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UNRAVELING THE MOLECULAR UNDERPINNINGS OF PHILADELPHIA-NEGATIVE MYELOPROLIFERATIVE NEOPLASMS: IMPLICATIONS FOR FUTURE THERAPEUTIC STRATEGIES

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Summary

The present thesis encompasses the two researches projects I conducted during my PhD program in Molecular Biology and Pathology. The common thread is represented by the analysis of the signaling pathways implicated in the pathophysiology of the two most aggressive Philadelphia-negative myeloproliferative neoplasms, namely, atypical chronic myeloid leukemia (aCML) and primary myelofibrosis (PMF).

In the last decade, since the description of the *JAK2*V617F mutation in 2005, the field of the molecular characterization of Philadelphia-negative myeloproliferative neoplasms has experienced an astonishing implementation that led to the discovery of 16 new mutations involving signal transduction, epigenetic modifiers, cell cycle regulators. Nevertheless, their pathogenetic relevance and whether they could represent good "druggable" candidates have to be proved yet.

In the first section I provide the first report of the signaling cascade down-stream the rare cytogenetic lesion t(8;9)(p22;p24)/*PCM1-JAK2* associated with aCML, finding that it selectively activates the ERK1/2 signaling without affecting JAK/STAT phosphorylation.

In the second part, I investigated the implication of the ε isoform of novel Protein kinase Cs (PKCs) in the pathophysiology of the aberrant megakaryocytopoiesis in PMF, concluding that the over-expression of PKCε detains a crucial relevance in the aberrant behavior of PMF megakaryocytes and its inhibition is capable to restore their normal differentiation and abrogate the anti-apoptotic signaling.

Both results are discussed in the view of their therapeutic implications.

In case *PCM1/JAK2*-related hematologic neoplasms, ERK-inhibitors rather than JAK-inhibitors (i.e. ruxolitinib) should be considered as a "tailored" drugs.

In case of PMF, PKCε-inhibitors (i.e. εV1-2 peptide) configure as an appealing strategy to re-direct the megakaryocytic neoplastic clone.

Preface

During my PhD program in Molecular Biology and Pathology, I studied the molecular underpinnings of Philadelphia-negative myeloproliferative disorders.

Specifically, I focused my research on two rare hematologic malignancies whose pathobiology is still controversial: atypical chronic myeloid leukemia (aCML) and primary myelofibrosis (PMF).

aCML and PMF are aggressive and rapidly progressive diseases characterized by a poor outcome and very limited therapeutic options. The only curative approach is represented by allogenic bone transplant, which, however, is approachable only to a minority of patients because of age and co-morbidities. Consequently, a better understanding of their molecular aspects is mandatory in order to identify new molecular therapeutic targets for novel, "tailored" pharmacological strategies.

In case of aCML, I explored the signaling pathways down-stream an extremely rare cytogenetic lesion, the translocation t(8;9) leading to *PCM1-JAK2* fusion, that, so far, has been described only in 34 hematologic neoplasms.

In case of PMF, I investigated the role of Protein Kinase C ε in the pathogenesis of the aberrant megakaryocytopoiesis that typifies this disorder.

These projects have been conducted in the laboratories of the Unit of Anatomy, Histology and Embryology of the Department of Biomedical, Biotechnological and Translational Sciences of the University of Parma in collaboration with the Hematology and BMT Unit of Parma University Hospital.

In the present thesis, I am presenting the results of these two research topics, that have been published on peer-reviewed journal and have been subject matter of communications at national and international symposia (see page 73).

Introduction

Philadelphia-negative myeloproliferative neoplasms

Myeloproliferative neoplasms (MPN) are clonal stem cell diseases, first conceptualized in 1951 by William Dameshek, and historically included chronic myeloid leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF) and the so called "Di Guglielmo syndrome" (erythroleukemia)[1].

The association of the Philadelphia chromosome with CML in 1960 by Nowell and Hungerford[2], and the subsequent recognition of erythroleukemia as a variant of acute myeloid leukemia, distinguished the other three diseases (PV, ET and PMF) as "classic" Philadelphia-negative myeloproliferative disorders. Dameshek was the first, in his seminal editorial published in 1951 in *Blood*, to recognize that these diseases should be classified as a set of phenotypically related "myeloproliferative disorders" characterized by bone marrow proliferation, *"perhaps due to a hitherto undiscovered stimulus"*[1].

Although the nomenclature and definitions of the different MPN have changed in the ensuring decades, the seminal observation that these are clinically and biologically related proliferative bone marrow syndromes has been substantiated by the recent elucidation of the genetic basis of MPN.

The current 2008 WHO classification for myeloid neoplasms incorporates novel information derived from molecular discoveries in *BCR-ABL* negative "classic" myeloproliferative and clonal eosinophilic disorders, including five major entities as follows: the acute myeloid leukemias (AML), the myelodysplastic syndromes (MDS), the myeloproliferative neoplasms (MPN), the category of overlapping myelodysplastic/myeloproliferative neoplasms (MDS/MPN) and the myeloid/lymphoid neoplasms associated with eosinophilia and specific molecular abnormalities (Table 1).

1	**Acute myeloid leukemia and related precursors neoplasms**
2	**Myeloproliferative neoplasms (MPN)** **i) Classic MPN** a) Chronic myelogenous leukemia (CML), *BCR-ABL1* positive b) Polycythemia vera (PV) ***c) Primary myelofibrosis (PMF)*** d) Essential thrombocythemia (ET) **ii) Non-classic MPN** a) Chronic neutrophilic leukemia b) Chronic eosinophilic leukemia c) Mastocytosis d) MPN, unclassifiable
3	**Myelodysplastic syndromes (MDS)** i) Refractory cytopenia with unilineage dysplasia (RCUD) ii) Refractory anemia with ringed sideroblasts iii) Refractory cytopenia with multi-lineage dysplasia (RCMD) iv) Refractory anemia with excess blasts (RAEB) v) MDS associated with isolated del(5q) vi) MDS, unclassifiable
4	**MDS/MPN** i) Chronic myelomonocytic leukemia (CMML) ***ii) Atypical chronic myelogenous leukemia (aCML), BCR-ABL1 negative*** iii) Juvenile myelomonocytic leukemia iv) MDS/MPN, unclassifiable
5	**Myeloid and lymphoid neoplasms associated with eosinophilia and abnormalities of *PDGFRA*, *PDGFRB* and *FGFR1*** i) Myeloid and lymphoid neoplasms with *PDGFRA* rearrangement ii) Myeloid neoplasms with *PDGFRB* rearrangement iii) Myeloid and lymphoid neoplasms with *FGFR1* abnormalities

Table 1. WHO classification of hematologic malignancies. Neoplasms discussed in the present thesis are highlighted in red. (Modified from *Tefferi, AJH 2014*[3])

The substitution of the attribute "neoplasm" for "disease" is a relevant, formal recognition that both "classic" and non-classic" myeloproliferative diseases share a common stem cell-derived clonal heritage. The incorporation of clonal markers such as *JAK2*V617F

and similar activating and *PDGFRA, PDGFRB,* or *FGFR1* rearrangements is consistent with the spirit of this revision [4].

Although the 2008 WHO classification has undoubtedly the merit of promoting a systematic approach to these entities, taking into account clinical, morphological and molecular features, it presents controversial aspects such as the actual reliability of the proposed clinical parameters required for diagnosis, and, particularly, the diagnostic relevance and reproducibility of bone marrow morphology criteria. Moreover, recently evolving molecular features have provided novel diagnostic and prognostic tools[5].

In its upcoming revision (2016), the WHO document addresses these issues formally integrating the assessment of the so called "driver mutations" (namely, *JAK2*V617F, *MPL*W515K/L and *CALR* mutations) as one of several major criteria in the diagnosis of PV, ET and PMF. The proposed amendments regard also the upgrading of bone marrow morphology as another major criterion for the diagnosis of these three MPN[6].

Primary myelofibrosis

PMF is a chronic, progressive, malignant hematologic disorder characterized by leukoerythroblastosis, hepatosplenomegaly, bone marrow fibrosis, increased marrow macrovessel density and extramedullary hematopoiesis. It was first described in 1879 by the German surgeon Gustav Heuck (1854-1940), who reported the presence of marrow fibrosis and extramedullary hematopoiesis in the liver and spleen of two patients[7]. Over the years, PMF has also been referred to by a variety of other terms, such as agnogenic myeloid metaplasia, myelosclerosis, idiopatic myeloid metaplasia and idiopatic myelofibrosis[8].

The current designation PMF reflects our greater understanding of the origin of this disorder, and differentiates a *de novo* disease from myelofibrosis which is preceded by a history of PV or ET (post PV/ET myelofibrosis).

PMF is the least common of the "classic" Philadelphia-negative MPN, with an annual age- and sex-adjusted rate of 1.46 (ranges from 0.5 to 1.3) per 100,000 persons per year, and carries the poorest prognosis.

PMF is a disease affecting older age, with an average at diagnosis of 65 years, with men having been affected more frequently than women. Exceptionally, PMF has been reported in the pediatric age group[9].

PMF, and the phenotypically related post-PV/ET myelofibrosis are associated with anemia, left-shift leukocytosis, thrombocytosis/thrombocytopenia, often progressive splenomegaly, hepatomegaly, and various debilitating symptoms, including constitutional symptoms such as fever, weight loss, and night sweats. PMF is associated with worsening cytopenias with eventual bone marrow failure and transformation into a blast phase, which is most often of myeloid phenotype, and mirrors poor-risk AML. Portal or pulmonary

hypertension can also develop as a consequence of massive splenomegaly and extramedullary hematopoiesis, respectively, and cause significant morbidity. PMF is associated with a shortened survival, with a median life expectancy of approximately 5 years in aggregate [10].

Current diagnosis of PMF is based on WHO-criteria and involves a composite assessment of clinical (exclusion of other myeloid neoplasm and causes of reactive marrow fibrosis, presence of palpable splenomegaly) and laboratory (anemia, increased LDH levels, bone marrow megakaryocytic proliferation and atypia with reticulin/collagen fibrosis, detection of *JAK2*V617F or other clonal markers). For post-PV/ET myelofibrosis, documentation of a previous diagnosis of PV/ET is mandatory[3, 10].

Risk stratification

Robust prognostic modeling in PMF started with the development of the International Prognostic Scoring System (IPSS) in 2009. The IPSS for PMF is applicable to patients being evaluated at time of initial diagnosis and uses five independent predictors of inferior survival: age > 65 years, hemoglobin < 10 g/dL, white blood cell count > $25x10^9$/L, circulating blasts ≥ 1%, and presence of constitutional symptoms. The presence of 0, 1, 2, and ≥ 3 adverse factors defines low, intermediate-1, intermediate-2, and high risk disease, with median survivals of 11.3, 7.9, 4, and 2.3 years, respectively[11].

The IWG-MRT subsequently developed a dynamic prognostic model [dynamic international prognostic scoring system (DIPSS)] which is based on the same prognostic variables used in IPSS but can be applied at any time during the disease course. DIPSS assigns two, instead of one, adverse points for hemoglobin < 10 g/dL and risk categorization is accordingly modified: low (0 adverse points), intermediate-1 (1 or 2 points), intermediate-2

(3 or 4 points), and high (5 or 6 points). The corresponding median survivals were not reached, 14.2, 4, and 1.5 years[12].

DIPSS was then up-graded to DIPSS-plus, adding to the five above-mentioned parameters, unfavorable karyotype (i.e. complex karyotype or sole or two abnormalities that include +8, -7/7q-, i(17q), inv(3), -5/5q-, 12p- or 11q23 rearrangement), red cell transfusion need and platelet count < $100x10^9$/L. Based on these eight parameters, four DIPSS-plus risk categories were generated: low (no risk factors), intermediate-1 (one risk factor), intermediate-2 (two or 3 risk factors) and high (four or more risk factors) with respective median survivals of 15.4, 6.5, 2.9 and 1.3 years[13].

In view of the recent discoveries concerning the impact that peculiar "molecular signatures" (combining the presence of *JAK2, MPL, CALR, IDH1/2, EZH2, SRSF2* or *ASXL1* mutations) have on overall survival and leukemia-free survival, Dr. Vannucchi's group recently proposed a new molecular-IPSS that recapitulates both clinical and molecular parameters and that is currently under validation[14].

Molecular aspects

In the last decade, our understanding of the molecular aspects of Philadelphia-negative MPN has been dramatically revolutionized by the genetic discoveries that began in 2005 with the identification of the *JAK2*V617F mutation[15-17]. Since then, several somatic mutations have been described, and their pathogenetic role is currently under investigation, but none of them appear to garner the disease specificity or pathogenetic relevance otherwise displayed by the Philadelphia chromosome in CML.

Overall, it is now clear that among the numerous genetic culprits that can affect the neoplastic clone, three mutations (namely, *JAK2*V617F, *MPL*W515K/L and *CALR* mutations) play a crucial role in driving the disease phenotype and affecting the clinical course of PMF, so that they have been recently re-defined as "driver mutations"[6].

JAK2V617F mutation

JAK2 is a tyrosine kinase that engages with multiple cytokine receptors, such as erythropoietin (EPO)-, thromobopoietin (TPO)-, granulocyte colony stimulating factor (G-CSF)-, granulocyte-macrophage colony stimulating factor (GM-CSF)- and interleukin-3- (IL-3) receptors, and becomes activated upon ligand binding[18].

In 2005, Dr. Skoda's, Dr. Vainchenker's and Dr. Gilliland's labs, using different approaches, discovered a recurrent acquired somatic mutation in the *JAK2* gene in a significant proportion of patients with MPN[15-17]. This dominant gain-of-function mutation is a guanine-to-thymidine substitution at nucleotide 1,849 of the JAK2 gene that results in a valine-to-phenylalanine substitution at codon 617 of *JAK2*. The *JAK2*V617F mutation maps to the JH2 domain of *JAK2*, which has significant homology to the tyrosine kinase (JH1) domain but it lacks catalytic activity and it is involved in the auto-inhibition of JAK2 activity. The mutant *JAK2*V617F is constitutively activated and consequently promotes pro-survival and anti-apoptotic signals as well as cytokine-independent growth. In fact, when *JAK2*V617F is expressed in hematopoietic cells, several signaling pathways that are important for proliferation and survival are activated, including STAT3, STAT5 (which can act as transcription factors in the nucleus), mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (ERK) and the phosphoinositide 3-kinase (Pi3K)–AKT pathway.

