagulant imbalance in patients with MPNs. Available data are, however, quite scant.

In 2013, Tripodi et al. evaluated thrombin generation in platelet-rich plasma and thromboelastometry in 111 patients with MPN and in 89 controls [71]. The endogenous thrombin potential (ETP) revealed to be higher in patients than in controls. ETP directly correlated with platelet counts, while there was an inverse correlation with free protein S, protein C, and antithrombin. Patients under hydroxyurea treatment showed lower ETP ratios than those on other treatments.

In 2016, Giaccherini et al. performed a study on ROTEM using INTEM and EXTEM reagents in patients with ET (N = 39) and PV (N = 23), while nineteen healthy subjects acted as the controls [72]. The ROTEM analysis showed a hypercoagulable state in MPN patients, with shorter CFT and higher MCF in comparison to the controls, with both reagents. A statistically significant correlation was found between platelet count and MCF or CFT. Platelet count resulted independently as associated to ROTEM parameters at the multivariate analysis. MCF values, corrected for the platelet count, revealed a lower platelet reactivity in the enrolled patients.

Plasma samples from 36 patients with MF (JAK2 V617-positive, 53%; CALR-positive, 31%; MPL-positive, 14%; and triple negative, 2%) and from 20 healthy volunteer blood donors were assayed by Tthrombin generation in PRP and in a fully automated system. Results were analysed for their correlation with clinical and laboratory parameters of the enrolled patients. Differences in ETP between the two groups were found, as ETP was lower in the patient group ($p = 0.0003$). Multivariate analysis confirmed a significant correlation between thrombin generation and platelet counts, with higher thrombin generation in patients with thrombocythemia $> 400 \times 10^9$/L ($p = 0.04$). ETP was higher in earlier stages of MF and lower in CALR-mutated samples. Authors concluded that thrombin generation in MPNs is mainly influenced by platelet counts and thrombocythemia may be a potential risk factor for thrombotic complications [63].

In another study, thirty-eight patients with MPN were enrolled (median age: 65 years), including 26 patients with essential thrombocythemia (68.4%), eight with PV (20.5%), three with MF, and one with unclassifiable MPN; blood samples were evaluated by thromboelastography and thrombin generation (CAT) [73]. Compared with the controls, there was no difference in the maximum amplitude and lysis time (LY30) was significantly higher in the thromboelastography. The CAT showed a higher thrombin peak and velocity index with comparable ETP. Fibrin generation parameters were significantly reduced with preserved overall fibrinolytic potential and P-selectin was markedly increased. This study showed specific differences between subjects with MPN and normal controls. A high lysis time (LY30) with reduced fibrin generation in MPN patients apparently did not fit with the prothrombotic characteristics of MPN, probably because they may mirror a compensatory attempt to balance haemostasis. It is most likely that the study included large percentages of patients on antiplatelet prophylaxis (95% of subjects) and/or undergoing cytoreductive therapy (60%). As already mentioned, the contribution of these treatments in promoting hyperfibrinolysis and dampening thrombin generation is widely conceivable. A small number of patients were receiving phlebotomies but we do not know the contribution of this strategy to the modification of the thromboelastographic parameters.

The circulating procoagulant activity (CPA) of microparticles in polycythaemia vera (PV) and in essential thrombocythemia (ET) has also been determined by a thrombin generation test performed in the absence and presence of thrombomodulin (TM), wherein TM-resistance was observed and postulated to contribute to the hypercoagulable state of MPN [74].

Sain et al. evaluated the role of Rotational Thromboelastometry (ROTEM) to screen the coagulation profile of patients with MPNs. Authors found higher mean maximum clot firmness values in patients affected by ET and PV compared to the healthy controls [75]. The authors highlighted how thromboelastography is able to finely detect those states of the hypercoagulability characteristic of MPNs. Furthermore, since clotting time is more influenced by coagulation factors rather than by platelet hyperaggregability, the hypothesis of a greater plasma contribution is supported. This implies the potential usefulness of tests such as ROTEM to dynamically measure global coagulation and to be combined with routine cell counts.

7. Current Therapies and Rising Therapeutic Alternatives

Low-dose ASA is the antithrombotic strategy that can be commonly used in MPN, thus making use of it is probably excessive compared to the real needs [76–79]. While the advantage in the management of PV is evident [80], in other pathological contexts such as MF or ET (especially in the presence of a CALR mutation), antiplatelet prophylaxis is at least questionable also because of the possible bleeding complications [76–79]. In ET patients receiving ASA, the increments in f-MLP-induced PMN-CD11b and in PMN-platelet aggregates were significantly lower versus the ET subjects not treated with ASA [81]. Resistance phenomena are not infrequent, often manifesting themselves with microcirculation disorders, which can be overcome with the administration of low doses twice a day (more effective than doubling the dosage by maintaining a single administration) [82–84]. In our study on platelet fibrinogen receptor expression in MPNs, by focusing on the group of patients under ASA and with no history of thrombosis, it was found that subjects with persistent microcirculatory disorders show a higher PAC-1-binding capacity if compared to the asymptomatic ones [29]. As said before, the fibrinolytic and hypoprothrombinemic properties could be more important than the pure antiplatelet properties; for this reason, we have often wondered if ASA is really to be considered the best strategy in MPNs.

Hydroxyurea remains the most used drug in cytoreduction. Long-term experiences with this compound show excellent safety characteristics [76]. Compared to other drugs, moreover, it can dampen the generation of thrombin [85]. To determine the efficacy of the treatment, the determination of the platelet count is used, as it seems to be a good surrogate

for TG [63]. However, it could be important to introduce periodic functional tests during treatment, starting with studies that evaluate their usefulness in the long term, given the incidence of vascular events in patients under combined treatment (cytoreduction in addition to antiplatelet or anticoagulant prophylaxis). The phenomenon of resistance or intolerance to hydroxyurea also opens the problem of the absence of similarly effective strategies for controlling TG [86].

The precision therapies currently available in MPNs—namely JAK inhibitors—are capable of exerting effects on GP-VI-mediated platelet functions but have no influence on the platelet response to thrombin. This evidence also confirms the need for new and targeted treatment options [87].

Theoretically, an anti-PAR molecule could offer more complete coverage, targeting the hypercoagulability state induced by impaired thrombin generation, as well as the inflammation and promotion of fibrosis resulting from the platelet activation and crosstalk of the previously mentioned TGF-beta/ALK5 and PAR-2/PAR-1 pathways [88–95]. Such an approach could perhaps offer a contribution to slowing the evolution of the disease. However, the only drug of this class approved by the FDA for the prevention of cardiovascular events—the anti-PAR-1 Vorapaxar—seems to have clinical use limits linked to the risk of bleeding, including intracranial bleeding in patients with previous stroke [96–98]. Several authors have suggested that translational and clinical research be focused on studying anti-PAR-4, also in consideration of the PAR-4 multifaceted role [98–100]. Numerous anti-PAR-4 inhibitors have been synthetised but few have been considered for clinical trials, probably due to the difficult selection of their targets [101]. However, anti-PAR4 antibodies have been recently experimentally evaluated for their targeted action on the thrombin cleavage site of PAR4, showing, in vitro, a specific and effective action on a human thrombosis model. Among them, we found function-blocking antibodies, peptidomimetics, low molecular weight compounds, and pepducins [99]. From a preclinical perspective, the combination of THE PAR-4 and PAR-1 inhibitors has been evaluated, showing promising results [102].

Dabigatran also acts as a PAR-1 inhibitor, combining anticoagulant and antiplatelet effects [103,104]. Among the direct oral anticoagulants (DOACs), this molecule could be the subject of specific studies, even if burdened by a higher rate of haemorrhagic events in MPNs [105]. It should be noted that the Dabigatran-dependent thrombin inhibition is useful

in enhancing the inhibition of the growth and dissemination of pancreatic tumour cells by means of a synergistic effect with gemcitabine (the use in monotherapy seems instead deleterious) [106]. The safety data of DOACs, with the limitations of a retrospective study, are comparable to those of vitamin K inhibitors and these drugs appear to effectively protect against the risk of thrombotic recurrence. In patients with atrial fibrillation and MPN, the low rate of cerebrovascular ischemic events may, however, be conditioned by the concomitant use of hydroxyurea [105].

8. Conclusions

Platelets seem to significantly contribute to the pathogenesis of thrombosis within the course of MPNs. Here, we have reviewed available molecular evidence on the role of platelets, gene polymorphisms, and receptors in contributing to hypercoagulability and sustaining thrombosis. We focused on a particular pathogenic mechanism as we believe that the platelets produced by the clonal process are responsible for the increased thrombin generation and that the consequent perpetual activation of the TGF-beta/ALK5 and PAR-2/PAR-1 pathways is a central element both for the risk of vascular events and for the progression of the disease.

Here, we propose a new conceptual model, which we believe could help in identifying the next goals. In fact, even though we have significantly improved our knowledge regarding the pathogenesis, risk stratification, and effective biologic treatments of Ph-neg MPNs, arterial thrombosis and venous thromboembolism (VTE) still represent the main reasons for the morbidity and mortality of patients with MPNs [107], thus an accurate and sensitive laboratory evaluation of the risk of thrombosis onset and recurrence may positively impact the management of MPNs.

The biological complexity behind the thrombotic risk in MPNs is evidenced by the numerous potential biomarkers that can be used in the context of a liquid biopsy [108]. Our model, therefore, does not claim to be exhaustive with respect to the various mechanisms underlying hypercoagulability, but it aims to interconnect a part of them and to highlight sensitive elements for targeted therapeutic strategies. A tailored approach to MPN-related thrombosis should help to prevent bleeding complications during long-term anticoagulant treatment. Molecular therapeutic targets interfering with platelet activation may be effective in the primary prophylaxis of VTE in patients with MPNs. Furthermore, treating

coagulation disorders in MPNs in a less empirical way could have an important impact on other pathogenetic aspects and on the transformation of these diseases. The interconnection described between coagulation and fibrosis is a clear example of this. Prospective ad hoc studies based on a combined molecular and clinical approach should be designed to answer a still open “wound” in MPNs.

