ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

DOTTORATO DI RICERCA IN

Scienze Biomediche e Neuromotorie

Ciclo 36°

**Settore Concorsuale: 05/ H2 Istologia**

**Settore Scientifico Disciplinare: BIO/17 – Istologia**

# Contribution of cytokines to the etiology and progression of Primary Myelofibrosis

**Presentata da:** *Maria Mazzarini*

**Coordinatore Dottorato**
**Prof.ssa Matilde Young Follo**

**Supervisore**
**Prof. Rocco Liguori**

**Co-Supervisore**
**Prof.ssa Anna Rita Migliaccio**

Esame finale anno 2024

## Index

## *Abstract*

Primary Myelofibrosis (PMF) is the end-stage of Philadelphia-negative myeloproliferative neoplasms (MPN) and is characterized by fibrosis and hematopoietic failure in bone marrow, with a consequential migration of the malignant hematopoietic stem cells (HSC) in the spleen where they induce ineffective haematopoiesis. To date, available therapies for PMF are still palliative and do not halt the progression of this neoplasm.

During my PhD years, our laboratory investigated the factors promoting the onset and progression of PMF. In our PMF mice model, *Gata1$^{low}$* mouse, we studied the role of the interaction of HSC niche with megakaryocytes and HSC localization in the bone marrow during their division and cycle. We observed the inflammation and the main protagonists (LNC-2, CXCL1, and TGF-β) of this process and how their level changes before and after the onset of the disease. We investigated the different megakaryocyte populations in the fibrotic environment in different organs (lung and bone marrow) to define the megakaryocytes implicated in this process. In human samples, we described different ultrastructural abnormalities of megakaryocytes from the bone marrow and the spleen, identifying a possible different metabolism in those two populations.

In conclusion, we highlighted the intricated crosstalk between the megakaryocytes, the niche and HSC in PMF. We identified megakaryocytes-dependent cytokines altering the homeostasis of the niche and HSC. Those cytokines could be used as alternative therapeutic targets. Furthermore, we observed different megakaryocytic populations in different organs, providing new prospective on the role of megakaryocytes in different microenvironments.

# *Introduction*

## Primary Myelofibrosis

Philadelphia-negative myeloproliferative neoplasms (MPN) are a group of heterogeneous clonal disease derived from a malignant hematopoietic stem cells (HSC) including Polycythemia Vera (PV), Essential Thrombocythemia (ET) and pre-fibrotic MF (pre-MF) and Myelofibrosis (MF). The onset of MF could occur spontaneously, without previous condition (Primary Myelofibrosis, PMF), or could be the end-stage of other MPN, such as PV or ET [1,2]. PMF is characterized by the presence of abnormal megakaryocytes in the bone marrow, driving the progression of the diseases, the presence of fibrotic tissue, leading to the destruction of the bone marrow environment, and the consequential hematopoietic failure, increasing the migration of progenitor cells in the spleen as an extramedullary hematopoietic site[3].

### *Genetic Landscape of Primary Myelofibrosis*

ET, PV and PMF have been widely investigated in the last decades and, to date, three principal mutual exclusive mutations were recognized: Janus kinase 2 (*JAK2*), Calreticulin (*CALR*), and Myeloproliferative Leukemia Virus Oncogene (*MPL*) mutations. The percentage of those three mutated genes is different for each neoplasm. Mutation in *JAK2* gene is the most common in MPN: ~98% in PV, ~ in 50% in ET and in PMF 50-60% patients[1,4]. *CALR* mutation is under 1% in PV, 20-25% in the ET and 25-30% in PMF [4]. The other more common and important mutation *MPL* is still less than 1% in PV, 6-7% in ET and 7-10% in MPN [4]. About the 10% of the PMF and 5% of ET patients do not express those mutation and are defined as *triple negative*[5–8].

In the *JAK2* exon 14 a G>T base substitution in 1869 position lead to an amino-acid substitution of a valine in 617 position with a phenylalanine (*JAK2*$^{V617F}$) [9]. This gain of function mutation causes a constitutively activation of JAK2. This tyrosine kinase receptor is constantly phosphorylated and transducing JAK/STAT signal that increase the cytokines production, promotes the myeloproliferation, inhibits apoptosis and de-regulate all signaling from other receptor implicated in megakaryocytic or erythroid differentiation [9–11]. CD34$^{pos}$ from PMF peripheral blood with *JAK2*$^{V617F}$ mutation increased the production of immunoproteasome genes, *INF-*γ(interferon gamma) pathway and other immune protein respect to healthy control or PMF patients without the *JAK2*$^{V617F}$ [9,12]. Mutation on the exon 12 of *JAK2* is just a small percentage of the PMF patients (2/3%) [13] and the

mutated cells are more skewed towards erythrocyte phenotype, with upregulation JAK2-STAT1 signaling instead of JAK2-STAT3[14].