Both wild type and mutated JAK2 are also active in the nucleus, where they phosphorylate histone H3 at tyrosine 41[19].

The incidence of the *JAK2*V617F mutation, as determined by allele-specific PCR in granulocytes from patients with MPN, was 35–50% in PMF, 32–57% in ET and over 95% in PV[15-17].

MPL mutations

MPL gene encodes for the TPO receptor (TPO-R; MPL) that also binds JAK2. *MPL* mutations are present in 5–10% PMF patients and 2–5% of those with ET. They (*MPL* W515L/K/N/A) occur in a stretch of five amino acids (K/RWQFP) adjacent to the transmembrane domain of this receptor. These five amino acids prevent spontaneous activation of the receptor. *MPL*W515 mutations activate the TPO-R and are characterized by spontaneous megakaryocyte growth in the absence of endogenous erythroid colony formation. A murine *MPL*W515L expression model results in a JAK2-dependent lethal MPN in the mouse characterized by thrombocytosis and myelofibrosis[20-22].

CALR mutations

CALR gene encodes for calreticulin, a highly conserved protein with chaperone properties that promotes adequate glycoprotein folding within the endoplasmic reticulum and modulates calcium homeostatsis. Calreticulin is also found in the other cell compartments, where it modulates several biologic processes, including proliferation, apoptosis and immunogenic cell death.

In 2013, whole-exome sequencing analyses contemporary performed by Dr. Green's[23] and Dr. Kralovics's[24] groups led to the identification of *CALR* exon 9 mutations in most ET and PMF patients (70%-80%) with non-mutated *JAK2*. Indeed, calreticulin mutations are mutually exclusive with mutations in both *JAK2* and *MPL* and, overall, affect about 20% to 25% of all patients with PMF.

About 80% of *CALR* mutated patients harbor one of two mutation variants: type-1, a 52-bp deletion (p.L367fs*46) or type-2, a 5-bp TTGTC insertion (p.K385fs*47). In PMF, type-2 *CALR* mutation is associated with higher risk category, circulating blast percentage, leukocyte count, and inferior survival. *CALR* variants that are neither type-1 nor type-2 are operationally classified into "type-1-like" and "type-2-like" variants, based on their structural similarities to type-1 and type-2 *CALR* variants, respectively, which in turns relies on alpha-helix content of the mutant C-terminus. Most noteworthy is the survival advantage shown by PMF patients with type-1 or type-1-like *CALR* variants, compared to all other genotypes[23-24].

Other mutations

Mutations or DNA sequence variants other than *JAK*2, *MPL* or *CALR* occur in a substantial number of patients with all three classic Philadelphia-negative MPN. The pathogenetic role of these other mutations has to be clarified yet and might include cooperation with the driver mutations in facilitating disease progression. Frequencies of these mutations are relatively low and do not overcome the 5% of PMF patients.

Specific genes affected in this regard include those relevant to epigenetic (e.g. *ASXL1, TET2, EZH2, IDH1, IDH2, DNMT3A*), RNA splicing (e.g. *SRSF2, U2AF1, SF3B1*) or

transcriptional (*TP53, IKZF1, NF-E2, CUX1*) regulation[3]. Mutations other than *JAK2, CALR* or *MPL*, which have been shown to have prognostic relevance in PMF, include *ASXL1, SRSF2, EZH2* and *IDH1/2*. In fact, Vannucchi et al. found that PMF patients presenting ≥ 2 mutations in any of the above listed genes, harbor the so-called "high molecular risk signature", characterized by a reduced overall survival and increased risk of leukemic transformation[25].

In addition, *TP53, IDH2, SRSF2* and *SH2B3* mutations have been recently reported to be over-represented in blast-phase MPN, suggesting their relevance in disease progression. Most noteworthy in this regard is *TP53* loss, which is believed to make *JAK2*-mutated patients vulnerable to leukemic transformation[26-27].

Atypical chronic myeloid leukemia

Chronic myeloid malignancies that share both myelodysplastic and myeloproliferative features define the myelodysplastic/myeloproliferative group, which includes atypical chronic myeloid leukemia (aCML). aCML is an extremely rare hematologic neoplasm with an estimated incidence of 1% that of typical *BCR-ABL*-positive CML. In fact only a few aCML patient cohorts have been reported, with the largest series consisting of 65 patients[28]. It was initially described as a subtype of myeloid neoplasm resembling CML, but lacking the pathognomonic Philadelphia chromosome.

In the 2008 WHO classification, aCML is defined by persistent leukocytosis ($\geq 13\times10^9$/L) with immature circulating myeloid precursors ($\geq$ 10% of leukocytes) and marked dysgranulopoiesis, with absent/minimal monocytosis ($< 1\times10^9$/L and < 10% of leukocytes) or basophilia (often < 2%). Diagnosis of aCML relies mainly on leukocyte counts and morphology, and requires also the exclusion of *BCR-ABL* and rearrangements of *PDGFRA, PDGFRB* or *FGFR1*[29].

Median age at the onset is estimated at 72 years, with a male : female ratio of 2 : 1. Patients tend to present severe anemia, thrombocytopenia, neutrophilic leukocytosis with granulocytic dysplasia and splenomegaly, while monocytosis and basophilia are not prominent in the peripheral blood[30].

According to the report of Dr. Orazi's group that considered the largest cohort of aCML cases studied so far (65 cases), these patients have an aggressive disease course, with a poor prognosis and an overall survival of 12.4 months. Median leukocyte count was $40.8\text{x}10^9$/L and bone marrow samples revealed hypercellularity and dysgranulopoiesis with variable fibrosis and osteosclerosis[28].

Risk stratification

Wang et al.[28] made also the first attempt to identify specific risk factors related to prognosis, finding that a higher leukocyte count - either as a continuous variable or a cut-off of 50×10^9/L - or a higher number of circulating myeloid precursors predicts an inferior overall survival and leukemia-free survival. A higher number of bone marrow blasts was a significant hazard for leukemia-free survival but not for overall survival. Other factors, such as platelet count, cytogenetic categories and circulating blasts that are used, for example, in PMF prognostic scores, became surprisingly not significant for survival[28].

Molecular aspects

The molecular pathophysiology of aCML is much less characterized than PMF, and our understanding of the molecular events leading to this disease is extremely scant.

Although no specific molecular abnormality has been described in aCML, recurrent mutations in SET binding protein 1 (*SETBP1*), located on chromosome 18q21.1, have been observed in 25% of aCML. The precise downstream effects of *SETBP1* mutations are unknown, but they may attenuate the activity of the tumor suppressor phosphatase PP2A through abrogation of a ubiquitination site (functionally equivalent to over-expression).

Additionally, recurrent somatic mutations in *JAK2, NRAS, IDH2, CBL, CSF3R* and *ETNK1* can also be detected in aCML, although at a much lower frequency; anecdotal cases with fusion genes such as *BCR-JAK2* and *PCM1-JAK2* have been reported[29].

In 2013, Maxson and colleagues[31] recorded the presence of the growth factor receptor *CSF3R* mutations, typically associated with CNL (90% cases), in 40% of patients with aCML, but subsequent publications were unable to confirm the high prevalence of these mutations.

Future studies are eagerly awaited to provide novel insights into the potential impact of such analyses on precision-medicine therapeutic approaches.

PCM1-JAK2/t(8;9)(p22;p24) down-stream signaling in aCML

Background

Chromosomal translocations involving *JAK2* have been described in hematologic malignancies involving both lymphoid and myeloid lineages. Better characterized translocation partners are *ETV6/TEL* on chromosome 12[32-33], *BCR* on chromosome 22[34-36] and the auto-antigen pericentriolar material-1 *(PCM1)* on chromosome 8.

While on one side the clinical phenotype of *JAK2*-fusion-related hematologic malignancies closely recalls Philadelphia-positive CML (onset as chronic disease, progression to an accelerated phase and, eventually, blast transformation) than *JAK2*-mutated classic MPN, on the other hand it appears to be characterized by a more aggressive course and unfavorable outcome than typical CML [37].

The *PCM1* gene is located on chromosome band 8p22-p21.3 and encodes for a 228-kDa centrosomal protein, containing multiple coiled-coil motifs, that is ubiquitously expressed in mammalian tissues. PCM1 plays a pivotal role in the correct assembly of the centrosome and in the organization of a radial microtubule network, that is critical for cell division. The disruption of PCM1 function leads to improper functioning of pericentriolar satellites, impaired protein integration to the centrosome and loss of microtubule organization.

Finally, the role of PCM1 in the regulation of cell cycle, has been elegantly demonstrated by injecting anti-PCM1 antibodies into murine fertilized zygotes, experiments that revealed how PCM1 is essential for completion of interphase and for cell cycle progression[38].

PCM1-JAK2 fusion events are extremely rare and, to our best knowledge, 34 clinical cases have been reported so far in the literature[39-59]. Although the clinical onset is extremely heterogeneous since the disease may hit both the myeloid and the lymphoid lineage, several

cases present with a MDS/MPN disease with striking dysplastic features of the erythroid compartment.

Overall, seven patients presented with aCML, seven patients with AML, six patients with a chronic eosinophilic disorder, three patients with an unclassifiable MDS/MPN neoplasm, three patients with a unclassifiable MPN, one of whom with concurrent myeloid sarcoma, four patients with myelofibrosis, two patients with *de novo* acute lymphoblastic leukemia, one T-cell lymphoma and one erythroid leukemia[60].

Young adults are affected more than elderly (median age is estimated at 47 years, ranging from 12 to 75 years), although a few cases of people aged over 70 have been described. A striking male predominance can be seen (85%). The reasons of this gender skewing are unknown.

One of the most intriguing aspect of these heterogeneous group of diseases is related to the peculiar abnormalities of the erythroid lineage that typifies a remarkable percentage of the reported cases[45-46, 49, 53, 55]. This observation is even more relevant if we consider that the cytogenetic abnormality involves *JAK2*, which detains a pivotal role in signal transduction down-stream the EPO receptor. Histopathological features may range from erythroid dysplasia to overt erythroid leukemia. Erythroid dysplasia is characterized by large nodules of immature erythoid precursors (mainly proerythroblasts and early erythroblasts), with high mitotic activitiy and impaired differentiative capacity. They are typically localized in paratrabecular areas with a pronounced microvessel density. Since normal erythropoiesis is not physiologically found at the paratrabecular spaces, the unique distribution of these clusters of morphologically abnormal proerythroblasts can be interpreted, as observed by Heiss et al.[45], as a sign of dysplasia, similarly to what described for myelopoiesis in

myelodysplastic syndromes (the so called "ALIP", Abnormal Localized Immature Precursors).

If the pathophysiological bases of this erythroid dysplasia might be related to the involvement of the JAK2-signaling is not clear yet.

Chromosome break points are identified by FISH as p22 on chromosome 8 and p24 on chromosome 9 in the vast majority of the cases, followed by p21 on chromosome 8 and p24 on chromosome 9. Only two cases of break points located at p23 on chromosome 8 and p24 on chromosome 9 and at p22 on chromosome 8 and p23 on chromosome 9 have been described.

A complex karyotype, with the *PCM1-JAK2* translocation being part of multiple chromosomal abnormalities, is not uncommon[43, 46, 56]. No phenotype-specificity can be described, but similarly to other hematologic malignancies (AML and MDS in particular), a rapidly progressive clinical course is observed, such as death early after diagnosis, or during induction chemotherapy, early relapse and association with other tumors.

All the translocation variants with alternative break-points on the short arms of chromosome 8 and 9 that have been described so far preserve the N-terminal domain of PCM1 and the C-terminal domain of JAK2. The N-terminal domain of PCM1 is characterized by the presence of multiple coiled-coil motifs, while the C-terminus of JAK2 contains the kinase (JH1) and pseudokinase (JH2) domain. Bousquet et al.[44] and Murati et al.[46] hypothesized, in 2005, that this peculiar structure of the fusion protein facilitates oligomerization of the PCM1-JAK2 chimera resulting in a constitutive activation of JAK2.

However, if PCM1 multiple coiled-coil domains serve as dimerization motifs to induce JAK2 autophosphorylation has never been proved yet.

In fact, although recent studies showed an activation of the JAK/STAT axis in a *PCM1-JAK2*-transformed murine fibroblast[51] or human lymphoma cell lines[42], little is known about signaling in primary cells from *PCM1-JAK2* patients.

This aspect is extremely relevant if we consider the availability of new drugs targeting the JAK/STAT pathway (i.e. ruxolitinib).

Aim of the study

Given this complex background, the aim of this research was to determine, in a patient carrying t(8;9)(p22;p24)/*PCM1-JA*K2 aCML, referred to our Hematology and BMT Unit:

(i) the erythroid differentiation capacity of ex-vivo expanded $CD34^+$ cells as compared to healthy donors and *JAK2*$V617F^{pos}$ PVs;

(ii) the signaling pathways activated in circulating neoplastic cells (CNC) harboring the fusion transcript.

Matherials and methods

Patients and controls

aCML

The patient, a 29-year-old Caucasian male, presented with the typical stigmata of a chronic myeloproliferative disorders: leukocytosis ($67.1x10^9$/L) with circulating myeloid progenitors (manual differential count showed: 64% neutrophils, 7% lymphocytes, 2% eosinophils, 14% metamyelocytes, 13% myelocytes), mild anemia (12.4 g/dL) and thrombocytopenia ($130x10^9$/L), splenomegaly (15x8 cm), elevated lactate dehydrogenase and vitamin B_{12} levels (815U/L and 1352 pg/mL, respectively). Bone marrow (BM) histology was intensely hypercellular with marked granulocytic hyperplasia reaching terminal differentiation. Blast count was less than 5%. Megakaryocytopoiesis was abnormally hypoplastic. The erythroid lineage was the one primarily affected by the dysplastic process, showing abundant large peritrabecular clusters of proerythroblasts associated with marked reduction of the mature erythroid compartment

PVs

Three JAK2V617F^{pos} PV patients were recruited. Diagnosis was posed according to the WHO 2008 criteria[4].