References

Complete list available at the following hyperlink: https://www.mdpi.com/1422-0067/22/21/11343

Phase 3: Correlation studies between genetic determinants of disease, thromboelastometry assays, evaluation of platelet fibrinogen receptor expression.

FINAL DISSERTATION

1. Abstract

Classical myeloproliferative neoplasms (MPNs) are hematopoietic stem cell disorders that manifest with inflammation, promotion of atherosclerosis, hypercoagulability, fibrosis, and clonal evolution. The complex biological background lends itself to multi-omics studies. We have previously shown that reduced platelet fibrinogen receptor (PFR) expression may follow hyperactivation of plasma-dependent mechanisms, such as tissue factor (TF) release, unbalanced thrombin generation, involvement of protease-activated receptors (PARs). Acetylsalicylic acid (ASA) helped to restore the expression of PFRs.

In this study, we enrolled 53 MPN patients, subjecting them to advanced genetic testing (panel of 30 genes in NGS), global coagulation testing (Rotational Thromboelastometry - ROTEM) and cytofluorometric determination of PFRs. ROTEM parameters appear to differ considerably depending on the type of pathology under investigation, cell count, and selected mutations. Essential thrombocythemia (ET) and CALR mutation appear to correlate with increased efficiency of both classical coagulation pathways, with significantly more contracted clot formation times (CFTs). In contrast, primary myelofibrosis (PMF) and polycythemia vera (PV) show greater imbalances in the hemostatic system. PV, probably due to its peculiar hematological features, shows a lengthening of the CFT and, at the same time, a selective contraction of parameters in INTEM with the increase of platelets and white blood cells. PMF - in contrast - seems to exploit the extrinsic pathway more to increase cell numbers.

The presence of DNMT3A mutations is associated with reduced clotting time (CT) in EXTEM, while ASXL1 causes reduced maximal lysis (ML). EZH2 could be responsible for the elongation of CFT in INTEM assay.

In addition, increased PFR expression is associated with history of hemorrhage and sustained CT time in FIBTEM under ASA prophylaxis.

Our findings corroborate the existing models on the connection between fibrosis, genetic complexity, clonal progression, and hypercoagulability. Global coagulation assays and PFR expression are potentially useful tools for dynamic evaluation of treatments' outcomes.

2. Introduction

Philadelphia-negative myeloproliferative neoplasms (MPNs) are a heterogeneous group of clonal hematopoietic stem cell disorders characterized by an abnormal proliferation of mature myeloid cells. This increase is independent of the stimulation by cytokine growth factors and derives from a gain of function of proteins with receptor tyrosine kinase function[1]. The triggering of the myeloproliferative process often begins with one of the "driver" point mutations in the Janus kinase 2 (JAK2), or calreticulin (CALR), or thrombopoietin receptor (MPN) genes. The constitutive activation of the JAK/STAT pathway and the progressive loss of heterozygosity (with consequent increase in the allelic mutation burden) are the basis of the proliferative advantage of clonal myelopoiesis[2].

Although biologically linked in a continuum, as theorized by William Dameshek in 1951 who described them as being guided by a single indefinite stimulus, the three classical forms of MPNs are clinically and phenotypically distinguishable from the histological, immunological, hemorheological and clinical points of view[3]. The evolution of the classification systems has taken into consideration aspects not only linked to increases in cell counts (erythrocytosis in Polycythemia Vera - PV and thrombocytosis in Essential Thrombocythemia - ET), restoring value to the bone marrow biopsy and affirming that of the search for clonality through molecular biology[4].

Although the three driver mutations are common to classical MPNs, their allelic burden together with a substantial genetic heterogeneity with respect to a wide range of accessory mutations, make these diseases equally heterogeneous from the point of view of clinical manifestations and the risk of progression. The purely mechanistic model of clonal progression, based on the incremental number of mutations, has now been overcome, as we

will discuss later. Nevertheless, studies on the genetic heterogeneity of MPNs, conducted mainly in the decade 2005-2015, have been of vital importance for a better understanding of both the biological mechanisms underlying clinical manifestations, and the prognostic aspects and consequently the most appropriate therapeutic choices[5].

2.1 Advanced genetics: overview

In approximately one half of patients with MPNs, additional somatic mutations in key genes for carcinogenesis may be found. In particular, there are altered sequences related to the processes of DNA methylation, chromatin modification, signalling and mRNA splicing[5,6]. Some of the most frequent additional mutations are now fully included in the diagnostic phase at the onset and during the evolution of the disease, as their detection has a strong prognostic impact, particularly in PMF[7]. In particular, a mutational profiling for the so-called "high molecular risk" (HMR), including ASXL1, EZH2, SRSF2 and IDH1/2 mutations, is useful to identify patients with a radically worse prognosis. In patients eligible for allogeneic bone marrow transplantation, the therapeutic flow-chart is now established thanks to MIPSS70 scores (the first system to include HMR) and MIPSS70-plus version 2.0 (enriched by the karyotype and the U2AF1 mutation)[8,9]. Having to identify subclones of disease, and sequence a number of genes of clinical interest, the most appropriate method to perform a molecular profile is currently the Next Generation Sequencing (NGS)[10]. The method is also useful to exclude mutations in non-conventional sequences (which is not possible by Sanger) and to enrich the information on the complex biology of the disease. Some of this information, as we will see shortly, are valuable to determine the risk of clinical events (particularly vascular) or genetic alterations that might precede the driver mutations. It is precisely the high molecular risk subclones that drive the evolution of the disease, even in more indolent forms such as ET or PV[11]. Therefore, despite the accessibility of the service, the NGS is to be considered an integral part of the diagnosis of chronic myeloproliferative neoplasms[10,11]. As proof of this, we can examine PV, which is the form most associated with Val617Phe mutation of JAK2 with a high allelic burden, such as to justify the typical manifestations: a pronounced erythrocytosis in the presence of panmyelosis, and a higher thrombotic risk. The most recent evidence shows forms of PV related to alternative mutations, which can be located within the JAK2 gene (of which it is recommended the complete sequencing) or in other genes such as SH2B3/LNK[12]. It is also

worth mentioning that some mutations in epigenetic regulators (commonly called DTA mutations), among which TET2, can be a strong indication of clonal erythrocytosis even before the appearance of classical driver mutations[13]. The coexistence of JAK2 and TET2 mutations contributes to an increased aggressiveness of the clone[14].

The clinical significance of the multiple genetic alterations found in NGS, in terms of magnitude of symptoms and splenomegaly or incidence of thrombo-haemorrhagic events, needs to be clarified. HMR mutations have been fully integrated in the IPSET score, during its validation phase for pre-fibrotic myelofibrosis (pre-PMF)[15]. In fact, they were found to be an independent risk factor for arterial thrombosis, possibly aggravated by the presence of leukocytosis. However, also DTA mutations seem to have a determinant role on thrombotic risk. In a cohort of 68 MPN patients studied with NGS by a Spanish research group, DTA mutations were associated with both older age and a higher occurrence of vascular events in PV patients. As further evidence of the association, the initial cohort was expanded to a total of 55 PV patients who were examined in an age-matched case-control study, comparing those who had experienced thrombosis with those who had a negative history of events. The presence of at least one DTA mutation, particularly TET2, was found to be associated with increased risk[16]. On the other hand, clonal hematopoiesis of undetermined potential (CHIP), characterized by DTA mutation (most often DNMT3A) is also considered state-of-the-art as an important risk factor for promoting atherosclerosis[17]. Interestingly, those who gain this mutation develop chronic pro-inflammatory conditions that appear to have their greatest effect on endothelial function[18]. Platelet dysfunction has also been hypothesized to underlie these phenomena, but at present there is no clear evidence. Finally, CHIP is found more frequently in cases of unprovoked pulmonary embolism[19].

All this evidence points to the need to evaluate the genetics of MPNs dynamically and extensively, given the growing information on the correlation between genotype and predictable clinical manifestations. However, such assessments must be placed within a paradigm that includes perpetual inflammation, dysimmunity and promotion of atherosclerotic disease as being responsible for further genotoxicity.

2.2 Modern paradigms: the "vicious circle"

In 2015, the Danish researchers Carl Hans Hasselbalch and Mads Emil Bjørn published an important perspective on MPNs as inflammatory diseases[20]. The proposed model is very convincing: a pathogenic noxa (either exogenous or of autoinflammatory nature) insists on a genetic polymorphism endowed with instability, causing a point mutation and a consequent gain of function of a protein complex with kinase activity. The uncontrolled proliferative stimulus is associated with a - more or less rapid - but incremental release of cytokines, whose most known long-term effects are atherosclerosis and the risk of carcinogenesis[21,22]. The further genetic damages insist negatively on an already compromised ground (the one occupied by the proliferating clone), worsening the pathology (**Figure 1**). Accomplice to all this is the imbalance of the mechanisms of immunosurveillance, caused by an abnormal trigger of the innate immune system. We have discussed this aspect at length in a recent review by our group[23]. The involvement of NLRP3 and AIM2 inflammasomes, protein scaffolds capable of facilitating the maturation of IL-1b and IL-18 interleukins, make MPNs very similar to rheumatic diseases for aspects closely related to chronic inflammation. These biological elements are components of innate immunity and are triggered by pathogen-associated molecular patterns or by nucleic acids of apoptotic cells (in the same way as double-stranded anti-DNA antibodies in Systemic Lupus Erythematosus - SLE)[24]. The result is inflammatory cell death as a consequence of a phenomenon commonly known as pyroptosis, that is usually associated with vascular risk conditions. Pyroptosis also influences immunosurveillance and may paradoxically lead to tumor growth or the acquisition of greater aggressiveness[25,26]. This perpetuates a mechanism of self-promotion of clonal progression and organ damage (especially vascular), which is defined by the Danes as a "vicious circle"[20].