Control subjects

Leukoapheresis bags from 2 G-CSF-mobilized donors and buffy-coats from 4 healthy subjects were utilized as controls. Controls are collectively referred to as HDs (healthy donors).

Cell isolation, liquid culture of human erythroblasts and immunophenotypic analysis

After approval by the Ethical Committee of Parma University Hospital, aCML circulating neoplastic cells (CNC) and peripheral blood mononuclear cells (PBMC) from 4 HD buffy-coats and 2 HD leukoapheresis were isolated by standard Ficoll-Hypaque gradient.

Primary $CD34^+$ cells were isolated by immunomagnetic positive selection from aCML, PVs and leukoapheresis bags using the $CD34^+$-cell isolation kit in the magnetic field of an autoMACS® separator (all from Miltenyi Biotec, Bergisch Gladbach, Germany), according to manufacturer's protocol.

$CD34^+$ cells were cultured up to 14 days in serum free X-vivo medium supplemented with 5 U/mL EPO, 50 ng/mL stem cell factor (SCF) and 3 ng/mL IL-3 (PeproTech, London, UK), renewed every 72 hours. Cell number was determined at day 9, 11 and 14 of culture and vitality checked by Tripan Blue staining. Erythroid differentiation was assessed by flow-cytometry using RPE-conjugated anti-Glycophorin A (Gly-A) antibody (Dako, Glostrup, Denmark). Analysis was performed by Epics XL flow cytometer and the Expo ADC software (Beckman Coulter, Fullerton, CA).

Fluorescence In Situ Hybridization (FISH)

Translocation was detected by FISH analyses, performed in the Laboratory of Cytogenetic and Molecular Genetics, Hematology Unit, University of Perugia, headed by Dr.

Cristina Mecucci. FISH tests with genomic clones for genes known to be involved in Philadelphia-negative chronic myeloid neoplasms (*BCR*/22q11, *ABL*/9q34, *PDGFRA*/4q12, *C-KIT*/4q12, *TET2*/4q24 *PDGFRB*/5q33, *FGFR1*/8p11, *ETV6*/12p13) were first undertaken. Break-apart FISH assays for *JAK2*/9p24, RP11-125K10 (5'*JAK2*) and RP11-39K24 (3'*JAK2*) and for *PCM1*/8p22, RP11-156K13 (5'*PCM1*) and RP11-484L21 (3'*PCM1*) was then performed.

Polymerase chain reaction (PCR)

To confirm the presence of the fusion transcript, I performed nested-RT-PCR on patient CNC and on HD-PBMC, as negative control. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse transcribed (1 µg) using ImProm-II TM Reverse Transcription System (Promega, Wisconsin, USA). using the following primers:

- *PCM25/1:*5'-CCATGTTTGAAGCTTTGCGAGATA-3',
- *P*CM25/2: 5'-CTCTTCCATGAGCTGCAGCTAC-3';
- *JAK9/1*: 5'-GGCTTTGGGGGACAGCATTTAG-3';
- *JAK9/2*: 5'-GAGCGAACAGTTTCCATCTGGTA-3'

Amplification reactions were performed for 32 cycles with an annealing temperature of 60°C.

Western Blot (WB)

CNC and HD-PBMC were resuspended in lysis buffer supplemented with fresh protease inhibitors as described. Fifty µg proteins/sample were run in 10% SDS–acrylamide gels and blotted onto nitrocellulose membranes. Blotted filters were blocked and incubated over-night with the following primary antibodies: anti-JAK2 (Cat. N. 3230), anti-phophoJAK2 (Tyr 1007/1008) (Cat. N. 3771), anti-STAT3 (Cat. N. 4904), anti-phosphoSTAT3 (Tyr 705) (Cat. N. 9145), anti-STAT5 (Cat. N. 9358), anti-phosphoSTAT5 (Tyr 694) (Cat. N. 3230), anti-Akt (Cat. N. 9272), anti-phopshoAkt (Ser 473) (Cat. N. 9271), anti-P44/42 ERK1/2 (Cat. N. 4695), anti-phosphoP44/42 ERK1/2 (Thr202/Tyr 204) (all from Cell Signaling Technology, MA, USA) and anti-GAPDH (Upstate, Lake Placid, NY), diluted as indicated in the manufacturers' protocols. Filters were washed and further incubated for 1.5 hours at room temperature with 1:2000 peroxidase-conjugated anti–rabbit or anti–mouse IgG (Pierce) in the primary antibody working solution. Specific reactions were revealed with the ECL SupersignalWest Pico Chemiluminescent Substrate detection system (Thermo Scientific, MA, USA).

Results

Patient CNC harbor PCM1-JAK2 fusion transcript

Chimeric *PCM1-JAK2* transcript was detected in patient CNC by nested RT-PCR. Specificity of the transcript was confirmed by the absence of signal in HD-PBMC (Figure 1)

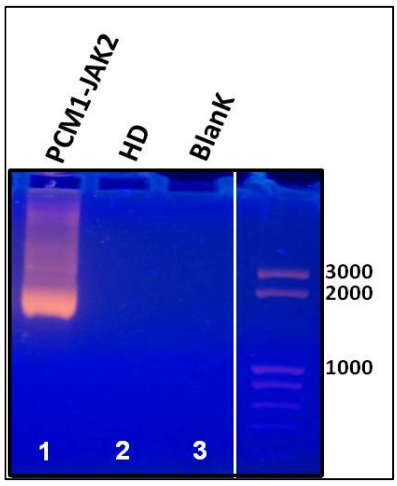


Figure 1. *PCM1-JAK2* fusion transcript. Chimeric transcript was detected by nested RT-PCR in circulating neoplastic cells (CNC) from the patient. Reaction was performed using primers derived from *PCM1* exon 25 and *JAK2* exon 9 on cDNA extracted from CNC (lane 1) and from PBMC of an healthy donor (HD, lane 2). Lane 3: negative control (H_2O).

Ex-vivo erythroid differentiation capacity of aCML $CD34^+$ cells is markedly impaired

Given the prominent dyserythropoietic picture of patient's BM histology, I first studied the erythroid differentiation capacity of *ex-vivo* expanded $CD34^+$ cells from the *PCM1-JAK2* patient as compared to $CD34^+$ cells from 3 PVs (in which *JAK2* is constitutively activated by the V617F somatic mutation) and 2 G-CSF

mobilized donors (HDs). Erythroid differentiation capacity was evaluated by the expression of Gly-A at day 14 of culture in erythroid differentiation medium.

Consistently with the *in-vivo* findings, $CD34^+$ from the *PCM1-JAK2* fusion patient showed: i) an impaired growth capacity when cultured in erythroid differentiation medium, as demonstrated by the low fold increase (F.I.= 0.6) as compared to PV- and HD-$CD34^+$ cells (mean F.I.= 18.21±18.55 and 4.98±2.11, respectively) at day 14 of culture (Figure 2, panel A); ii) an impaired erythroid differentiation capacity, as demonstrated by the low percentage of Gly-A^{pos} cells generated at day 14 of erythroid culture (3.1%) as compared to a representative PV and HD (96.6% and 66.6%, respectively) (Figure 2, panel B).

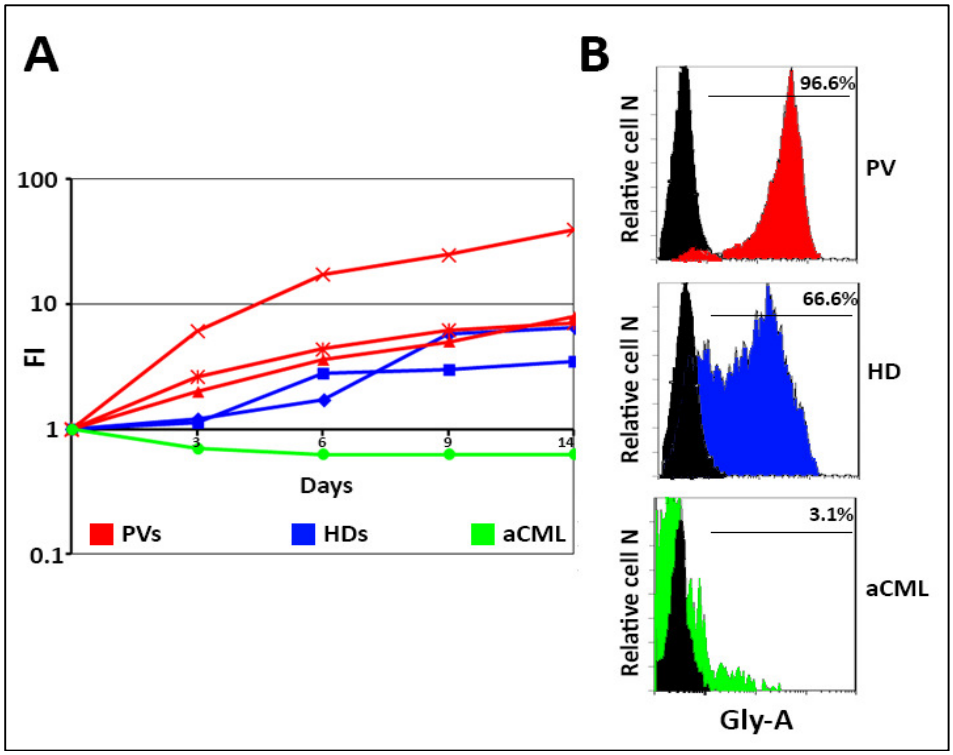


Figure 2. Erythroid growth and differentiation from PV, HD and aCML $CD34^+$ cells.
(A) Ex-vivo expansion capacity in erythroid differentiation medium of $CD34^+$ cells from 3 PVs, 2 HDs and the aCML; data are expressed as fold increase (F.I.) with respect to the number of cells seeded in culture ad day 0. **(B)** Percentage of Glycophorin-A (Gly-A)-positive cells after 14 days of culture of $CD34^+$ cells isolated from a representative PV (upper panel), HD (middle panel) and the aCML (bottom panel). Coloured histograms indicate specific fluorescence; Black histograms isotype-matched irrelevant Ab, negative control.

PCM1-JAK2 chimera leads to activation of ERK1/2 pathway

This prompted me to investigate, in CNC harboring the fusion transcript, the activation levels of the main signaling cascades down-stream the EPO-Receptor [i.e. i) Extracellular signal-Regulated Kinase (ERK)1/2 pathway; ii) Akt pathway; iii) JAK/STAT pathway] known to be activated by the V671F point mutation of the *JAK2* gene. HD-PBMC (n.4) were utilized as control.

Surprisingly, I found that patient CNC expressed reduced levels of total JAK2, STAT5 and Akt, while only total ERK and STAT3 were detectable at levels comparable to healthy donors. Additionally, while ERK1/2 showed a robust phosphorylation in patient CNC with respect to controls, intermediates of the JAK/STAT signaling axis (JAK2, STAT3 and STAT5) were not phosphorylated, indicating that the ERK pathway is selectively activated in CNC harboring the fusion transcript. (Figure 3)

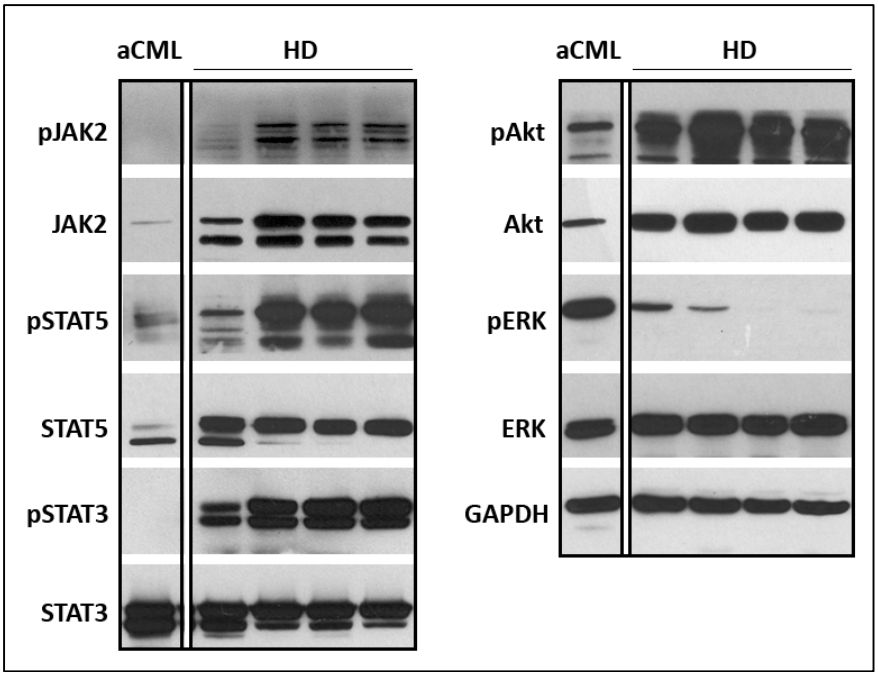


Figure 3. PCM1-JAK2 down-stream signaling. Western Blot analyses of phosphorylation levels of pJAK2, pSTAT5, pSTAT3, pAKT and pERK1/2 in patient CNC and in PBMC from 4 HDs. Total JAK2, STAT5, STAT3, AKT, ERK1/2 and GAPDH expression levels are shown as quantitative control.

Discussion

MDS/MPN resulting from an acquired t(8;9) that leads to *PCM1-JAK2* fusion are rare diseases. Heterogeneous clinical presentation, rapid and aggressive course, poorly defined pathology make them a scantly characterized group of hematologic malignancies[60]. In the present research, I provided the first functional characterization of *ex-vivo* expanded $CD34^+$ cells from a t(8;9)(p22;p24)/*PCM1-JAK2* fusion case presenting with aCML, describing the intracellular signaling pathways activated in patient CNC.

Consistently with the prominent diserythropoietic picture of BM histology, patient $CD34^+$ cells showed impaired eryhroid growth and differentiation as compared to $CD34^+$ cells from 3 *JAK2*V617F^{pos} PVs and 2 control cases. This led me to investigate, in CNC harboring the fusion transcript, the activation levels of the main signaling cascades down-stream EPO-R (i.e. ERK1/2, Akt and JAK/STAT signaling axes), known to be activated by the V671F point mutation of the *JAK2* gene[61].