As previously reported, there are genetic susceptibilities that allow more easily to "turn on the switch". Masselli et al. in a pivotal review have effectively recalled the role of germline variants in predisposing a subject to the onset of MPNs[27]. The polymorphisms that constitute susceptibility can be divided into five categories depending on the primary function of the gene involved: hematopoiesis, epigenetics, inflammation, DNA damage repair, cellular aging. Among these, however, stands out the haplotype 46/1 of JAK2 gene, which besides being characterized by hypermutability (probably related to an overexposure of nucleic acids to repair errors, in turn due to an increased expression), is a promoter of inflammation and immunological disorders. Part of the haplotype - always inherited en bloc as an effect of a marked linkage disequilibrium - are also the Insulin-Like (INSL) 4-6 genes,

capable of creating an inflammatory environment and at the same time permissive for the replication of emerging clones[28].

In summary, the biological continuum theorized by Dameshek is progressively proving to be concrete. MPNs can be occult diseases, with an ancestral origin, and with the simultaneous acquisition of elements that drive their emergence. These elements are primarily genetic but supported by chronic inflammation and alterations in immunoregulation. All the studies suggest the need to make the diagnostic phase more articulate and deeper, or even to anticipate the biomolecular investigations in NGS to a pre-diagnostic phase if there is a clear familiarity.

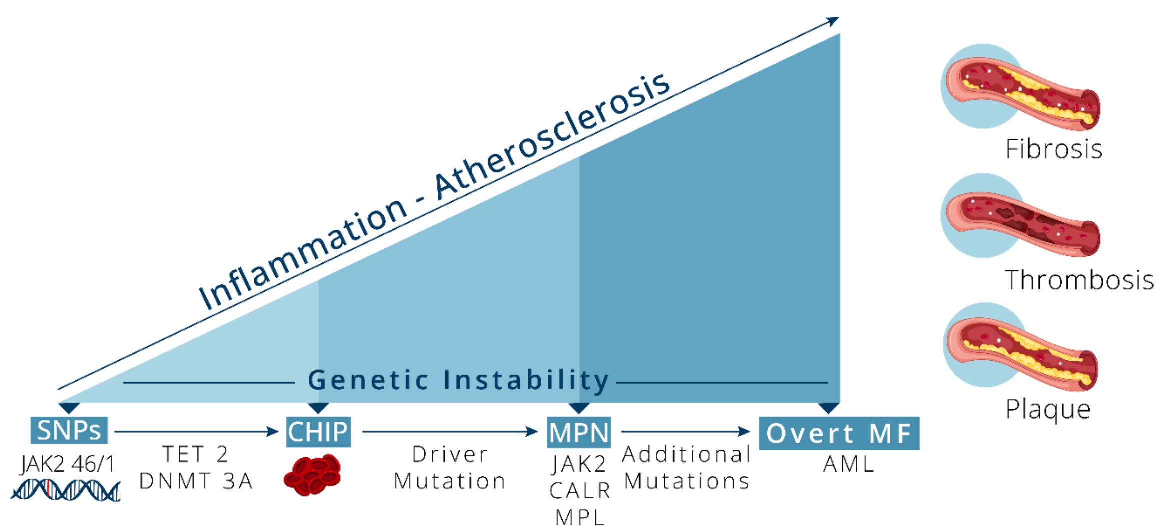


Figure 1. Pattern of clonal progression. Genetic complexity conditions the degree of inflammation, which in turn is responsible for promoting atherosclerosis and fibrosis. In turn, inflammation and dysimmunity are major genotoxic stimuli.

2.3 Modern paradigms: the "circulating wound"

The treatment of MPNs has for many years had a radically empirical approach, based on the association of cytoreductive drugs (with hydroxyurea in the first line) and antiplatelet agents (usually acetylsalicylic acid - ASA)[29]. The goal of containing platelet count is however challenged by the fact that vascular events can occur independently of it[30], while for the use of ASA there is convincing evidence on the benefits of prophylaxis are in patients with PV, thanks to the study of the European Collaboration on Low-dose Aspirin in Polycythemia Vera (ECLAP)[31]. Although many studies on platelet function have been proposed, the results are often inconclusive and sometimes misleading, also for reasons

related to the population under examination and the reproducibility of the experiments[32]. Even more likely, the genetic heterogeneity influences the "quality" of the cellular components put into circulation, as well as the state of endothelial inflammation[33,34]. There is undoubtedly more evidence of an increased interaction between white blood cells and platelets, through the monocyte integrin CD11b[35,36]. It is probably on these mechanisms that standard therapeutic combinations work best[37], along with modulation of some procoagulant factors (as we will see later), but in clinical practice the focus continues to be on cell counts rather than functional aspects.

Our research group recently focused on the role of the platelet fibrinogen receptor (PFR)[38]. This aptene is in fact expressed on the surface of the platelet only after a conformational change of glycoprotein IIbIIIa (GPIIbIIIa), a process that occurs only when the platelet is activated[39]. PFRs are hyperexpressed under conditions of shear stress or rolling friction: their evaluation was therefore particularly appropriate in view of the microcirculation disorders that often characterize MPNs and that seem to be attenuated using ASA[40]. For evaluation, we used cytofluorometric analysis with the PFR-selective PAC-1 antibody. Unexpectedly, we found a significantly reduced expression of PFR in patients with MPNs and naïve to any treatment - both cytoreductive and antiplatelet - if compared to values recorded in healthy volunteers (HV).

Paradoxically, the intake of ASA was able to restore values very close to those observed in HV. The explanation of this phenomenon is provided by Moore et al[41], since the authors describe an altered binding to fibrinogen by platelets in ET. This alteration would be due to a rapid disappearance of fibrinogen binding sites, caused by the activation of Protease Activated Receptors (PARs), capable of rapidly converting functional fibrinogen into fibrin. This activation is operated by thrombin in its protease function, whose role in MPNs is quite well known[42–44]. The ASA, also known for its hypoprothrombinemic and hyperfibrinolytic properties[45,46], would therefore allow a partial restoration of the balance, trying to slow down the effects of a marked generation of thrombin in these diseases. In this context, there are three other important aspects to consider:

1) platelets produced by the clonal process in MPNs are rich in tissue factor (TF), the release of which is effectively reduced by the use of hydroxyurea[47];

2) the rapid disposal of mature cells is associated with an abundant release of extracellular vesicles, which also have a TF-dependent procoagulant action, as well as proinflammatory, immunoregulatory and microenvironmental conditioning functions[48,49];

3) PARs have a series of activities favoring hemostasis, ranging from the regulation of vascular tone to platelet activation, but above all they present a signaling pathway that interacts with that of TGF-beta, making themselves promoters of fibrosis[50].

There is therefore a close connection between hypercoagulability, inflammation, and fibrosis, in a model that simulates perpetually the attempt to repair a non-existent wound. Our group, which has schematically reconstructed these processes, has termed this paradigm the "circulating wound"[51].

Taking a cue from the models mentioned so far, **Figure 2** below shows some of the mechanisms of interconnection between inflammation, initiation of hemocoagulative processes, vascular damage, fibrosis, and dysimmunity.

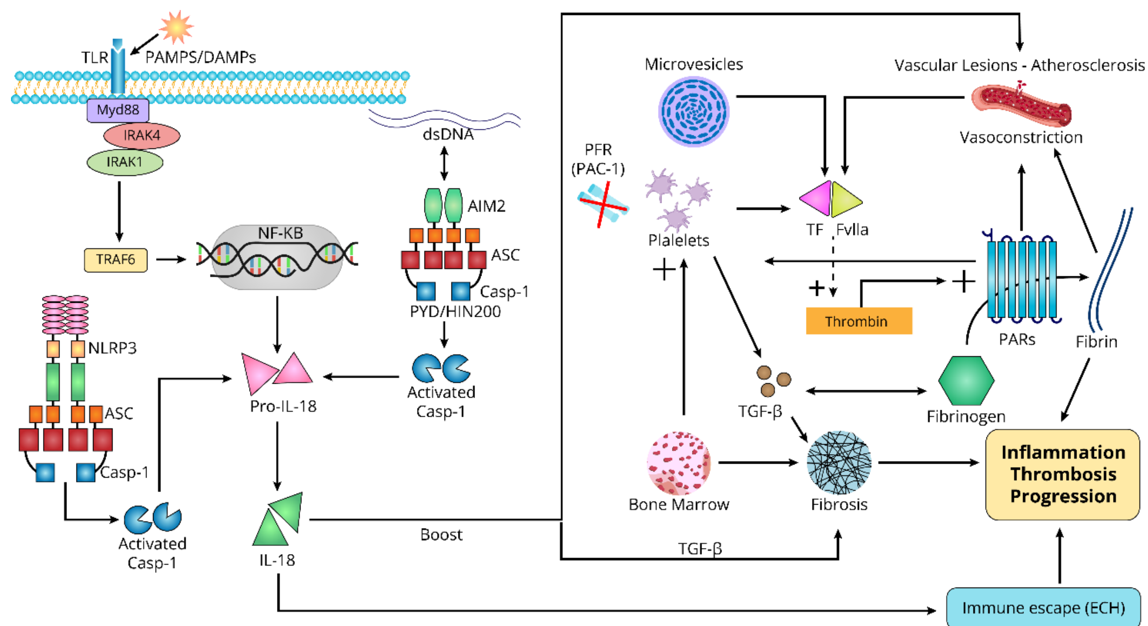


Figure 2. The triggering of inflammasomes and caspase-1 matures pro-inflammatory cytokines such as IL-18, a known promoter of atherosclerosis and immune escape, and enhancer of TGF-beta actions. However, the TGF-beta pathway is also triggered by coagulation imbalances and hyperactivity of PARs. Fibrosis, thrombosis, and clonal progression are the ultimate consequences.

2.4 Global coagulation assays: existing evidence in MPNs

Despite their propensity for vascular events, MPNs do not exhibit abnormalities of conventional coagulation tests (CCTs). This is probably related to the fact that CCTs are not able to evaluate complex systems of interaction between platelets, white blood cells, vascular endothelium, coagulation factors, blood viscosity. The state of hypercoagulability or hemorrhagic diathesis often becomes manifest with clinical rather than laboratory elements. In clinical practice is spreading the use of instruments capable of assessing any imbalances in the viscoelastic properties of whole blood, verifying the integrity of the classic pathways of coagulation and the contribution of fibrinogen to the formation of the clot. Thromboelastography (TEG) and rotational thromboelastometry (ROTEM) are the most widely used tests. They are part of the so-called global coagulation assays (GCAs) and are currently most useful in hemorrhagic emergencies and post-traumatic coagulopathies[52,53]. GTAs have already been used in MPNs, but data are still rather scant.

Studies to date with ROTEM have predominantly identified a higher maximum clot firmness (MCF) indicating a hypercoagulable state in MPNs compared to healthy volunteers. MCF and clot formation time (CFT) values correlated with platelet count[54]. The lengthening of the clotting time (CT) found by Sahin et al, highlights an instability in the contribution of plasma factors of coagulation, which would then become a pro-coagulant condition only with the contribution of the cellular component[55]. The study by Trelinski et al, focusing on patients with ET, shows a marked difference in CT and CFT values compared to healthy controls, a difference that was smoothed out by the administration of hydroxyurea. The article also highlights two interesting parameters that can be analyzed: FIBTEM (which evaluates the contribution of fibrinogen independently from that of platelets) and NATEM. The latter is the least specific, since it does not use coagulation activators, but it can offer an overview of the initiation and propagation of the processes. Although with respect to different parameters for the different assays, all tests were compatible with a state of hypercoagulability. In virtue of these preliminary findings, we can think of ROTEM as one of the instruments to be studied in the best way for the dynamic evaluation of hemostatic balances in MPNs.

2.5 Aim and design of the study

Starting from existing models of clonal progression, and from their parallelism with inflammation and haemostatic imbalances, the study aims to establish whether in patients

with MPNs there is a relationship between genetic determinants of the disease and any differences in the outcome of global coagulation assays. Thromboelastometry allows us to "didactically" separate the contribution of the two classic pathways of coagulation and fibrinogen turnover to clot formation. Contextually, we repeated the experience of assessing the platelet fibrinogen receptor to determine whether there was a relationship between a reduction in it and the change in global hemocoagulative pattern. Discovering possible discrepancies in the ignition of hypercoagulability states between more "indolent" diseases from more aggressive ones is important both for differentiating the antithrombotic strategies and for confirming the validity of the paradigms theorized so far. We decided to focus on the treatment-naïve population and on subjects who were receiving antiplatelet therapy only, given the known influence of cytoreductive therapy on TF-induced haemostatic processes and in order to obtain as much information as possible on the conditions prior to clinical intervention.

3. Materials and methods

3.1 Sample collection

Samples from 53 MPN patients who never received cytoreductive agents or targeted therapy were collected at the IRCCS Istituto Romagnolo per lo Studio dei Tumori (IRST) "Dino Amadori", Meldola, Italy. For some of the enrolled patients, additional blood draws were taken to assess the changes after introduction or discontinuation of ASA. Therefore, there were a total of 63 laboratory determinations. All patients provided informed consent in accordance with the Declaration of Helsinki. The study was approved by the Institutional Review Board (IRB). Subjects who met the study criteria were consecutively enrolled for a total period of three years (September 2018-September 2021). Four subcategories of MPNs out of seven were included, according to the new edition of the 2016 World Health Organization (WHO) classification system for tumors of the hematopoietic and lymphoid tissues: polycythemia vera (PV), primary myelofibrosis (PMF), essential thrombocythemia (ET) and MPN - unclassifiable (MPN-U).

Table 1 shows the complete list of inclusion and exclusion criteria, while patients' characteristics are shown in **Table 2a and 2b**.

Table 1. List of inclusion and exclusion criteria

Inclusion and exclusion criteria	**Inclusion criteria**: 1. Ability to understand and the willingness to sign a written informed consent document. 2. Male or Female 3. Age ≥ 18 years 4. Diagnosis of MPN (ET, PV, PMF, MPN-U) according to the WHO 2016 classification **Exclusion criteria**: 1. Age < 18 years 2. Subjects treated with cytoreduction, target therapies, or other disease-specific treatments (excluding antiplatelet agents) 3. Other malignant neoplasia with a disease-free interval of less than 5 years. 4. Liver disorder or alanine aminotransferase more than 2 upper limit range. 5. Known coagulation disorders (both congenital and acquired) 6. Surgery in the past 4 weeks

Table 2a. Subjects characteristics (n=63)

Variable	Overall patients N=53 (%)
Age	
Median (range)	57.9 (24.5-85.6)
Gender	
Female	27 (50.9)
Male	26 (49.1)
Aspirin	
No	26 (50.0)
Yes	26 (50.0)
Unk	1
Disease	
MF	15 (28.3)
MPN-U	4 (7.6)
PV	14 (26.4)
TE	20 (37.7)
JAK 2	
Not mut	19 (35.9)
Mutati	34 (64.1)
CALR	
Not mutated	46 (85.8)
Mutated	7 (13.2)
MPL	
Not mut	49 (92.4)
Mutati	4 (7.6)
EXH2	
Not mut	50 (94.3)
Mutati	3 (5.7)
DMNT	
Not mut	41 (77.4)
Mutati	12 (22.6)
ASX	
Not mut	47 (88.7)
Mutati	6 (11.3)
TET	
Not mut	44 (83.0)
Mutati	9 (17.0)

Table 2b. Associations between driver mutations and DTA/HMR.

Driver/additional	EZH2 (=3)	DNMT3A (=12)	ASXL1 (=6)	TET2 (=9)*
JAK2	2	8	3	8
MPL	-	1	2	2
CALR	-	2	1	-

*one patient was detected as carrier of 2 driver mutations (JAK2 + MPL)

Although the mutational picture detected with the NGS panel was sometimes more complex and characterized by additional mutations, we considered only those suitable for an investigation of comparison with the parameters of PFR and ROTEM. The frequency of mutations follows the already known one[56], while the selection of patients who were not receiving cytoreductive therapy slightly influenced the median age, which is reduced. The low percentage of patients treated with ASA is instead due to the enrollment in the diagnostic phase and to the inclusion of patients who, due to very low thrombotic risk, were not candidates for any treatment.

3.2 Flow cytometry

Our aim was to verify the expression of platelet fibrinogen receptors (PFRs). In analogy to our previous experiments[38], both sodium citrate and heparin tubes were collected from the same patient (positive control of platelet activation). Within 10 min from blood sampling, 5 µl of whole blood from each tube was incubated for 20 min at room temperature in the dark with a saturated concentration of CD61 peridinin-chlorophyll proteins (PerCP), CD62P phycoerythrin (PE) and PAC-1 fluorescein isothiocyanate (FITC). Positive control was also incubated with PAC-1 in the presence of Arg–Gly–Asp–Ser (RGDS) in order to test the specific antibody binding. Samples were fixed with paraformaldehyde 1% for 30 min at 4°C in the dark and analyzed on a flow cytometer. Flow cytometric analyses were performed using a FACSCanto flow cytometer (Becton–Dickinson, Franklin Lakes, NJ, USA) and 50 000 events were recorded for each sample.

3.3 Isolation of Mononuclear Cells

Bone marrow mononuclear cells (BMMCs) and peripheral blood mononuclear cells (PBMCs) were isolated from EDTA tubes by Ficoll density-gradient using Lymphosep (Biowest) and lysed in RLT buffer (Qiagen, Ltd) additioned of 1% ß-mercaptoethanol.

3.4 Genomic DNA isolation

DNA was extracted from primary mononuclear cells (BMMCs or PBMCs) using the Maxwell® RSC Blood DNA Kit (Promega Corporation) according to the manufacturer's instructions. DNA from T cells (invitrogen) lysed in TRIzol™ reagent was obtained according to the manufacturer user guide.

3.5 Next Generation Sequencing: libraries preparation and variant annotation

The mutational profile of XX patients was determined using SOPHiA Myeloid Solution™ (SOPHiA GENETICS), a CE-IVD marked molecular diagnostic application. Libraries were prepared according to the manufacturer's instructions and quantified using the Qubit® dsDNA High Sensitivity Assay on a Qubit® Fluorometer (Thermo Fisher Scientific, Inc.). The median size of amplicons was determined by capillary electrophoresis using Agilent High Sensitivity DNA kit (Agilent Technologies, Inc.). The pooled libraries were paired-end (2×301) and sequenced with v3 chemistry on a MiSeq™ instrument (Illumina, Inc.), as described in the manufacturer's protocol. FASTQ sequencing files were uploaded onto the SOPHiA DDM® platform (version 4), which uses patented advanced technologies for variant calling and annotation. Human Genome Build 19 (Hg19) was used as the reference for sequence alignment. A minimum coverage depth of 1,000× was recommended. A filtering tool was set to exclude known single-nucleotide polymorphisms, variants localized in intronic and UTR regions and synonymous variants. Only exonic and splice site variants with a variant allele frequency (VAF) $\geq$ 1% were evaluated.