By analogy with PV[61], I expected an activation of the JAK/STAT signaling pathway. By contrast, I surprisingly found a selective activation of the ERK1/2 pathway, while neither JAK2, nor STAT3/STAT5 were activated, suggesting that PCM1-JAK2 fusion protein is not capable to activate JAK/STAT axis. These data support the clinical and hystopathological findings in our patient, in which the sustained myeloproliferative stimulus was associated with impaired erythroid differentiation. Whether *PCM1-JAK2* fusion-associated malignancies are “stem-cell diseases” or rather hit committed hematopoietic progenitors has not been determined yet, but the in-vitro behavior of primary $CD34^+$ cells from our patient faithfully

reproduce the in-vivo findings. Since diseases harboring *PCM1-JAK2* fusion are rare hematologic malignancies, patient population is scant and treatment choices are primarily based on physician's personal expertise and anecdotal reports, and we are aware of the difficulty to generalize individual experiences. Given the fact that allogenic stem cell transplant is the only curative option in eligible patients, several therapeutic approaches have been suggested as a bridge to transplant or in unfit patients: interferon-α, hydroxyurea, conventional chemotherapy and, more recently, JAK inhibitors[42, 51, 55].

This latter therapeutic approach is primarily based on the laboratory data from Lierman et al.[51], showing that ruxolitinib, a JAK1/2 inhibitor approved for treatment of intermediate-high and high risk myelofibrosis, is able to inhibit in-vitro growth and JAK2/STAT5 phosphorylation of the *PCM1-JAK2*-transformed Ba/F_3 murine cell line. However, JAK/STAT activation has never been proved in primary cells from *PCM1-JAK2* patients and my data rather suggest the opposite.

Moreover, the molecular dissection of the signaling cascade in patient neoplastic cells, not only provides a coherent explanation of the *in-vivo* and *in-vitro* observations, but has immediate therapeutic implications in this subset of disease.
In fact, by demonstrating that the *PCM1-JAK2* fusion product does not lead to JAK/STAT axis activation would suggest that these specific patients would not benefit from therapy with JAK-inhibitors, but rather could be considered for different treatment options, i.e. MAP-kinase inhibitors.

Consistently with my research, Dr. Reiter's group recently documented the limited duration of complete remission on ruxolitinib in myeloid neoplasms with *PCM1-JAK2* and *BCR-JAK2* fusion genes[62].

Overall, my data provide a unique contribution to the pathophysiological characterization of these neoplasms and consequently a robust rationale for treatment strategy, immediately pinpointing to a potential druggable target in the subset of patients resistant to JAK-inhibitors represented by the MAP-kinase pathway.

Protein Kinase Cε as a novel therapeutic target in PMF

Background

Abnormalities in the process of megakaryocytes (MKs) differentiation are a hallmark of PMF. PMF megakaryopoiesis is characterized not only by a disturbance of bone marrow histologic topography (extensive clustering of MKs with loose to dense groupings and abnormal localization of these towards the endosteal borders) but also by striking abnormalities in their morphology and maturation. These include: i) a high degree of cellular pleomorphism, with variations in size from small to giant forms, with a prevalence of the so called "micromegakariocytes"; ii) atypical nuclear lobulation (extent and shape of nuclear foldings; i.e., hypolobulation), often described as cloud-like, leading to bulbous (plump, clumsy) nuclei, *versus* hyperlobulation with marked segmentation and anomalies of the chromatin pattern (mostly hyperchromasia) (Figure 4)[9, 63].

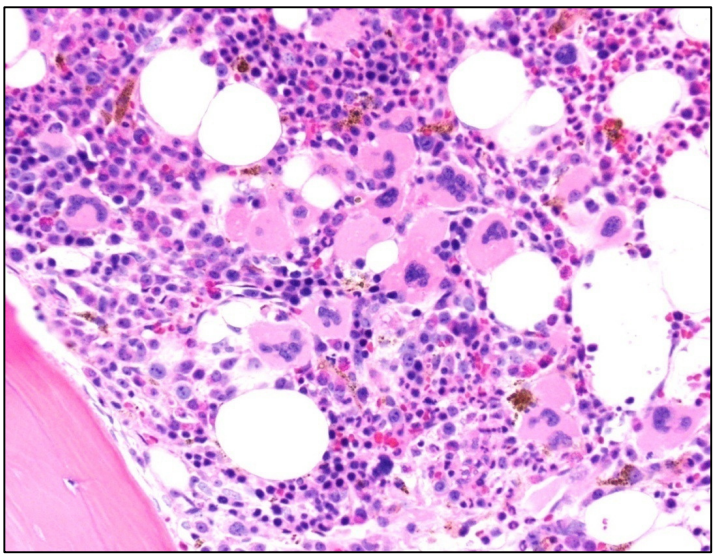


Figure 4. Bone marrow histology in PMF.

Overall, MKs in PMF are regularly characterized by a more pronounced degree of cytologic atypia (megakaryocytic dysplasia, dysmegakaryopoiesis) than in any other subtype of MPN, especially of ET, and are one of the most important features discriminating prefibrotic, early-stage PMF from true ET[64].

Dr. Hoffman's group demonstrated that MKs generated in-vitro from PMF $CD34^+$ cells cultured in presence of TPO and SCF, recapitulate the same atypia of PMF MKs in-vivo, such

as smaller size, lower ploidy and increased proliferative capacity associated with a decreased rate of apoptosis likely due to Bcl-xL over-expression[65]. More recently, Balduini et al. showed that PMF MKs display an impaired capacity to generate proplatelets in-vitro when compared to MKs derived from ET, PV and healthy subjects[66].

Collectively, these data suggest that PMF MKs display enhanced proliferation and impaired differentiation both *in-vivo* and *in-vitro*, which most likely accounts for reactive proliferation of fibroblasts, collagen deposition and consequent BM structural changes.

The molecular events that lead to aberrant megakaryocytopoiesis have not been clarified yet and in the present study I investigated the role of Protein Kinase C ε (PKCε) in this process and its therapeutic implications.

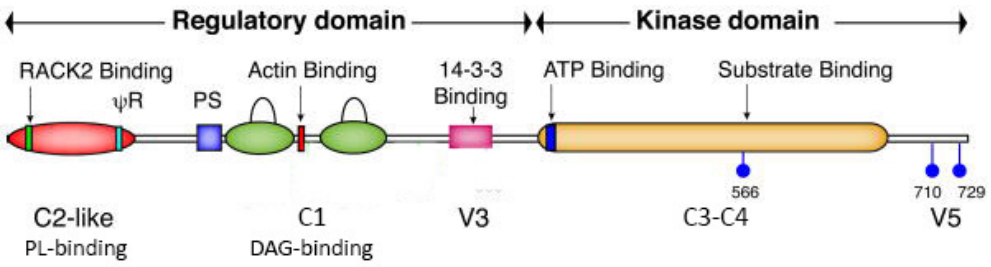


Figure 5. PKCε structure. Modified from *Newton et al., Biochem J. 2011*[67]

PKCε is a member of the PKC family, a subset of serine/threonine kinase enzymes that are involved in many cellular processes including cell proliferation, differentiation, apoptosis, adhesion, migration, gene expression modulation and ion secretion. Specifically, PKCε is novel, DAG-dependent and Ca^{2+}-independent PKC, which shares many structural features with other members of the PKC family including a C1 domain containing two cysteine-rich motifs that bind DAG, a C2-like phospholipid binding domain, a

pseudosubstrate domain, C3 and C4 catalytic domains that contain a purine binding site for ATP, and a substrate recognition site[68]. Like other PKCs, PKCε must be primed through phosphorylation to display full enzymatic activity and respond to lipid-derived second messengers: the main phosphorylation sites are Thr566, Thr710 and Ser729 located in the catalytic kinase core. PDK1 phosphorylates Thr566, while the other two sites likely undergo autophosphorylation similarly to what described in classic PKCs[69] (Figure 5).

Upon activation, PKCε translocates from the cytosolic to the particulate cell fraction via interaction with its anchoring proteins εRACKs (Receptors for Activated C Kinase), which provide access to PKCε substrates[70-71]. Consequently, interaction between PKCε and εRACK is a required step for PKCε function[72-73].

Among different PKC family isoforms, PKCε is now emerging as the one displaying the greatest oncogenic potential[74]. Indeed, PKCε was found over-expressed in a variety of both solid and hematologic malignancies, such as breast[75], gastro-intestinal[76-77], prostate[78-79], thyroid[80] and lung[81] cancers, aggressive gliomas[82], hairy cell leukemia[83] and AML[84].

Focusing on hematopoiesis, PKCε plays a crucial role in both murine and human models of *ex-vivo* hematopoiesis. More in details, Dr. Vitale's lab demonstrated that, during in-vitro erythroid maturation of normal $CD34^+$ cells, PKCε levels are low till day 6 of culture and then progressively increase, protecting late erythroid precursors from TRAIL-mediated apoptosis via Bcl-2 up-regulation, eventually allowing their full maturation[85]. By contrast, during megakaryocytic differentiation, PKCε levels display an opposite kinetic, peaking at day 6 and then progressively decreasing. PKCε down-modulation is necessary for MK full maturation, since a forced expression of PKCε from day 8 onward prevents MK full differentiation and favors Bcl-xL-mediated survival[86] (Figure 6).

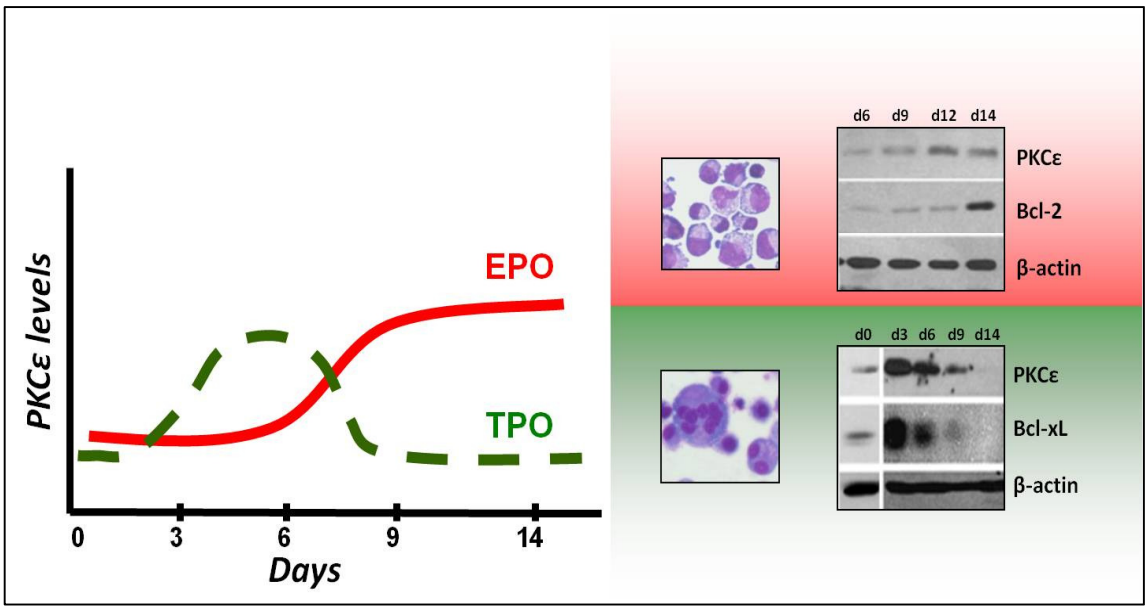


Figure 6. PKCε expression during erythroid *vs.* megakaryocytic differentiation of primary human CD34$^+$ (healthy subjects). Kinetic of expression of PKCε during EPO- and TPO-driven differentiation at various time points of culture (left panel). Western blot analysis of PKCε, Bcl-2 and Bcl-xL levels at various time points of erythroid and megakaryocytic differentiation (right panel). Modified from *Mirandola et al. Blood 2006*[85] and *Gobbi et al. Stem Cells 2007*[86].

The same team also used an in-vitro model of murine platelet production to investigate the expression and function of PKCε during murine proplatelet formation. Gobbi et al.[87] found that, in the mouse, PKCε expression escalates during megakaryocytic maturation reaching the highest levels of expression in terminal differentiated MKs; PKCε inhibition resulted in lower proplatelet-forming MKs and larger platelet diameter, proving an antithetical role of PKCε in murine *vs*. human thrombopoiesis.

Additionally, during human malignant hematopoiesis, it has been demonstrated that PKCε is over-expressed in AML blasts and that its down-modulation sensitizes AML cells to TRAIL-mediated apoptosis while inducing their full differentiation along the MK lineage[84].

Given this background, it is reasonable to hypothesize PKCε involvement in the molecular pathophysiology of PMF aberrant megakaryocytopoiesis.

Aim of the study

The aim of the present research was to:

(i) Analyze in-vitro MK differentiation of PMF hematopoietic progenitors as compared to healthy control subjects;

(ii) Assess whether PMF MK maturation defects are attributable to an over-expression of PKCε and its down-stream effector Bcl-xL;

(iii) Test if pharmacological inhibition of PKCε restores normal *in-vitro* megakaryocytopoiesis from PMF hematopoietic progenitors;

(iv) Investigate a potential interaction between PKCε and JAK/STAT signaling.

Material and methods

Patients and controls

After approval of the Ethical Committee of Parma University Hospital, ~30 mL of peripheral blood from 15 PMF patients were collected in ETDA tubes. Diagnosis was established according to WHO 2008 criteria[4]. Leukoapheresis bags from 10 G-CSF mobilized donors were utilized as controls (healthy donors, HD).

PMF patients' samples were collected at the time of diagnosis (PMF#: 1-5, 8-9, 11, 12, 14 and 15) or after cytoreductive-treatment wash out (PMF#: 6-7, 10 and 13).

Patient data were retrospectively collected from clinical charts. Prognostic risk category was assessed according to IPSS[11] for newly diagnosed patients or according to DIPSS[12] for patients with ongoing disease at the time of sample collection.

Cell cultures

Human Erythroleukemia Cell line (HEL) was grown in 10% FBS-enriched RPMI medium at the optimal density of 0.5×10^6 cells/mL.