3.6 Rotational thromboelastometry (ROTEM®)

Global Coagulation Assays (GCAs) data were recorded and analyzed with a ROTEM® delta thromboelastometry analyzer (Werfen). INTEM, EXTEM and FIBTEM assays were performed and coagulation time (CT, min), Clotting Formation Time (CFT, min), Clot

amplitude at progressing timepoints expressed as A5, A10, A20 (mm), Maximum Clot Firmness (MCF, mm) and Maximum Lysis (ML, %) were recorded. We used whole blood, collected in a 3-mL sodium citrate tube, at room temperature. Although basic results can be extrapolated within 10 minutes with this instrument, we observed thromboelastographic curves and associated data for 1 hour in total.

In the ROTEM® system, the sample is placed into a cuvette and a cylindrical pin is immersed. Between pin and cuvette remains a gap of 1 mm, which is bridged by the blood or the blood clot. The pin is rotated by a spring alternating to the right and the left. As long as the blood is liquid, this movement is unrestricted. As soon as the blood clots, the clot restricts the rotation of the pin increasingly with rising clot firmness. Thus, the rotation of the pin is inverse proportional to the clot firmness. It is detected optically. An integrated computer calculates the ROTEM® curve as well as its numerical parameters.

The most important parameters that can be evaluated with the instrument are the following:

- ✓ CT (clotting time): time from start of the measurement until initiation of clotting o initiation of clotting, thrombin formation, start of clot polymerization;
- ✓ CFT (clot formation time): time from initiation of clotting until a clot firmness of 20 mm is detected o fibrin polymerisation, stabilisation of the clot with thrombocytes and FXIII;
- ✓ MCF (maximum clot firmness): firmness of the clot o increasing stabilisation of the clot by the polymerised fibrin, thrombocytes as well as FXIII;
- ✓ ML (maximum lysis): reduction of clot firmness after MCF in relation to MCF o stability of the clot (ML< 15%) or fibrinolysis (ML > 15% within 1h).

3.7 Statistical methods

Continuous data were expressed as median and range or interquartile range (IQR range). For patient samples, normality distribution of ROTEM parameters values was tested using Shapiro-Wilk test. Nonparametric tests were used: the association between ROTEM parameters and ASA, mutations or other factors was assessed by Mann-Whitney or Kruskal Wallis test, depending on number of groups. Spearman's correlation index was calculated to analyze the relationship between ROTEM parameters white blood cell count, neutrophils, platelets, hemoglobin. Univariable and multivariable linear regression analysis

will be used to evaluate potential independent factors among white blood cell count, neutrophils, platelets, hemoglobin respect to ROTEM parameters.

Box-plots or scatter plots have been presented as descriptive purposes.

A p-value of 0.05 was adopted for all statistical analysis. All statistical analyses were performed using Stata/SE version 15.1 for Windows (StataCorpLP, College Station, TX, USA).

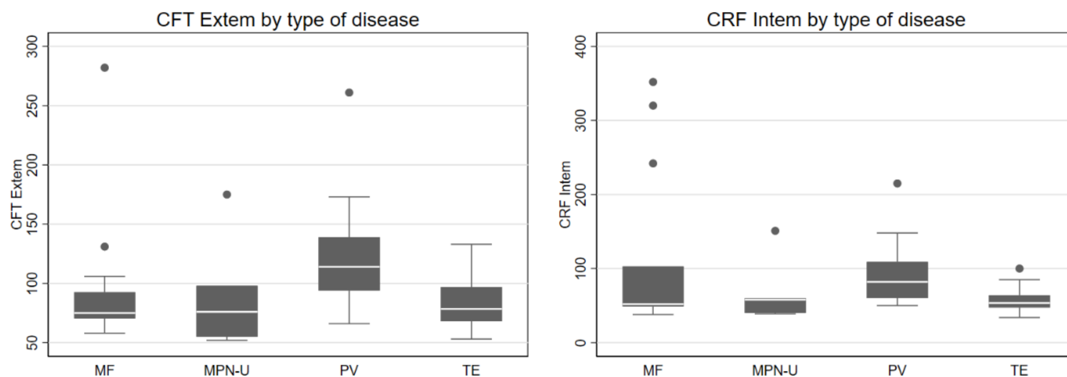
4. Results

The results of the experiments show that ROTEM parameters, with particular reference to CFT relative to INTEM and EXTEM, are conditioned by the type of myeloproliferative disease, certain key mutations, and the number of white blood cells (rather than that of platelets or other cell counts). The contextual analysis of PFR expression allowed to establish under which conditions they were more reduced or more modifiable with the use of ASA, as well as their relationship with the GCAs.

4.1 Disease-specific parameters

Comparing the CFT (EXTEM) and CRF (INTEM) among diseases, only patients with PV had higher median CFT values than those found in the other MPNs (**Figure 3a and 3b**). Those measured in EXTEM (Figure 3a) have median values of 114 sec (IQR range 94-139) in PV, while in ET, PMF and MPN-U the median CFT was of 78.5 (IQR range:70.5-92.5), 75.0 (IQR range:70.5-92.5) and 76.0 sec (IQR range:55.0-98.0) respectively (p= 0.008). The alteration is also found using the INTEM test (Figure 3b), with median CFT values of 82 sec (IQR range: 60-109) for PV and 53.5 (IQR range:47.5-64.0), 52 (IQR range:49.0-103.0) and 58.0 (IQR range:40.0-60.0) for ET, PMF and MPN-U respectively (p=0.027). No differences with statistical significance were found for the other parameters (clot amplitude, MCF, ML).

Figure 3a and 3b. Clot formation time (CFT) variability among different MPNs.



4.2 ROTEM parameters in patients on prophylaxis with acetylsalicylic acid

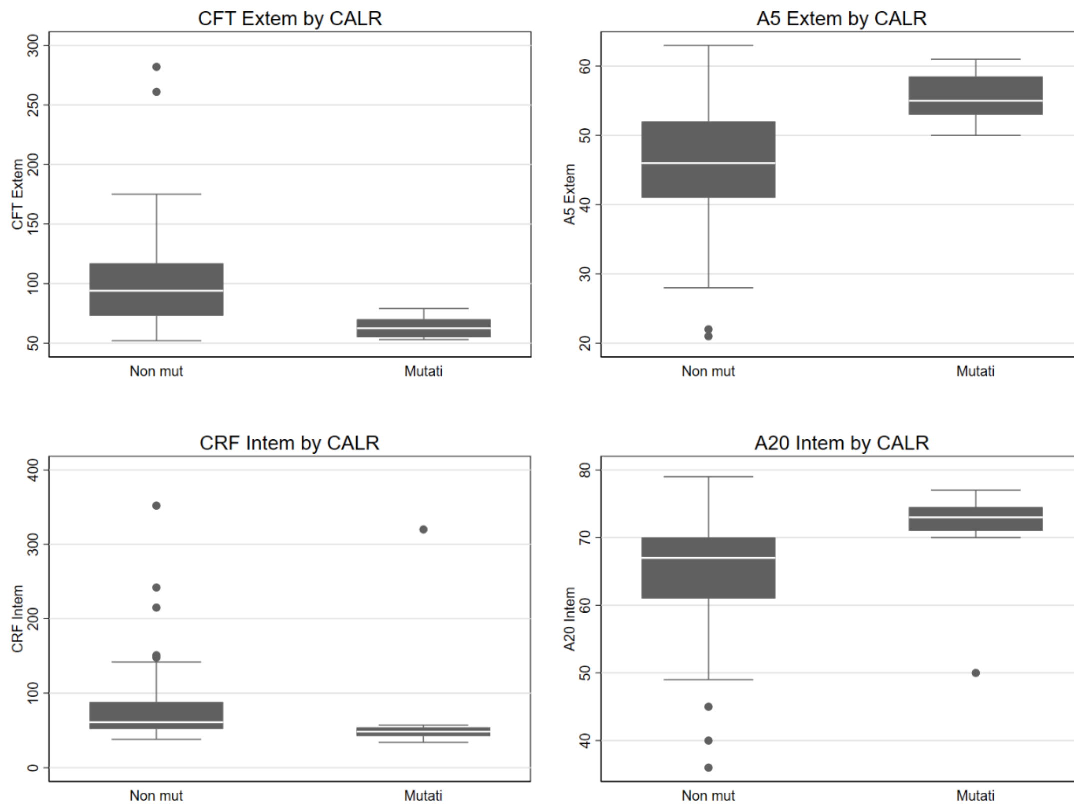
In the standard thromboelastometric analysis, the contribution of the antiplatelet was generally irrelevant. However, since our initial hypothesis considered a role of ASA in modulating some functions of the hemostatic system (promotion of fibrinolysis and hypoprothrombinemic effect), patients treated with this drug were compared with those not yet under prophylaxis. Statistical analysis did not provide significant differences, not even among PV patients, who were equally divided with respect to ASA intake. Considering the totality of patients, and consistent with the underlying hypothesis, however, ASA appears to contribute to an increase in the rate of maximum lysis (ML), raising it from 4% to 6% ($p=0.003$). The usefulness of this information is however doubtful, given that the results are contracted to very low values compared to those defined as significant by the instrument (15%).

4.3 Mutation-specific parameters

The presence of selected mutations seems to affect - across all diseases - the parameters of thromboelastometry.

As shown in **Figure 4 a-d**, the patients with CALR mutation (7/53), a shortening of CFT (median 62.5 sec, IQR range: 55.0-70.0 vs 94 sec, IQR range:73.0-117.0, $p<0.001$) and an increase in clot amplitude have been observed, with values that at INTEM become more and more significant with increasing observation time (A20, 73.0, IQR range 71.0-74.4) vs 67 mm, IQR range, $p=0.005$), and at EXTEM find the highest significance at A5 (55 mm, IQR range:53.0-58.5, vs 46 mm, IQR range:41.0-52.0, $p<0,001$). The variant allele fraction (VAF, %) of the CALR mutation was not directly related to specific changes.

Figure 4a-d. Significant variations of ROTEM parameters in CALR mutated subjects.