Mononuclear cells from PMF and HD were obtained by Ficoll-Hypaque gradient. Primary $CD34^+$ cells were isolated by immunomagnetic positive selection as described in the previous chapter and cultured up to 14 days, at an optimal cell density of $1x10^6$/mL, in serum free X-vivo medium supplemented with 2 ng/mL IL-3 (only day 0), 200 ng/mL TPO and 50 ng/mL SCF (renewed every 72 hours) (all from PeproTech, London, UK). Cell number and viability were determined by Tripan Blue staining. Fold increase (FI) was calculated with

respect to the number of $CD34^+$ cells plated at day 0. At day 6, 9, 11 and 14 cells were collected for subsequent analyses.

MK isolation

At day 11 of culture, $CD61^+$-MKs were isolated using $CD61^+$ Isolation Kit (Mylteni Biotech) according to the manufacturer's recommendations, in the magnetic field of autoMACS® separator. A purity ≥ 90% was verified flow cytometrically labelling cells with RPE-conjugated anti-CD61 antibody (BD Pharmigen, San Diego). Cell lysates were then prepared for WB analyses.

Assessment of MK differentiation

At day 14 of culture, MK differantiation was evaluated by:

(i) assessing cell dimension by ImageJ software analyzing 100 cells per representative high power field (HPF) images (Leica IM DL, magnification 40x/0.5 NA);

(ii) assessing proplatelet formation by immunofluorescence as follows:

day 14 MKs were transferred on poly-l-lysine-coated coverslips and cultured overnight. Samples were then fixed in 4% formaldehyde, washed with phosphate-buffered saline (PBS), permeabilized with 0.5% Triton, and eventually blocked before antibody labeling. Samples were incubated with mouse monoclonal antibodies against α-tubulin (1:1000) and subsequently treated with secondary goat anti-mouse antibodies, conjugated to an Alexa Fluor 488 (all from Sigma-Aldrich,

St. Louis, MO). MK nuclei were counterstained with 4,6 diamidino-2-phenylindole (DAPI) (Sigma-Aldrich).

Specimens were examined with ECLIPSE 80i microscope (Nikon Corporation, Tokyo, http://www. nikon.co.jp/main/eng) and images acquired by Nikon Digital sight DS-L1 camera. ImageJ software was utilized for analyses.

(iii) determining the number of proplatelet-forming MKs/100 cells per representative HPF images obtained by Leica IM DL (magnification 40x/0.5 NA);

(iv) evaluating of the expression of early (CD41-RPE, Chemicon, Temecula, CA.) and late (CD42b-Cy5, BD Pharmigen) megakaryocytic markers. For this purpose, aliquots of $0.1x10^5$ cells were labeled.

Pharmacological inhibition of PKCε activity

PKCε activity was inhibited by the εV1-2 peptide (AnaSpec Inc, Fremont, CA, U.S.), which blocks PKCε-εRACK interaction and is delivered into cells by a cargo peptide (TAT_{47-57}); efficacy and specificity of this compound have already been tested previously[88-90]. From day 3, PMF cultures (n. 3) were treated with the εV1-2 peptide (1μM, renewed every 48 hours) or with the same concentration of sole εV1-2-delivery-peptide (TAT_{47-57}), as control. Untreated cultures were utilized for comparison.

shRNA cell infection

HEL were treated with shRNA against human-PKCε and "scrambled" shRNA, as control. Briefly, pLKO.1 lentiviral vector encoding shRNA against human PKCε (NM_005400; shPKCε) were obtained from Open-Biosystem (Thermo Fisher Scientific). As control (shRNA CT), we used the MISSION pLKO.1-puro Non-Target shRNA Control

Plasmid, not targeting any known genes (Sigma-Aldrich). The shRNA expressing viruses were produced in 293TL cells according to standard protocols.

Cells were collected for protein expression analysis after 5 days of puromycin-selection.

Western blot (WB)

Fifty µg proteins were separated on 10% SDS PAGE, transferred to nitrocellulose and incubated with the following primary antibodies according to the manufacturers' protocol: rabbit polyclonal anti-PKCε (Millipore, Billerica, MA, cat. # 06-991), anti-Bcl-xL (Cell Signaling Technology, Inc. Lake Placid, NY, cat. # 2762), anti-JAK2 (Cell Signaling Technology, cat. # 3230), anti-phosphoJAK2 (Tyr 1007/1008) (Cell Signaling Technology, Inc., cat. # 3771), anti-STAT5 (Cell Signaling Technology, cat. # 9358), anti phosphoSTAT5 (Tyr 694) (Cell Signaling Technology, cat. # 3230) antibodies and mouse monoclonal anti-GAPDH (Millipore, cat. # MAB374) antibody. Filters were washed and further incubated for 1.5 hours at room temperature with 1:5,000 peroxidase-conjugated anti-rabbit or with 1:2,000 peroxidase-conjugated anti-mouse IgG in the primary antibody working solution at room temperature. Immune complexes were detected with the ECL SuperSignal West Pico Chemiluminescent Substrate Detection System (Thermo Fisher Scientific).

Statistical analysis

Statistical analysis was performed by t-test or using analysis of variance (ANOVA) and Dunnett's test or Tukey's test, when applicable.

Results

PMF patients characteristics

Complete clinical information were available for 13 out of 15 PMFs, as detailed in Table 2. Briefly, 8 of them (61.5%) were male. Median age at diagnosis was 69 years (range 30-89 years). Mean WBC, Hb and PLT were 15,750/mm^3, 8 g/dl and 428,000/mm^3, respectively. Peripheral blood blast count ranges from 0 to 10%.

The great majority of patients (11 out of 13) was splenomegalic, as expected. Approximately one third experienced constitutional symtoms. Concerning risk stratification, 2 patients fell into the low risk category, 5 into the intermediate-1, 2 into the intermediate-2 and 4 into the high one.

Code	Gender, age	*JAK2* V617F	WBC mm3	Hb g/dl	PLT mm3	Blast %	Spleen Øcm	IPSS*	DIPSS#	c. symptoms	Therapy
PMF1	*nd*	pos	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
PMF2	M, 89	pos	7,750	14.8	738,000	0	*nd*	Int-1	-	no	Aspirin, HU
PMF3	F, 69	neg	7,140	12.6	667,000	0	9	Int-1	-	no	Aspirin, HU
PMF4	M,82	pos	24,000	7.9	174,000	1	15	Int-2	-	no	HU
PMF5	*nd*	pos	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
PMF6	F, 66	pos	11,370	11.7	393,000	0	19	-	Int-1	yes	Aspirin, HU+da
PMF7	M,36	pos	33,680	11.4	286,000	3	28	-	High	yes	Aspirin, HU, ifn, ruxo
PMF8	M,73	pos	8,350	11.7	97,000	2	9	Int-2	-	no	watch&wait
PMF9	F,70	pos	12,110	11.5	107,000	0	17	Int-1		no	watch&wait
PMF10	M,70	pos	65,000	8	514,000	1	21	-	High	yes	Aspirin, HU
PMF11	M,33	neg	4,530	13.2	886,000	0	12	Low	-	no	Aspirin
PMF12	F,30	neg	4,910	10.6	618,000	0	15.5	Low	-	no	Aspirin
PMF13	M,63	neg	9,500	8.2	7,000	10	23	-	High	yes	Aspirin, ana, HU+da, melph, ruxo, CHT

PMF14	M,78	*nd*	8,210	9.7	984,000	2	16	High	-	no	Aspirin, HU
PMF15	M,74	neg	8,270	12.9	94,000	0	18	Int-1	-	no	watch&wait

Table 2. Clinical and laboratory characteristics of PMF patients.
*IPSS (International Prognostic Scoring System) has been calculated for patients with newly diagnosed disease at time of sample collection. #DIPSS (Dynamic International Prognostic Scoring System) has been calculated for patients with ongoing disease at time of sample collection.
Abbreviations: ana: anagrelide; c. symptoms: constitutional symptoms; CHT: conventional AML-like chemotherapy; da: danazole; dx: diagnosis; HU: hydroxyurea; ifn: interferon-α; Int-1: intermediate-1; Int-2: intermediate-2; melph: melphalan; neg: negative; nd: not determined; pos: positive;. ruxo: ruxolitinib.

PMF CD34$^+$ cells show in-vitro enhanced proliferation and impaired megakaryocytic differentiation

To evaluate proliferation and megakaryocytic differentiation of primary CD34$^+$ cells from PMFs and HDs, cells from each conditions were grown in serum-free megakaryocytic cultures up to 14 days.

Changes in cell growth were assessed as the FI in cell numbers from days 0-14. As expected, PMF CD34$^+$ cells showed a significantly higher expansion capacity at each time point of the culture analyzed (day 3, 6, 9, 11 and 14) (Figure 7A). Indeed, at the end of the culture (day 14), PMF CD34$^+$ cells displayed a 12.7 FI in cell expansion compared to HD CD34$^+$ cells (38.3±17.2 *vs* 2.7±2.9, p=0.006).

The differentiation state of PMF and HD megakaryocytic cultures was assessed at day 14 by MK surface antigen expression (Figure 7B), cell morphology (Figure 7C), cell diameter (Figure 7D), and proplatelet generation capacity (Figure 7C-E).

At the end of cultures, PMF MK culture appeared as a uniform population of small cells, poorly differentiated as compared to HD, in which bigger cells (i.e. MKs) sprouting

proplatelets could be detected (Figure 7C). To support this, mean PMF cell diameter was significantly smaller than HD (8.9±3.4 µm *vs* 12.1±5.5 µm, respectively, p=0.001, Figure 7D). Immunofluorescence studies confirmed that PMF megakaryocytic cultures showed significantly impaired proplatelet generation compared to HD (Figure 7C right panels) and the number of proplatelet-forming MKs in culture is significantly lower in PMF than in HD (1.16±0.64 *vs* 4.81±1.39, respectively. p=0.003, Figure 7E).

Day-14 MK maturation was also evaluated by flow cytometric expression of early (CD41) and late (CD42b) MK surface antigens (Figure 7B). Compared to HD, PMF specimens displayed significantly lower percentages of $CD41^+$ (PMF=25.7±18.2% *vs* HD=65.9±19.4% p=0.04), $CD42b^+$ (PMF=16.2±17.5% *vs* HD=53.3±19.7%, p=0.04) and $CD41^+/CD42b^+$ cells (21.9±11.6% *vs* 62.5±6.9%, p<0.001).

Collectively, these data provide a comprehensive characterization of *in-vitro* behavior of TPO-stimulated PMF $CD34^+$ cells in our serum-free based culture system, showing enhanced growth and impaired MK differentiation compared to $CD34^+$ cells derived from HD.

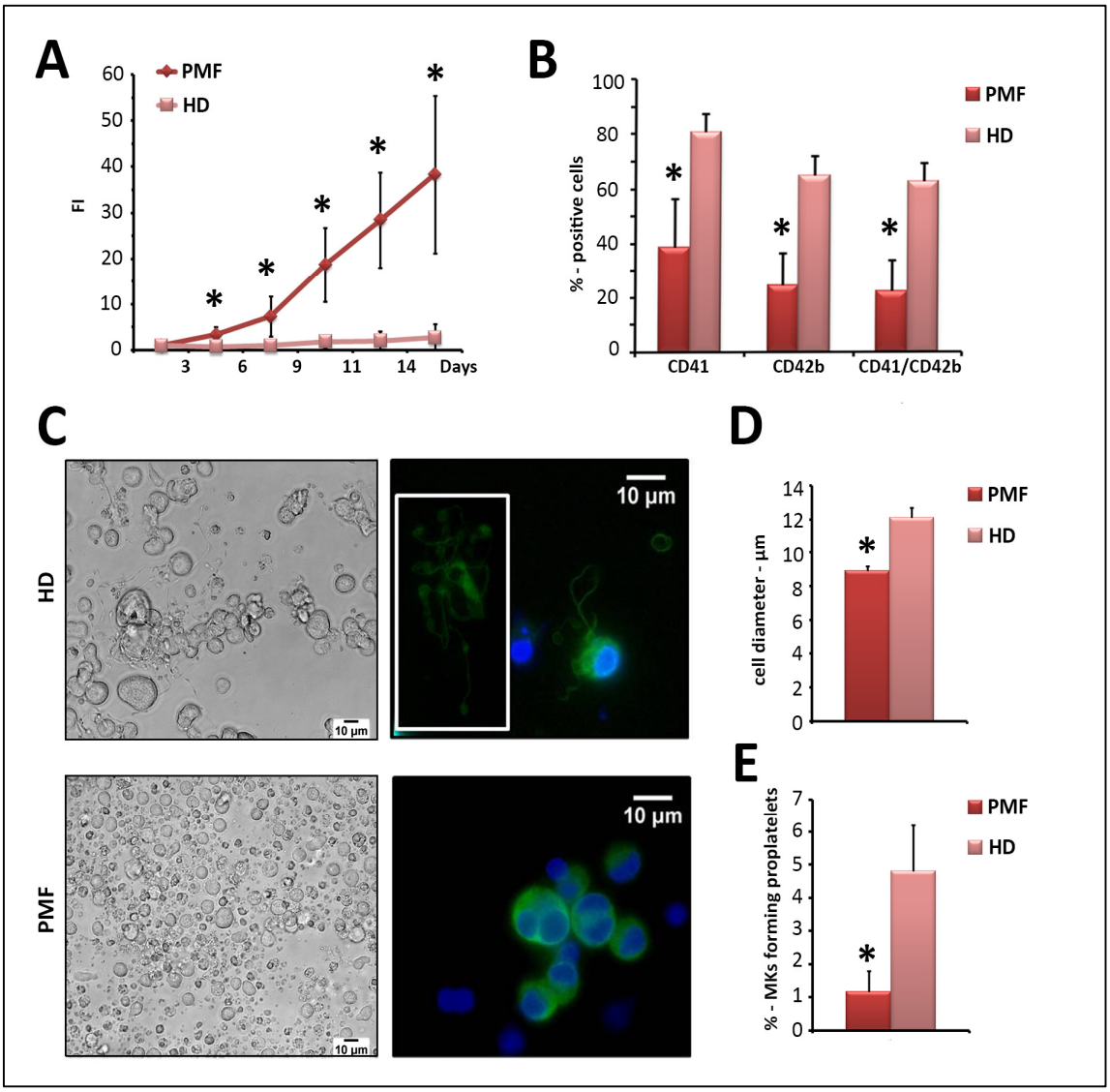


Figure 7. PMF CD34$^+$ cells display higher expansion capacity and impaired megakaryocytic differentiation than HD CD34$^+$ cells.
(A) Growth curves of CD34$^+$ cell cultures from 4 PMFs and 4 HDs cultured in the presence of IL-3, TPO and SCF. Cell count and viability was assessed by Tripan Blue staining. Data are expressed as FI with respect to the number of CD34$^+$ cells seeded in culture at day 0. *p<0.05 *vs* HD; *t*-test statistical analysis. **(B)** Flow cytometric assessment of megakaryocytic differentiation of 4 PMF and 3 HD cultures (day 14 days). Percentages of CD41$^+$ and CD42b$^+$ and double positive CD41$^+$/CD42b$^+$ cells are reported (mean±SD). *p<0.05 *vs* HD; *t*-test statistical analysis. **(C)** Inverted microscope representative images of PMF and HD MK culture at day 14 (left panels, 40x/0.5 NA magnification of Leica IM DL inverted microscope) and representative images of different proplatelet output from day-14 PMF and HD MKs after overnight cultures on poly-L-lysine coverslips. Proplatelets are revealed by α-tubulin staining (green) and nuclei are counterstained with DAPI (original magnification 100x/1.4 NA oil objective, fluorescent direct microscope) (right panels). **(D)** Mean cell diameter (from > 100 cells) and relative standard deviation (SD) from 3 PMF and 3 HD MK cultures (14 days). Cell plates were observed by inverted microscope and the acquired images analyzed by ImageJ software (original magnification: 400x/0.5 NA, *p<0.05 *vs* HD; *t*-test statistical analysis). **(E)** Proplatelet-forming MKs (expressed as % of total cells observed at 40x/0.5 NA magnification of Leica IM DL inverted microscope) at day 14 of 3 PMF and 3 HD cultures (mean±SD, *p<0.05 *vs* HD; *t*-test statistical analysis).

PMF megakaryocytic cultures display higher levels of PKCε and Bcl-xL

Gobbi et al. previously demonstrated that the expression of PKCε and, concurrently, of Bcl-xL, changes throughout the course of in-vitro MK differentiation of primary human $CD34^+$ cells, peaking at day 6 and then progressively decreasing till nearly undetectable levels at day 14 of culture. Therefore, I evaluated the expression levels of PKCε and Bcl-xL proteins during in-vitro MK differentiation of primary $CD34^+$ cells from PMF and HD in the unfractioned cell population (Figure 8A-C) and, at day 11 of culture, in PMF and HD $CD61^+$ cells (Figure 8D-E).

I found that PKCε and Bcl-xL levels are significantly higher in PMF megakaryocytic cultures compared to HD at each time point analyzed and that the two proteins are modulated with a similar kinetic during megakaryocytic differentiation (Figure 8A-C).

I then focused on the expression of PKCε and Bcl-xL in a selected, more differentiated cell population, comparing PKCε and Bcl-xL protein levels in day 11-$CD61^+$ cells immunomagnetically selected from PMF and HD cultures. Similarly to what observed in the unfractioned cell population, PMF $CD61^+$ cells retained higher levels of PKCε and Bcl-xL compared to HD $CD61^+$ cells (Figure 8D-E).

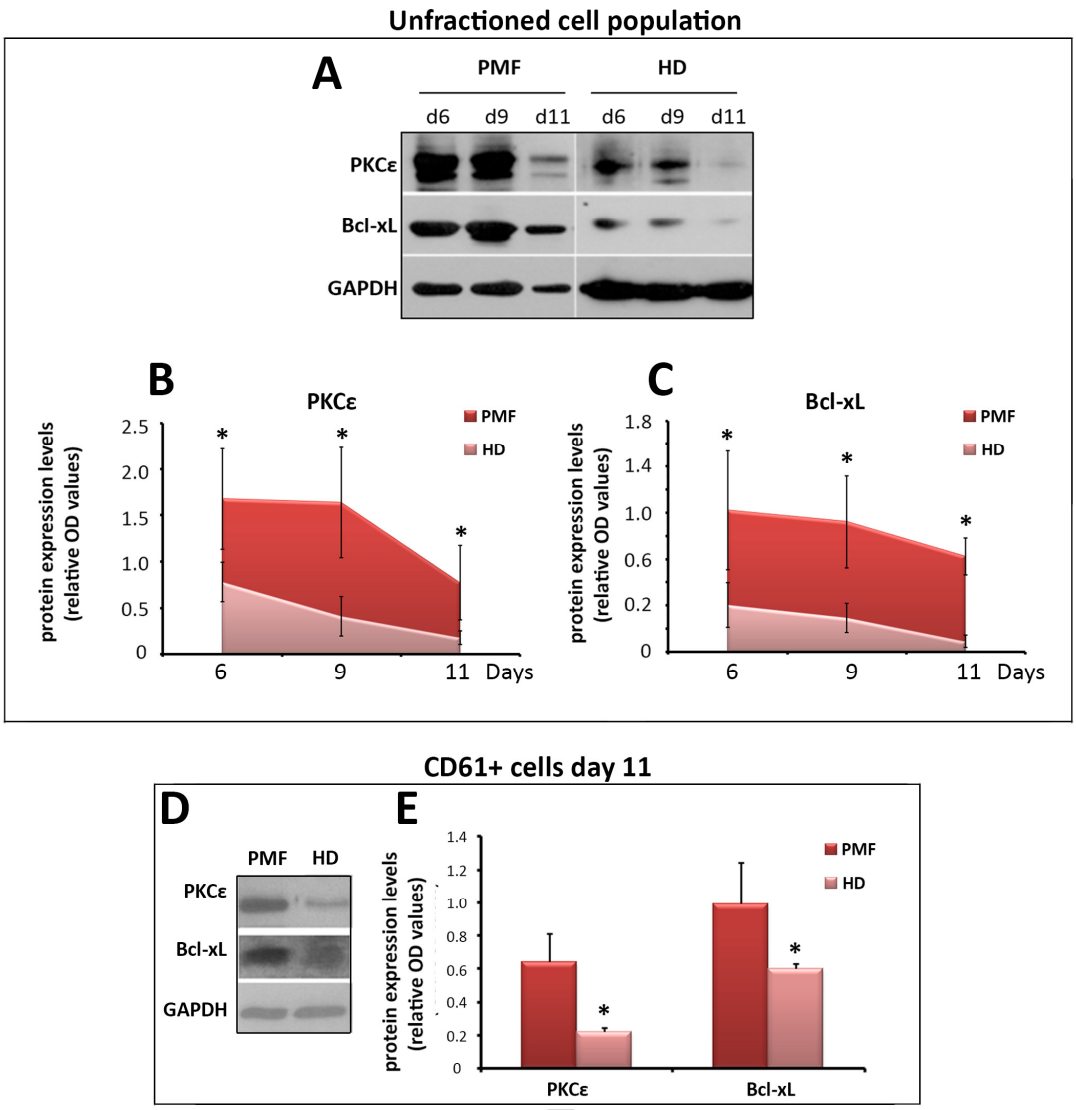


Figure 8. PKCε over-expression typifies PMF MKs and is associated with increased Bcl-xL levels.
(A) Representative western blot analysis of PKCε and Bcl-xL kinetics at day 6, 9, 11 of MK differentiation of PMF and HD $CD34^+$ cells. **(B-C)** Densitometric measurements (relative PKCε/GAPDH OD values and Bcl-xL/GAPDH OD values) of western blots from 4 PMFs and 4 HDs at day 6, 9, 11 of culture (means ±SD) (*$p<0.05$ *vs* HD; *t*-test statistical analysis). **(D)** Representative western blot analysis of PKCε and Bcl-xL expression in day-11 PMF *vs* HD $CD61^+$ cells. **(E)** Densitometric measurements (relative PKCε/GAPDH OD values and Bcl-xL/GAPDH OD values) of western blots of $CD61^+$ fractions from 3 PMF and 3 HD megakaryocytic cultures (means ±SD) (*$p< 0.05$ *vs* HD; *t*-test statistical analysis).

PMF MK cultures from high risk patients display enhanced growth, impaired differentiation and higher PKCε levels compared to low/intermediate risk patients

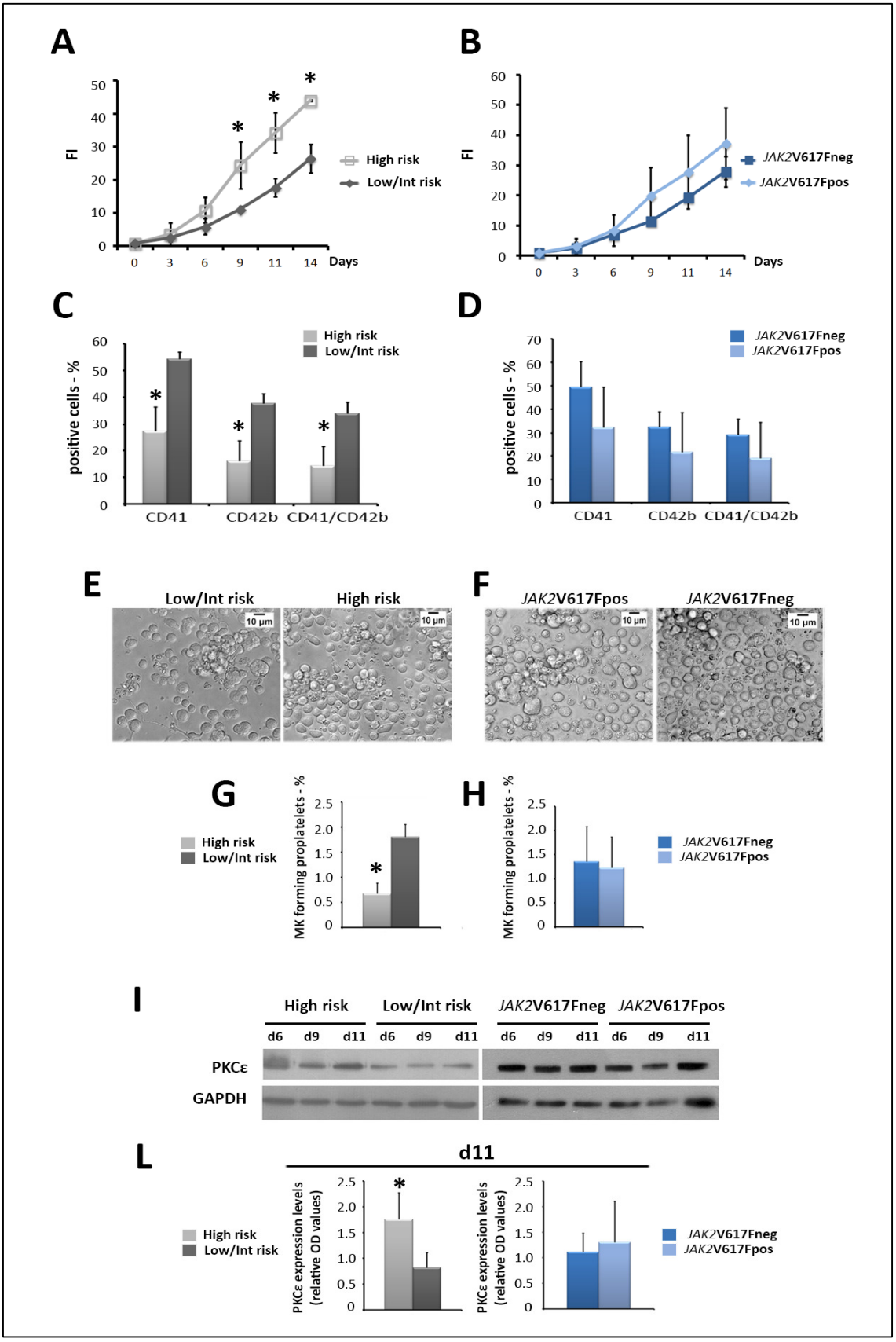
Although PMF $CD34^+$ cells clearly showed enhanced growth and impaired megakaryocytic differentiation as compared to HD, I did observed some degree of variability in the magnitude of these cellular characteristics among patients' samples. This prompted me to investigate whether clinical (included in the IPSS or DIPSS risk category) or biological (*JAK2* mutational status) variables might impact the in-vitro behavior of PMF hematopoietic progenitors.

Specifically, I stratified patients according to the IPSS or DIPSS risk category (low/intermediate *vs* high risk) and *JAK2* mutational status (*JAK2*$V617F^{pos}$ *vs* *JAK2*$V617F^{neg}$) and evaluated: (i) expansion capacity (expressed as FI), (ii) megakaryocytic differentiation (expressed as % of $CD41^+$, $CD42b^+$ and $CD41^+/CD42b^+$ cells and % of proplatelet-forming MKs) and (iii) PKCε levels at day 11 of TPO-stimulated cultures (by western blot analysis).

As shown in Figure 9A-B, $CD34^+$ cells from high risk patients clearly display an increased proliferative capacity from day 9 onwards as compared to low/intermediate risk patients (day 14-FI: 44±0.2 *vs* 26.5±4.3, p=0.012), while no difference could be observed between *JAK2*$V617F^{pos}$ and *JAK2*$V617F^{neg}$ patients (day 14-FI: 37.2±11.8 *vs* 27.9±5, p= 0.39) consistently with the observation of Ciurea et al.[65]. Additionally, high risk patients revealed impaired megakaryocytic differentiation potential, as indicated by the lower % of $CD41^+$, $CD42b^+$ and $CD41^+/CD42b^+$ cells (respectively: 26.3±9.4 *vs* 54.2.6, p= 0.008; 16.1±8 *vs* 38.2±3, p= 0.011; 14.2±7.5% vs 34±4.4% p=0.017, Figure 9C) and proplatelet-forming MKs (0.67±0.21 *vs* 1.8±0.25 p=0.004, Figure 9E and G). I did not observe any significant

differences by stratifying patients according to *JAK2* mutational status (for $CD41^+$: 49.4±10.8% of *JAK2*$V617F^{neg}$ *vs* 31.2±17.8 of *JAK2*$V617F^{pos}$ PMFs, p= 0.21; for $CD42b^+$: 32.8±6.7% of *JAK2*$V617F^{neg}$ *vs* 21.5±17.2% of *JAK2*$V617F^{pos}$ PMFs, p=0.35; for $CD41^+/CD42b^+$: 29.3±6.6% of *JAK2*$V617F^{neg}$ *vs* 19±15.7 of *JAK2*$V617F^{pos}$ PMFs, p=0.35, Figure 9D; for proplatelet-forming MKs: 1.36±0.73% for *JAK2*$V617F^{neg}$ *vs* 1.22±0.65% for *JAK2*$V617F^{pos}$ PMFs, p=0.82, Figure 9F and H).