No statistically significant differences were found in the outcomes of ROTEM assays with respect to the other two driver mutations (CALR, MPL).

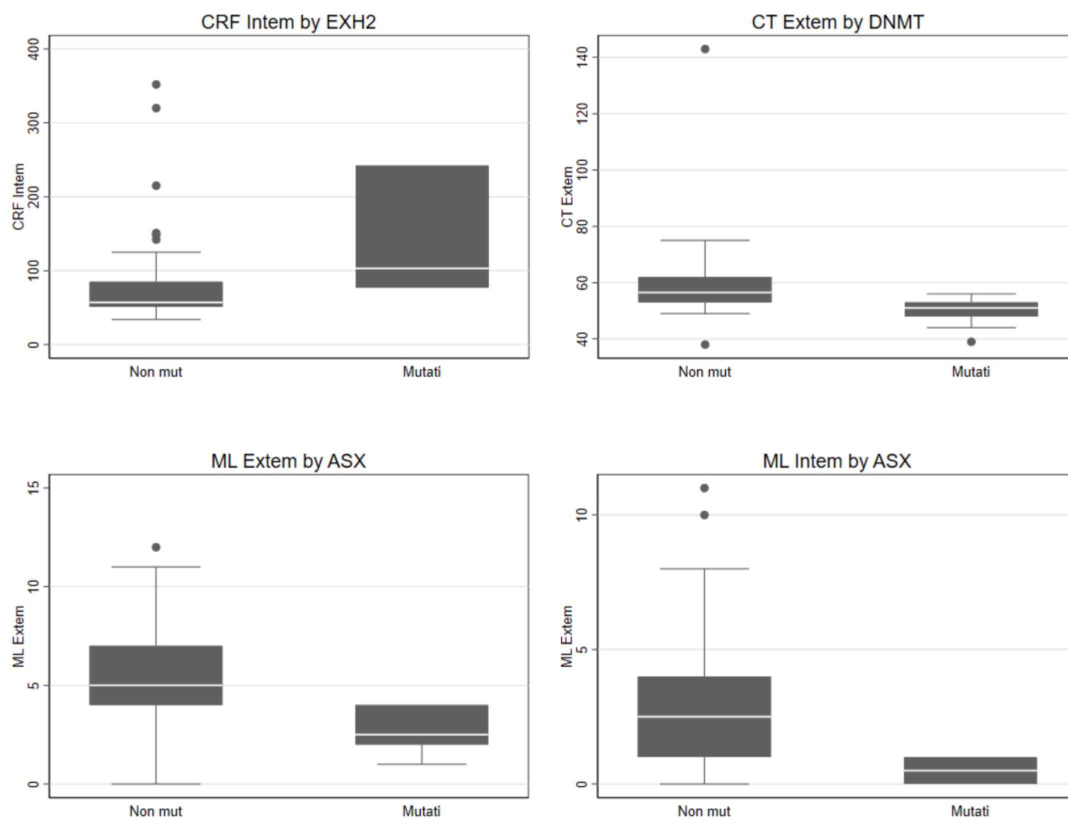
The mutational status of EZH2 appears to have an important impact on assessment in INTEM. In fact, the median CFT is almost doubled in mutated patients with respect to those without EZH2 mutation (103 sec, IQR range: 77.0-242.0 vs 57 sec, IQR range: 51.0-85.0, p=0.043). Contextually, a smaller clot amplitude is appreciated in the early stages (A5, A10, the latter with statistical significance). The already low number of EZH2 mutated patients is however affected by the presence of an elderly subject, with vascular history and a complex genetic picture, involving JAK2, MPL, EZH2 and TET2. This patient had pathologically prolonged CFT values, therefore the reliability of the overall data is questionable. More interestingly, in all patients analyzed ML remains stationary at 0% (**Figure 5a**).

Patients mutated for DNMT3A, which constituted 20.6% of the subjects enrolled in our study, presented a significant reduction in median EXTEM CT values (51 sec, IQR range 48-53 vs 56.5 sec, IQR range 53.0-62.0; p<0.001). The alteration is specific to this

parameter and does not affect at all the other indices of the thromboelastometric analysis (**Figure 5b**).

Finally, ASXL1 mutation appears to confer resistance to lysis, with significantly reduced ML values in both EXTEM (2.5%, IQR range 2.0-4.0) vs 5%, IQR range: 4.0-7.0, p=0.007) and INTEM (0.5%, IQR range:0.0-1.0, vs 2.5%, IQR range:1.0-4.0, p=0.003).

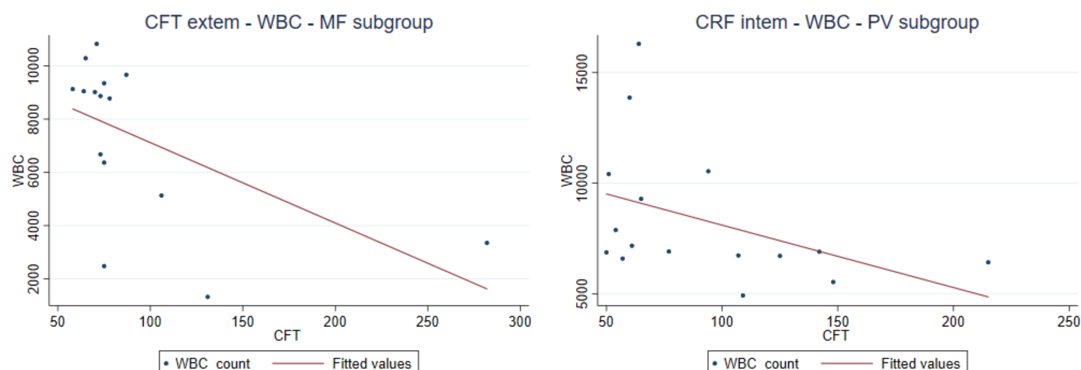
Figure 5a-c. Significant variations of ROTEM parameters in EZH2, DNMT3A and ASXL1 mutated subjects.



4.4 Relationship between cell counts and ROTEM parameters

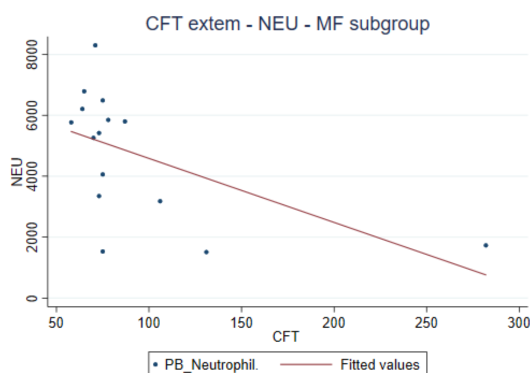
We identified two important inverse correlations between white blood cell count (WBC) and CFT in PMF and PV subgroup (**Figure 6a and 6b**). Remarkably, INTEM was involved in PV (rho= -0.61), while in PMF the modified value was that of EXTEM (rho= -0.52).

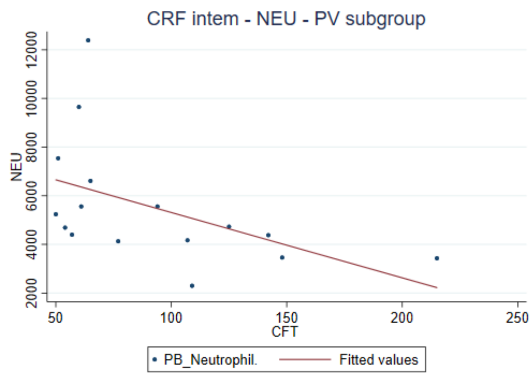
Figure 6a and 6b. Relationship between WBC and clot formation time (CFT) in patients with PMF and PV.

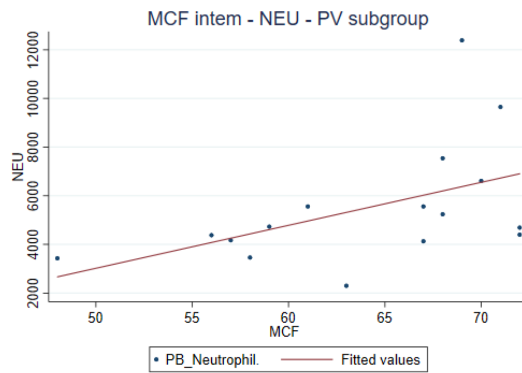


Given the evidence on the role of neutrophils (NEU) described above, we considered their count in the analysis, confirming the impact on CFT in EXTEM in PMF and in INTEM for PV (**Figure 7a and 7b**). In the latter, however, we could also verify an impact on the increase of MCF (rho= 0.57, p= 0.021, **Figure 7c**) and on clot amplitude parameters. For PMF, a higher number of neutrophils was associated with a decrease in MCF values when analyzed with FIBTEM (rho= -0.52, p=0.045).

Figure 7a-c. Impact of neutrophil count (NEU) on clot formation time (CFT) in PMF and PV.

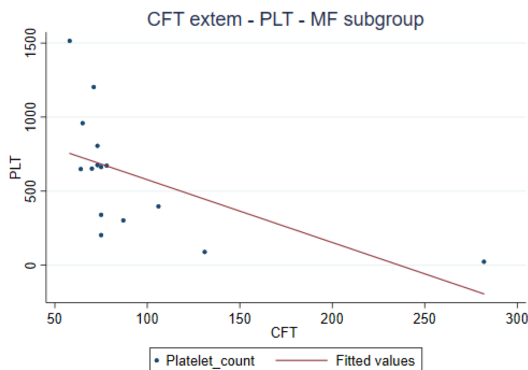


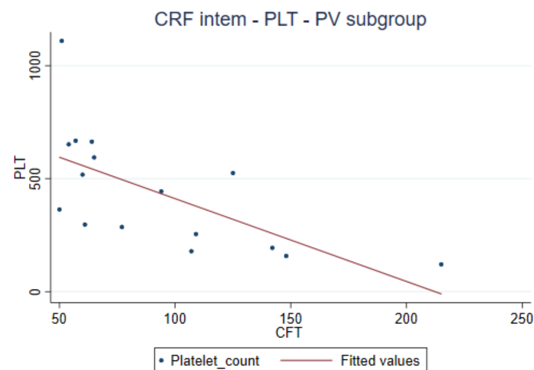


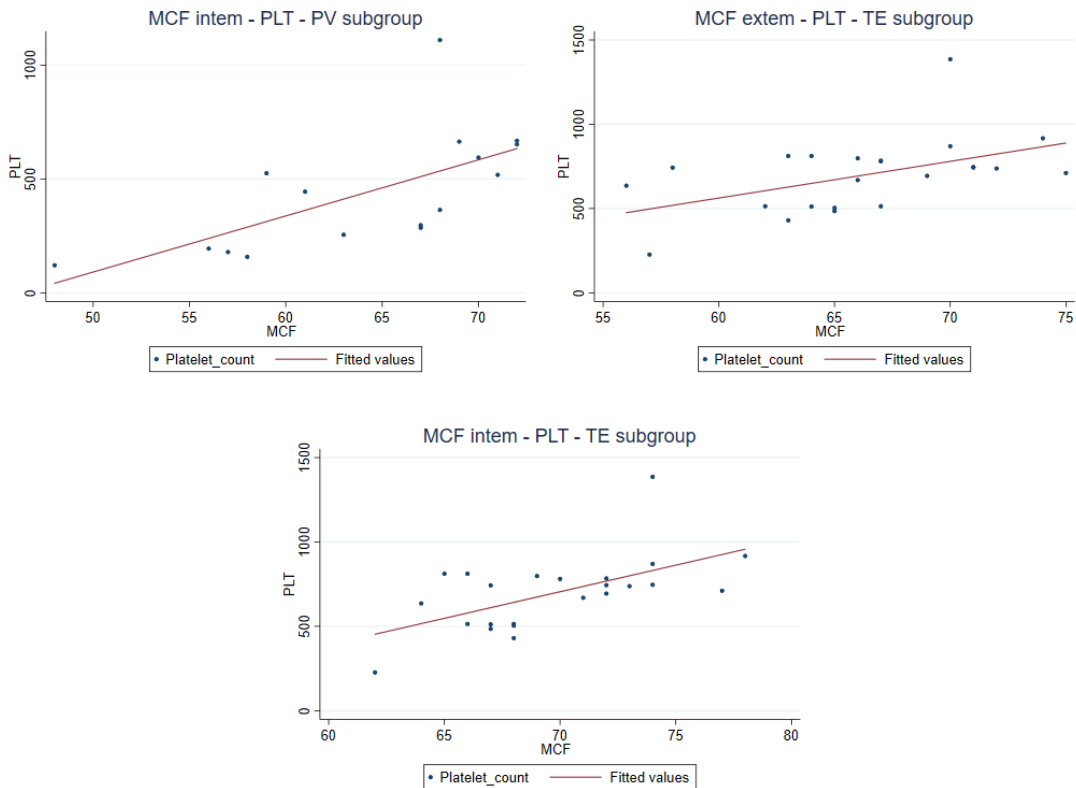


However, a significant impact on ROTEM analysis seems to be provided across all diseases by platelet counts. To confirm the validity of the observations, the CT was not significantly affected, while the CFT values on EXTEM were affected for all conditions, but particularly for PMF (rho= -0.75, p=0.001, **Figure 8a**), the CFT values on INTEM only for PV (rho= -0.73, p= 0.001, **Figure 8b**) and the MCF values for ET and PV (only for INTEM on PV and on both pathways for ET, **Figure 8c-e**).

Figure 8a-e. Main impact of platelet count on clot formation time (CFT) and maximum clot firmness (MCF) in different MPNs.







For PMF and PV, in which both WBC and Plt values were associated to CFT parameters; to evaluate the potential independent factors We carried out the univariable models and multivariable models with CFT as dependent variable. In the multivariable model for PMF patients, only the platelet value maintained statistical significance ($p<0.001$), as shown in **Table 3**. It is therefore possible to assert that the EXTEM CFT for PMF value depends primarily on the platelet count.

Table 3. Comparison of univariable and multivariable models for cell counts with respect to EXTEM clot formation time (CFT) in the PMF subgroup

	Univariable models			Multivariable model		
Variable	Coefficient	p-value	95% CI	Coefficient	p-value	95% CI
Plt (log2)	-0.33	<0.001	-0.42; -0.24	-0.41	<0.001	-0.56;-0.25
WBC (log2)	-0.39	0.017	-0.69; -0.08	0.08	0.728	-0.42;0.58
NEU (log2)	-0.46	0.007	-0.77-;-0.15	0.10	0.717	-0.51; 0.72

We repeated the analysis for PV, which had deviations in CFT values only on INTEM for WBC and in both tests when platelets were considered. Again, the contribution of platelets in multivariable analysis seems to be greater, but hemoglobin (Hb) levels can be added to it, offering an even more relevant contribution (**Table 4 and 5**). It is important to note that for the CFT related to EXTEM, only Hb turns out to be significant in multivariable analysis.

The impact of at least one DTA mutation, rather frequent than PVs, does not appear to be considerable.

Table 4. Comparison of univariable and multivariable models for cell counts with respect to EXTEM clot formation time (CFT) in the PV subgroup.

	Univariable models			Multivariable model		
Variable	Coefficient	p-value	95% CI	Coefficient	p-value	95% CI
HB (log2)	2.09	0.002	0.89; 3.29	1.51	0.022	0.25; 2.78
WBC (log2)	-0.17	0.577	-0.81 ;0.47			
NEU (log2)	-0.25	0.281	-0.75 ; 0.24			
DTA mutation (1 vs no)	0.01	0.976	-0.54 ; 0.55			
Plt (log2)	-0.38	0.007	-0.63 ; -0.12	-0.23	0.068	-0.48; 0.02

Table 4. Comparison of univariable and multivariable models for cell counts with respect to INTEM clot formation time (CFT) in the PV subgroup.

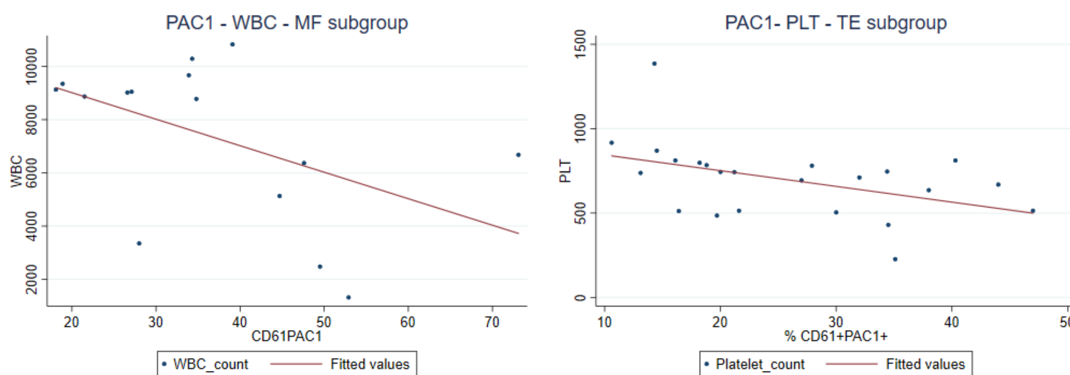
	Univariable models			Multivariable model		
Variable	Coefficient	p-value	95% CI	Coefficient	p-value	95% CI
HB (log2)	2.72	0.001	1.36 ; 4.08	1.73	0.007	0.58 ; 2.90
WBC (log2)	-0.66	0.060	-1.35 ; 0.03			
NEU (log2)	-0.63	0.018	-1.14; -0.12	-0.13	0.491	-0.53 ; 0.27
DTA mutation (1 vs no)	0.29	0.343	-0.34 ; 0.94			
Plt (log2)	-0.56	<0.001	-0.81 ; -0.31	-0.34	0.025	-0.62; -0.04

4.5 Platelet fibrinogen receptors

We firstly hypothesized the potential inverse relation between expression of PFRs and thrombin generation, by activation of PARs and rapid conversion of functional fibrinogen to fibrin. PFR expression was particularly low, confirming the results of the previous study: median 26.1% in 62 laboratory observations of the 53 patients in the study. The value is reduced especially in PVs in the absence of ASA, standing at 21.9%. In this study PV seems to be the only pathology in which the use of ASA seems to determine a statistically significant recovery of PFRs expression (median 33.2, IQR range: 27.0-37.4, p=0.046 vs median 22.8, IQR range: 18.8-29.4). The antiplatelet agent also seems to be able to reduce MCF and clot width at FIBTEM analysis in ET patients, at the limits of statistical significance (data not shown). The data are however very interesting because the test is conducted with a cytoskeletal inhibitor of platelet function and should selectively measure fibrinogen levels and the quality of fibrin polymerization.

Remarkably, expression of PFRs appears to have an inverse relationship with cell counts and CFT, confirming that its disappearance from the surface of circulating platelets has a bearing on hypercoagulable states. In PMF, for example, PFRs are less expressed in conjunction with higher WBC counts (rho=-0.52, p=0.044, **Figure 9a**), the same context in which a shortening of CFT is evident in EXTEM analysis. However, this finding is not confirmed if only neutrophils are considered. The correlation with returns to be significant - in an inverse way - with the platelet count in the ET (rho=-0.51, p=0.012, **Figure 9b**), and therefore with the shortening of the CFT of EXTEM.

Figure 9a and bb. Relationship between cell counts and expression of platelet fibrinogen receptors (PFRs) in PMF and ET.