Finally, I found that megakaryocytic cultures from high risk patients are characterized by higher expression of PKCε as compared to low/intermediate risk patients (PKCε/GAPDH OD values at day 11: high risk=1.75±0.52 *vs* low/int risk=0.82±0.29, p=0.023), while PKCε levels are comparable among *JAK2*$V617F^{pos}$ and *JAK2*$V617F^{neg}$ patients (PKCε/GAPDH OD values at day 11: *JAK2*$V617F^{pos}$=1.11±0.34 *vs JAK2*$V617F^{neg}$ =1.3±0.8, p=0.72), Figure 9I-L.


A
FI
Days
High risk
Low/Int risk
B
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Days
JAK2V617Fneg
JAK2V617Fpos
C
positive cells - %
CD41
CD42b
CD41/CD42b
High risk
Low/Int risk
D
positive cells - %
CD41
CD42b
CD41/CD42b
JAK2V617Fneg
JAK2V617Fpos
E
Low/Int risk
High risk
10 µm
F
JAK2V617Fpos
JAK2V617Fneg
10 µm
G
MK forming proplatelets - %
High risk
Low/Int risk
H
MK forming proplatelets - %
JAK2V617Fneg
JAK2V617Fpos
I
High risk
Low/Int risk
JAK2V617Fneg
JAK2V617Fpos
d6
d9
d11
PKCε
GAPDH
L
d11
PKCε expression levels (relative OD values)
High risk
Low/Int risk
JAK2V617Fneg
JAK2V617Fpos

Figure 9. MK cultures from high risk patients display enhanced proliferative capacity, impaired megakaryocytic differentiation and higher PKCε levels compared to low/intermediate risk patients.
PMF cultures were stratified according to the IPSS or DIPSS risk category (high vs low/intermediate) and the *JAK2* mutational status of the donors. **(A-B)** Growth curves of $CD34^+$ cell cultures from 3 high risk *vs* 3 low/intermediate (low/int) risk PMFs **(A)** and from 3 *JAK2*$V617F^{pos}$ *vs* 3 *JAK2*$V617F^{neg}$ PMFs **(B)** in the presence of IL-3, TPO and SCF. Cell count and viability was assessed by Tripan Blue staining. Data are expressed as FI with respect to the number of cells seeded in culture at day 0. *p<0.05 *vs* low/int; *t*-test statistical analysis. **(C-D)** Flow cytometric assessment of megakaryocytic differentiation of 3 high risk *vs* 3 low/int risk PMFs **(C)** and from 3 *JAK2*$V617F^{pos}$ *vs* 3 *JAK2*$V617F^{neg}$ PMFs **(D)** after 14 days of megakaryocytic culture. Percentages of $CD41^+$ and $CD42b^+$ and $CD41^+/CD42b^+$ cells are reported (mean±SD) *p<0.05 *vs* low/int; *t*-test statistical analysis. **(E-F)** Morphology (Inverted microscope representative images of high *vs* low/int. and *JAK2*$V617F^{pos}$ *vs JAK2*$V617F^{neg}$ PMFs at day 14 of culture (40x/0.5 NA magnification of Leica IM DL inverted microscope) **(G-H)** proplatelet-forming MKs at day 14 of culture (% of total cells observed at day 14 at 40x/0.5 NA magnification of Leica IM DL inverted microscope) Mean±SD of 3 high risk vs 3 low/int PMFs **(G)** and of 3 *JAK2*$V617F^{pos}$ *vs* 3 *JAK2*$V617F^{neg}$ PMFs **(H)** are presented. *p<0.05 *vs* low/int *t*-test statistical analysis. **(L-I)** PKCε protein expression [representative western blots kinetic **(L)** and relative PKCε/GAPDH OD values at day 11 **(I)**]. Mean±SD of 3 high *vs* 4 low/int PMF cultures and of 4 *JAK2*$V617F^{pos}$ *vs* 3 *JAK2*$V617F^{neg}$ are shown. *p<0.05 *vs* low/int; *t*-test statistical analysis.

PKCε pharmacological inhibition reduces cell growth and restores megakaryocytic differentiation of PMF $CD34^+$ cells

Given these data, I we sought to assess whether PKCε pharmacological inhibition was able to affect in-vitro growth and megakaryocytic differentiation of primary PMF $CD34^+$ cells. To test this, PKCε activity was specifically inhibited by a commercially available compound, the εV1-2 peptide.

I first observed that PKCε inhibition led to up to ~20% of reduction of PMF $CD34^+$ cell growth capacity (assessed as residual FI after the addition, from day 3 onward, of εV1-2), while the sole TAT_{47-57} did not produce any effect on cell growth (Figure 10A), indicating that PKCε inhibition significantly reduced PMF $CD34^+$ cells expansion capacity.

I then tested the effects of PKCε inhibition on PMF $CD34^+$ megakaryocytic differentiation, assessed by: expression of megakaryocytic differentiation antigens (Figure

10B), morphology (Figure 10C), cell diameter (Figure 10D) and proplatelet formation (Figure 10C-E).

I found that PKCε inhibition induced the generation of mature MKs: $CD42b^+$ and $CD41^+/CD42b^+$ cell populations increased by >30% ($p<0.01$, Figure 10B, data expressed as % of untreated cultures). No statistically significant increase of $CD42b^+$ and $CD41^+/CD42b^+$ cells was produced by TAT_{47-57}. PKCε inhibition led only to a slight increase of $CD41^+$ cells which is not surprising since CD41 is an early differentiation marker.

I observed that PKCε inhibition was capable to increase the mean cell diameter of PMF cultures (11.5±0.3µm *vs* 9.2±0.5µm of PMF UNTR and *vs* 9.5±0.3µm of PMF+TAT, $p<0.001$, Figure 10D) up to values that are comparable to those of HD cultures (12.1±0.6 µm, see Figure 7D).

Consistently, morphology of MKs generated from the εV1-2-treated cultures mirrors the more mature phenotype with respect to control cultures, as shown in Figure 10C, upper panels).

Immunofluorescence studies reveal that MKs from the εV1-2-treated cultures are capable of undergoing cytoskeleton modifications and generate proplatelets when compared to the PMF UNTR and PMF+TAT (Figure 10C, lower panels). Moreover, the number of proplatelet-forming MKs in εV1-2-treated cultures is 3-fold higher than in PMF UNTR and PMF+TAT conditions (Figure 10E, $p=0.006$).

Overall these data suggest that PKCε pharmacological inhibition hampers PMF cell growth and promotes restoration of PMF MK morphology, phenotype and ability to generate proplatelets, all relevant parameters of MK terminal differentiation.

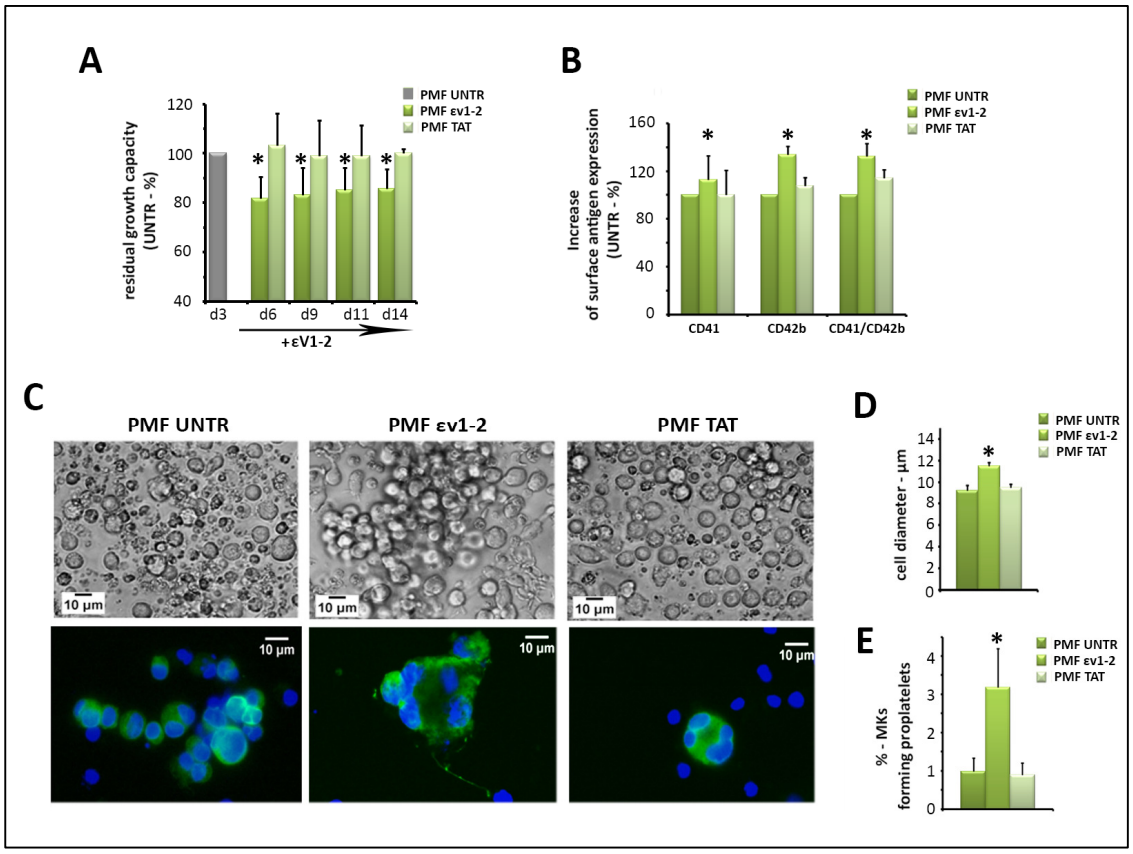

Figura 10. PKCε pharmacological inhibition reduces growth capacity and restores megakaryocytic differentiation of PMF CD34$^+$ cells.

(A) Residual growth capacity after treatment with PKCε inhibitor (PMF+εV1-2) and with the sole carrier TAT_{47-57} (PMF+TAT) of TPO-differentiated PMF cultures at different time-points (day 3, 6, 9, 11 and 14). Cell count and viability was assessed by Tripan Blue staining. Peptides were added from day 3 as described in the text. Data are expressed as percentage of untreated cultures (PMF UNTR) (mean±SD of 3 replicates). *p<0.05 *vs* UNTR, statistical analysis by ANOVA followed by Dunnett's test. **(B)** Flow cytometric assessment of CD41$^+$, CD42b$^+$ and CD41$^+$/CD42b$^+$ at day 14 of PMF UNTR, PMF+εV1-2 and PMF TAT. Mean ± SD of 3 replicates are reported (*p<0.05 *vs* UNTR, statistical analysis by ANOVA followed by Dunnett's test). **(C)** Upper panels: representative inverted microscope images of 14 days cultures from PMF UNTR, PMF+εV1-2 and PMF+TAT (Leica IM DL inverted microscope, original magnification 400x/0.5 NA). Lower panels: immunofluorescence detection of proplatelet formation by 14 days-MKs from PMF UNTR, PMF+εV1-2 and PMF+TAT cultures. Proplatelets are revealed by anti- α-tubulin staining (green) while nuclei are counterstained with DAPI (original magnification 100x/1.4 NA oil objective, Nikon ECLIPSE 80i microscope, Nikon digital sight DS-L1 camera). **(D)** Cell diameter (>100 cells) from PMF UNTR, PMF+εV1-2 and PMF+TAT at day 14 of culture (Leica IM DL inverted microscope, original magnification 400x/0.5 NA). Mean±SD from 3 replicates are reported (*p<0.05 vs UNTR, statistical analysis by ANOVA followed by Dunnett's test). **(E)** Proplatelet-forming MKs in PMF UNTR, PMF+εV1-2 and PMF+TAT cultures at day 14 (data expressed as % of total cells observed at 40x/0.5 NA magnification of Leica IM DL inverted microscope). Mean±SD of 3 replicates is shown (*p<0.05 *vs* UNTR; statistical analysis by ANOVA followed by Dunnett's test).

PKCε inhibition reduces the levels of the anti-apoptotic protein Bcl-xL in PMF megakaryocytic cultures

In order to demonstrate that PKCε inhibition was capable to switch off the down-stream anti-apoptotic signaling that contributed to PMF cells enhanced proliferation, I tested whether PMF MKs generated from εV1-2 treated cultures had reduced levels of the anti-apoptotic protein Bcl-xL. I found that inhibition of PKCε function by the εV1-2 peptide leads to a statistically significant decrease (~30%) in the amount of Bcl-xL protein (p=0.049), as shown by the representative western blot image and the relative Bcl-xL/GAPDH OD values (Figure 11).

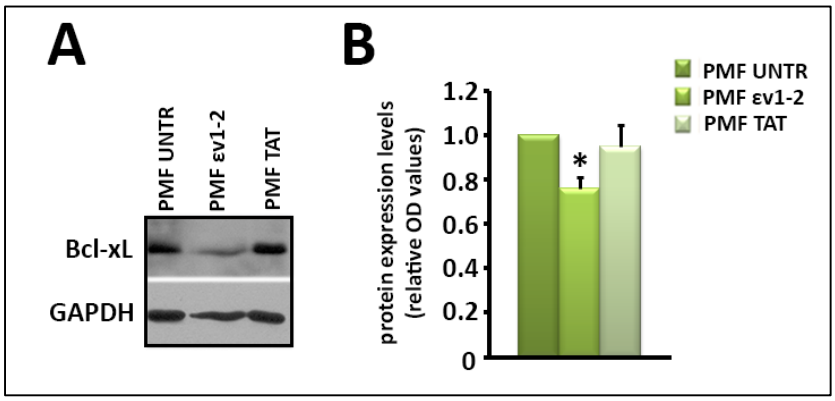

Figura 11. PKCε inhibition reduces Bcl-xL expression.
(A) Representative western blot analysis of Bcl-xL levels in day-11 PMF MK cultures. Untreated cultures (PMF UNTR) were compared to PMF+εV1-2 and PMF+TAT. **(B)** Densitometric measurements of Bcl-xL protein expression levels. Relative Bcl-xL/GAPDH OD values from western blots of 3 separate experiments are reported (mean±SD). Data are presented as % of PMF UNTR. *p<0.05, statistical analysis by ANOVA followed by Dunnett's test.