Regarding the relationship between PFRs expression and mutations, only CALR mutation seems to be associated with a reduction of events in cytofluorimetry (median 25.3%, IQR range: 20.2-30.7, vs 33.6%, IQR range:26.5-44.7, p=0.045). For all other mutations examined individually, the variation was not significant (data not shown).

4.6 Thrombosis

7/53 patients (13.2%) had as many thrombotic events during their clinical history. There were 3 arterial thromboses, two of which were major (acute myocardial infarction), and 4 venous thromboses, two of which were major (pulmonary thromboembolism, intracranial).

The genetic makeup of the subjects affected by thrombosis was peculiar: 6/7 had at least one DTA mutation, 2/7 more than one DTA mutation, 2/7 an HMR due to the presence of

ASXL1 or EZH2, 2/7 the simultaneous presence of two driver mutations (JAK2 and MPL). Taken individually, an impressive heterogeneity is evident among the data of the GCAs of these patients, with extremely contracted or prolonged values, but without a clear relationship with the type of thrombosis. Of note, probably due to this heterogeneity, no statistical significance emerged either for ROTEM or PFR parameters.

4.7 Haemorrhage

4/53 patients (7.5%) had bleeding in their medical history. Of these events, two were minor (epistaxis and ecchymosis) and two major (intracranial hemorrhage, without fatal outcome). 100% of patients were mutated for the JAK2 gene, 1 for TET2, 1 for MPL and CBL, 1 for EZH2.

Two important associations were found:

- significantly higher expression of PFRs in patients with a history of bleeding (41.6%, IQR range: 38.6-58.6, vs 29.4%, IQR range:22.8-38.9, p= 0.038);

- a prolongation of FIBTEM CT (64 sec, IQR range: 59.5-79.5, vs 56 sec, IQR range: 51.0-59.0, p=0.033).

All patients were taking ASA because of their JAK2 positivity.

No associations were found between hemorrhage and particular mutations, also because of the relatively low number of subjects who manifested the event.

5. Discussion

The study confirms a marked heterogeneity of MPNs under the hemocoagulative profile, in a manner dependent on the type of disease under investigation, its mutational profile and the cell line most affected by the proliferative drive.

Modern models linking clonal progression to the interplay between fibrosis and hypercoagulable states affirm the existence of a biological continuum between different forms of MPN. At the same time, however, they invite to investigate the differences in clinical behavior and variability of biological parameters.

It is known how tissue factor (TF) release can accelerate both thrombin generation and CFT in GCAs[57]. The paradigm of the "circulating wound" revolves around the TF-induced thrombin generation, and the consequences of the crosstalk between PARs and the TGF-beta pathway. The mechanisms contained therein are certainly more "credible" in the context of a transforming process. Therefore, it is not surprising the observation derived from the literature, which studying the generation of thrombin in PMF, finds an endogenous thrombin potential (ETP) more marked in the early and proliferative phases of the disease[58].

Our study shows how the extrinsic coagulation pathway in PMF can be studied by other - easily accessible - global coagulation tests. The contraction of EXTEM CFT appears to be primarily dependent on platelet count, as highlighted by previous studies on ETP[58,59], but also influenced by white blood cell and neutrophil counts. This finding suggests the involvement of a more complex system, which could include inflammation and a greater interaction between cells and endothelium[60]. Notably, EXTEM is selectively affected, whereas the other tests are not affected. The genetic picture of patients with more significant variations in EXTEM is often complex, characterized by the coexistence of drivers and DTA or HMR mutations. The heterogeneity of these patients does not allow to draw conclusions on the influence of a particular structure in conditioning the outcomes of GCAs. PMF appears to be the disease most characterized by the presence of platelet- and monocyte-derived circulating microparticles (MP) containing TF[61,62]. Their role in modifying EXTEM parameters could be relevant. In any case, the results of disease-specific assays seem to support the biological model previously proposed by our group.

In reference to PV, it is useful to refer to multivariable analytical models to understand how this disease can differ from the others. Panmyelosis does not selectively promote platelet proliferation, and hemorheological features change in relation to erythrocytosis. It may seem paradoxical that in the disease most characterized by thrombosis higher CFT values were found. In the multivariable analysis, however, the variations seem to be associated with the Hb values: the Plt (which tend to shorten the CFT) lose their impact on the data in EXTEM and preserve it in a weaker way in INTEM. The Hb values therefore condition the test, probably radically affecting the viscoelastic properties of the sample. Similarly - although the methods are not directly comparable, thrombin generation studies show an increased lag-time associated with higher Hb levels[63]. NEU also appear to play a pivotal role: they retain their significance on multivariable analysis for INTEM CFT, and as their count increases there is a consensual increase in MCF. Several publications mention NEU as co-responsible for thrombotic phenomena due to their adhesive properties to the endothelium and their ability to form aggregates with platelets[36,64,65]. It is also the elements of the white series that determine a release of TF in polycythemia, but the effects on the extrinsic pathway are probably masked by the dominant role of shear stress on the endothelium, with activation of von Willebrand factor, hypersensitive platelets, and recruitment of leukocytes[66,67]. The system appears overall different from other MPNs, as well as the only apparent one able to derive some benefit from ASA. The recovery of expression of PFRs (which we will discuss more extensively) supports this concept.

ET appears to be totally dominated by the effects of platelets on both classical coagulation pathways. The pattern is probably more linear, less characterized by the interference of inflammatory or rheological phenomena. As platelet count increases, PMF sees a shortening of CFT in EXTEM, PV in INTEM. The ET instead "does not make distinctions", and in a similar way sees the growth of MCF.

The key to the interpretation of our experiment is that the ROTEM is an instrument designed to investigate major imbalances in hemostasis, primarily in bleeding disorders. We identified significant differences in parametric oscillations that - in clinical practice and according to the indications of the instrument - in most cases should not be considered as abnormal. Setting the reasoning on trends and imbalances, the picture becomes sharper, and tends to turn into a biological continuum even on the front of coagulation, with the ET still endowed with a good hemostatic efficiency, while the PMF and PV lean respectively

towards the extrinsic and intrinsic pathways, for reasons that have strictly to do with the genotype-phenotype relationship.

Thus, the "contracted" CFT values found in subjects with CALR mutation become an indication of a preserved efficiency of the system, in accordance with the studies that identify this mutation as the one with the least impact on the risk of thrombosis[68,69]. In mutated CALR forms, megakaryocytopoiesis appears to be highly accelerated[70], resulting in pictures of extreme thrombocytosis, but recent experiments showed that mutated CALR platelets were less responsive to ADP activation test and to thrombin in the adhesion tests[71]. Thus, the effects of proliferative drive in the presence of this mutation appear to be self-limiting.

Among the "non-driver" mutations, DNMT3A seems to be of particular interest. The reduced EXTEM CT could in fact mean a greater ease in thrombin formation and clot polymerization. Since the finding does not strictly concern platelets, and is exclusive to the extrinsic pathway, the mutation could indeed be studied for TF-induced thrombin generation. It is worth mentioning that DTA mutations underlie endothelial damage and atherosclerosis in CHIP[72,73], an aspect that they likely retain during MPN. In our case series, most patients with a history of thrombosis had this type of genetic alteration. Among the other DTA mutations, ASXL1 showed very low percentages of ML, similarly to EZH2. Both mutations are associated with a tendency to CFT elongation, but only for EZH2 this was significant. The value of these data, although already reported in the results section, is however questionable and do not allow to draw conclusions. However, we are aware that HMR mutations have an impact on the IPSET score in prefibrotic myelofibrosis[15] and that the wild-type asset of these genes is associated with an increased risk of thrombotic events in ET[74]. The HMR patient is often characterized by a much greater genetic and clinical complexity, so further studies will be needed to understand the real impact of mutations on the coagulation profile.

The study did not identify parameters related to the history of thrombosis, except for the merely descriptive data on the shifting of values towards the extremes of the normal ranges, and what has already been mentioned on the particular genetic structure of the 7 patients of the case series.

In contrast, what was found for subjects with bleeding history highlights the potential usefulness of the assays used. Increased expression of PFRs and prolongation of FIBTEM

CT seem to be two characterizing features. All patients were on prophylaxis with ASA, so evidently the process of fibrin formation was excessively inhibited. The usefulness of ROTEM combined with cytofuorimetric analysis of PFR expression deserves to be further explored to dynamically define hemorrhagic risk.

Concerning PFRs, partial restoration of their expression under ASA was found to be a feature more associated with the use of the antiplatelet in PV, confirming more pronounced effects (and likely greater benefit) in this disease. In summary, with respect to the type of myeloproliferative neoplasm under investigation, antiplatelet use, and modulation of GCAs parameters, reduced expression of PFRs appears to be:

- ✓ a marker of pro-coagulant activity, especially of PV;
- ✓ a surrogate marker of the efficiency of the extrinsic pathway of PMF and in ET;
- ✓ an indirect index of the type of function exerted by the antiplatelet in different contexts.

6. Conclusions

The biological complexity of MPNs should be studied with dynamic tools that capture the nuances of their clinical behaviors. The instruments used in this research project served to underline the validity of old and new paradigms on the close connection between fibrosis, inflammation, hypercoagulability and clonal progression. Although not identifying frankly pathological values, the differences between the different forms of MPNs and their genetic aspects, clearly indicate the need to diversify the approach. The indolence of ET and CALR mutated neoplasms results in a greater efficiency of the classical pathways, the rheological characteristics and endothelial stress of PV in a greater involvement of the intrinsic pathway. In PMF, the "circulating wound" model seems solid, relying more on the extrinsic pathway. These aspects may open the way to more personalized therapies, and to the study of key elements such as PARs, whose inhibition could also have antifibrotic effects. Finally, the possibility of performing a dynamic monitoring of the efficiency of therapeutic choices and related risks - such as bleeding - could also be facilitated by the integration of global coagulation assessment methods.

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