PKCε silencing does not affect the activation status of JAK/STAT signaling

To investigate a potential correlation between PKCε signaling and JAK/STAT axis, I tested the effects of shRNA-mediated down-regulation of PKCε expression on a Human Erythroleukemia cell line model (HEL cells), and I found that PKCε silencing does not cause any statistically significant modulation of total and phosphorylated JAK2 and STAT5 (Figure 12).

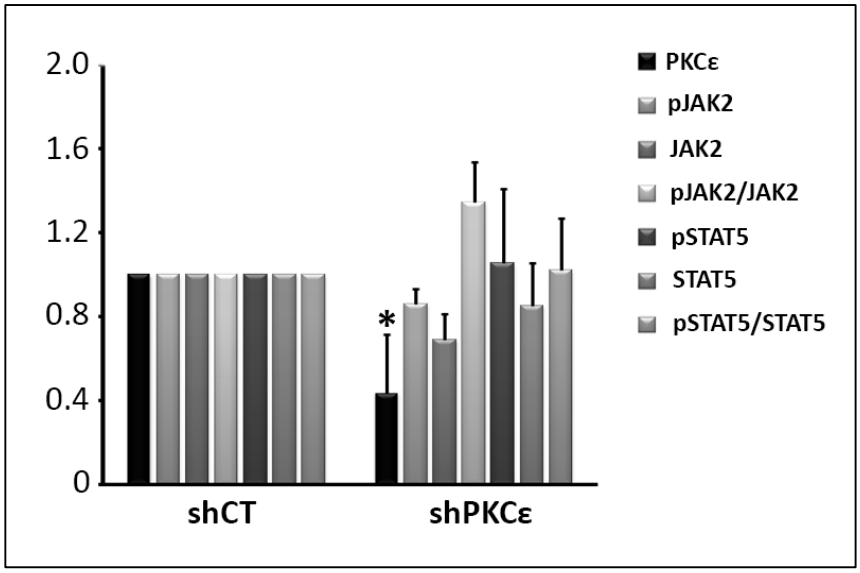

Figure 12. PKCε-silencing does not modulate JAK/STAT signaling.
PKCε, phospho-JAK2, JAK2, phospho-STAT5 and STAT5 protein expression of HEL cells treated with shCT and shPKCε. The degree of JAK2 and STAT5 phosphorylation with respect to total proteins is also represented (p-JAK2/JAK2; p-STAT5/STAT5). Protein expression levels are normalized for GAPDH and represented as arbitrary unit of shCT. (Mean ± SD of 3 independent experiments are reported, *$p < 0.05$; t-test statistical analysis).

Discussion

Hyperplasia of morphologically abnormal MKs, BM fibrosis and consequent extramedullary hematopoiesis are hallmarks of PMF. Current dogma holds that bone marrow changes as well as the clinical phenotype reflect a cytokine-mediated secondary inflammatory response that is triggered by the abnormal interaction between the neoplastic MK clone and polyclonal bone marrow stromal cells[91].

The molecular events that lead to disturbances of the normal development of megakaryopoiesis are still largely unknown. In the present study, I aimed to unravel one of the potential mechanisms that accounts for this pathognomonic aspect of PMF, identifying the ε isoform of novel PKCs as a crucial player in this process.

PKCε is finely tuned in in-vitro models of human megakaryocytopoiesis, reaching the highest levels in early MK progenitors and then decreasing in more differentiated MKs. Since a forced expression of PKCε in the later phases of megakaryocytopoiesis interferes with MK differentiation[86], I hypothesized that PKCε over-expression could underlie PMF MKs altered differentiation and tested whether its modulation could affect PMF MK maturation.

I assessed the megakaryocytic commitment of PMF $CD34^+$ cells in our serum-free based culture system and, consistently with previous findings[65-66], I observed that, respect to normal $CD34^+$ cells, PMF hematopoietic progenitors display increased proliferative capacity associated with impaired megakaryocytic differentiation as demonstrated by smaller size (micro-megakaryocytes), reduced expression of MK surface markers and markedly impaired proplatelet formation.

Additionally, since I observed some degree of heterogeneity in terms of cell growth and MK differentiation among different PMF donors - that could reasonably reflects the broad

clinical phenotype of the disease - I investigated a potential correlation between clinical (i.e. IPSS/DIPSS risk category) or biological (i.e. *JAK2* mutational status) variables and the in-vitro behavior of PMF hematopoietic progenitors.

I proved that enhanced expansion capacity and impaired megakaryocytic commitment of PMF $CD34^+$ cells are associated with a high IPSS/DIPSS risk category of the donors, while no correlation was found with the *JAK2*V617F mutational status.

I then demonstrated that PMF $CD34^+$ MK cultures were characterized by significantly higher levels of PKCε and Bcl-xL. Particularly, at day 11, when the kinase is supposed to be down-modulated, relative PKCε protein levels are as high as day 6 in HDs (0.77±0.4 *vs* 0.78±0.21, respectively). PKCε sustained levels drive, in PMF, a Bcl-xL-mediated pro-survival and anti-differentiation signal (consistent with a report from Ciurea et al.[65]).

Moreover, I demonstrated that the over-expression of PKCε and Bcl-xL in the late phases of megakaryocytic differentiation (day 11) is attributable to the fact that PMF-$CD61^+$ cells, differently from HD $CD61^+$ cells, retain high expression levels of both proteins. Consequently, we can assume that the over-expression of PKCε and Bcl-xL in PMF MKs plays a significant role in the pathophysiology of their aberrant phenotype and behavior.

Additionally, I found that PKCε levels are significantly greater in high *vs* low/intermediate risk patients while no correlation was established according to *JAK2* mutational status, indicating that PKCε expression correlates with high IPSS/DIPSS risk categories. This leads us to speculate that PKCε can be utilized as a marker of high disease burden and a more aggressive disease.

To clarify the contribution of PKCε over-expression to PMF MKs hyperplasia/dysplasia, we tested the effects of PKCε pharmacological inhibition on PMF

$CD34^+$-derived MKs. We found that PKCε inhibition was capable to improve significantly PMF MK maturation, to limit their expansion and to reduce the down-stream anti-apoptotic signaling (Bcl-xL).

Taken together, my data indicate that PKCε detains a central role in the pathogenesis of the abnormal megaryocytopoiesis that typifies PMF and that its over-expression may contribute to sustained proliferation and impaired differentiation of the MK neoplastic clone.

Overall, the present research provides a novel insight into the pathogenesis of PMF and represents a proof-of-concept for PKCε as a new potential "druggable" target in this disease, whose current therapeutic options are still scant.

Indeed, inhibition of PKCε in the clinical setting is not a distant scenario, since εV1-2 peptide – also known as KAI-1678 – has already been tested in the clinic *(www.clinicaltrial.gov)*.

KAI -1678 has been investigated phase I/II clinical trials testing its safety and efficacy in post-herpetic neuralgia, post-operative pain and pain due to spinal cord injury. The drug has been administered subcutaneously and, overall, resulted well tolerated[92-93].

Given this, therapeutic strategies based on PKCε inhibition may offer the basis for future treatment of PMF.

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List of publications in the course of PhD program

Articles in peer-reviewed journals

1. **PROTEIN KINASE Cε INHIBITION RESTORES MEGAKARYOCYTIC DIFFERENTIATION OF HEMATOPOIETIC PROGENITORS FROM PRIMARY MYELOFIBROSIS PATIENTS.**

   Masselli E, Carubbi C, Gobbi G, Mirandola P, Galli D, Martini S, Bonomini S, Crugnola M, Craviotto L, Aversa F, Vitale M.
   Leukemia. 2015 Nov;29(11):2192-201.

2. **PKCε IS A NEGATIVE REGULATOR OF PVAT-DERIVED VESSEL FORMATION.**

   Galli D, Carubbi C, **Masselli E**, Corradi D, Dei Cas A, Nouvenne A, Arcari ML, Bucci G, Mirandola P, Vitale M, Gobbi G.
   Exp Cell Res. 2015 Jan 15;330(2):277-86

3. **LABORATORY DIAGNOSTICS OF INHERITED PLATELET DISORDERS.**

   Carubbi C*, **Masselli E*,** Nouvenne A, Russo D, Galli D, Mirandola P, Gobbi G, Vitale M. *(*coauthors)*
   Clin Chem Lab Med. 2014 Aug;52(8):1091-106.

4. **CYTOFLUORIMETRIC PLATELET ANALYSIS.**

   Carubbi C*, **Masselli E***, Gesi M, Galli D, Mirandola P, Vitale M, Gobbi G. *(*coauthors)*
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5. **PROPLATELET GENERATION IN THE MOUSE REQUIRES PKCε-DEPENDENT RhoA-INHIBITION.**

   Gobbi G, Mirandola P, Carubbi C, **Masselli E**, Sykes SM, Ferraro F, Nouvenne A, Thon JN, Italiano JE, Vitale M.
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6. **IMPLICATION OF MAPK1/MAPK3 SIGNALING PATHWAY IN t(8;9)(p22;24)/*PCM1-JAK2* MYELODYSPLASTIC/MYELOPROLIFERATIVE NEOPLASMS.**

Masselli E, Mecucci C, Gobbi G, Carubbi C, Pierini V, Sammarelli G, Bonomini S, Prezioso L, Rossetti E, Caramatti C, Aversa F, Vitale M.
Br. J. Haematol. 2013 Aug;162(4):563-4.

Book chapters

***PCM1-JAK2* MYELODYSPLASTIC/MYELOPROLIFERATIVE NEOPLASMS.**

Masselli E, Vitale M, Aversa F.
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Editor: Antony M. Camden. Nova Publisher, Inc. 2014

Proceeding from international congresses

1. **MONITORING OF PCM1-JAK2 FUSION TRANSCRIPT IN A CASE OF t(8;9)(P22;24)-RELATED MYELODYSPLASTIC/MYELOPROLIFERATIVE SYNDROME.**

   Masselli E, Prezioso L, Mecucci C, Sammarelli G, Bonomini S, Lambertini C, Craviotto L, Rossetti E, Caramatti C, Vitale M, Aversa A.
   3rd International Congress Translational Research in Oncology. Forlì May 6-9, 2014. ***Poster Presentation***

2. **PROTEIN KINASE C EPSILON INHIBITION RESTORES IN-VITRO MEGAKARYOCYTE DIFFERENTIATION OF PRIMARY MYELOFIBROSIS HEMATOPOIETIC PROGENITORS.**

   Masselli E., Mirandola P., Gobbi G., Carubbi C., Bonomini S., Craviotto L., Crugnola M., Russo F., Cambò B, Plenteda C. Rossetti E, Caramatti C, Aversa F, Vitale M.
   55st Annual Meeting of the American Society of Hematology. New Orleans, December 7-10, 2013. ***Oral Presentation, Recipient of the ASH abstract achievement award***

3. **HAPLOIDENTICAL STEM CELL TRANSPLANTATION AFTER NEGATIVE DEPLETION OF T CELLS EXPRESSING THE αβ-CHAIN OF THE T-CELL RECEPTOR (TCR) FOR ADULTS WITH HEMATOLOGICAL MALIGNANCIES**

   Prezioso L, Bonomini S, Lambertini C, Rossetti E., Caramatti C, Monti A, Sammarelli G, Craviotto L, **Masselli E,** Schifano C, Todaro G, Sassi M, Aversa F.

55st Annual Meeting of the American Society of Hematology. New Orleans, December 7-10, 2013. ***Poster Presentation***

4. **t(8;9)/*PCM1-JAK2* FUSION HEMATOLOGIC MALIGNANCIES DISPLAY A PECULIAR SIGNALLING PATTERN COMPARED TO OTHER MYELOID NEOPLASMS: IMPLICATIONS FOR THERAPEUTIC STRATEGY.**

   Masselli E., Carubbi C., Reiter A., Vitale M, Aversa F.
   7th Biannual International Congress on Myeloproliferative Neoplasms and Myelodysplasia. Brooklyn, NY on November 7-8, 2013. ***Poster Presentation, Recipient of the Travel Grant Award***

Proceeding from national congresses

1. **IN-VITRO CHARACTERISTICS OF HEMATOPOIETIC PROGENITORS FROM PRIMARY MIELOFIBROSIS PATIENTS CORRELATES WITH IPSS/DIPSS RISK CATEGORY**

   Masselli E., Carubbi C., Gobbi G., Mirandola P., Galli D., Martini S., Pozzi G., Gildone M., Albanese C., Bonomini S., Crugnola M. Craviotto L., Aversa F., Vitale M.
   45th National Congress of the Italian Society of Hematology (SIE). Firenze, 4-7 October 2015. ***Poster Presentation***

2. **COMBINATION THERAPY OF HYDROXYUREA PLUS ANAGRELIDE IN A CASE OF ESSENTIAL THROMBOCYTHEMIA AT HIGH THROMBOTIC RISK FOR PREVIOUS ACUTE CORONARY SYNDROME.**

   Masselli E.
   MPD in Practice: Multidisciplinary approach to Essential Thrombocythemia. Milan, November 22nd 2013.

3. **ERK1/2 IS THE SIGNALING PATHWAY PRIMARILY ACTIVATED IN MYELOID NEOPLASMS CARRYING t(8;9)/PCM1-JAK2 FUSION. A TWO CASES REPORT.**

   Masselli E., Mecucci C., Gobbi G., Carubbi C., Reiter A., Vitale M., Aversa F.
   44th National Congress of the Italian Society of Hematology (SIE). Verona, 20-23 October 2013. ***Poster Presentation***