*CALR* most common mutations are in exon 9: a deletion of 52 bp (p.L367fs*46, type-1) and a insertion of 5 bp (p.K385fs*47, type 2) [15] . The result of these mutations is C terminus with the deletion of all or half of the negative ammino-acids (Type-1 mutation and Type-2 mutation respectively) [16] . This C terminus presents Calcium binding function and an endoplasmic reticulum retaining signal (KDEL motif), the loss of this disrupts the Calcium binding and its mobilization from the endoplasmic reticulum which regulate the megakaryocytic functions[17,18]. Interestingly, mice with the deletion mutation, Type-1, progress towards MF more frequently than the insert mutation[18,19].

*MPL* gene encodes for the thrombopoietin (TPO) receptor and its mutation in exon 10 sustains the uncontrolled proliferation of hematopoietic cell lineages, in particular the megakaryocytic lineage. The thrombopoietin axis is not just sustained by the mutation in its receptor, but both the *JAK2* and the *CALR* mutations can upregulate MPL, resulting into hyperproliferation of the malignant cells[2,4].

Further studies on the triple negative patients highlighted the presence of other mutation which influence their prognosis [5–8] . *TET2* (member of the ten-eleven-translocation 2) [2] gene was still under observation for evaluating its contribution on the prognosis of MPN patients, while ASXL1 (Additional sex combs like 1)[20], *SRSF2*[21] (Serine/arginine-rich splicing facto) or *U2AF1*[22] (U2 small nuclear RNA cofactor) genes are known to correlate with a poor prognosis.

## *Pathophysiology and Diagnosis of Primary Myelofibrosis*

The main features of PMF are the presence of a large amount of cluster of abnormal megakaryocytes and the deposit of fibers of reticulin and/or collagen that distinguish the PMF from the pre-MF [1,2,23] . The fibrotic tissue can be divided in different grades. In PMF patients, the fibrosis of grade 2 (dense fiber of reticulin diffuse, sparce focal bundles of thick fibers composed by collage deposit and focal osteosclerosis) or grade 3 (increase in the presence of extensive intersections and course bundles of thick fibers of reticulin and collagen and focal osteosclerosis)[24–26] can be observed. The presence of the fibrotic deposit and the upregulation of pro-inflammatory cytokines deplete the bone marrow of the erythropoietic tissue driving the onset of the anemic state in those patients[26]. To compensate, the erythropoietic progenitor cells migrate in others organ, such as the spleen, that do not present the optimal environment for erythropoiesis. The relocation of those cells and the request

for counterbalance the loss of the erythropoiesis lead to enlargement of the spleen [27]. Consequential to the splenomegaly, the spleen fails to produce functional and mature erythrocytes. Furthermore, amplyfied erythrocyte turn-over and the increase of the volume of produced plasma aggravate the anemic state. Another common (26%) feature in the PMF patients is thrombocytopenia. As the anemia, the factors of this phenomena are similar, and it can be related to some treatment too. It was observed that some mutation (for example in the *U2AF1*) are present in patients developing thrombocytopenia, but those genetic mutation are not necessarily correlated [22]. In PMF patients, thrombotic events are observed, though until now the mechanism is not clear. Those thrombotic events can be associated with the high activation of the patients' platelets, indicated by an elevated P-Selectin expression on their surface and to a greater interaction between the megakaryocytes with the leukocytes enhancing the production and activation of the platelets [28–30]. The thrombopenia and thrombosis can lead to a high risk of bleeding of those patients [31,32]. Correlated with the high inflammatory environment of the bone marrow by the amount of pro-inflammatory cytokines, the production of white blood cells increases and the presence of neutrophil leukocytosis or monocitosis can be observed in those patients [13,33,34]. In the peripheral blood of PMF patients, the presence of immature myeloid cells is due to the inefficient hematopoiesis from the extramedullary organs. The presence of those blast cells, with the onset of the fibrosis in the bone marrow, indicates the progression and aggravation of the PMF.

The diagnosis of PMF is based on the presence of three major criteria and at least one of the minor, that need to be confirmed in two separate consecutive determinations[1]. The major criteria are:
1) histologic analysis of bone marrow: presence of cluster of megakaryocytes with abnormal structure (different size with abnormal nuclear/cytoplasmic ratio, hyperchromatic and poorly developed nuclei), increase granulocytic population and cellularity but decreased erythropoietic tissue, paired with fiber of collagen and reticulin; 2) genetic analysis for the most common mutations (*JAK2*, *CALR*, or *MPL*) or other clonal marker for clonal hematopoietic neoplasms 3) no presence of WHO criteria for other MPNs, myelodysplastic syndromes or myeloid malignancy[1].

The minor criteria that need to be investigated are: leukocytosis (> $11x10^9$/L). leukoerythroblastosis, anemia not related to previous condition, increased LDH levels and palpable splenomegaly.

## *Available Therapies*

PMF present an incidence of 0.49/100,000 person-years [35–38], with a slight prevalence in the males and a median age of prognosis at 65 years[35]. In the case of elderly patients, the median overall

survival is 5 years, while for patients with the estimated prognosis before the 60 years of age the survive rates can reach 15 years after the diagnosis [2,23]. The primary causes of death in PMF are the progression from PMF to leukemia, the high risk of cardiovascular complication or infection and bleeding events due to cytopenia [2,23]. To date, PMF is an unmet clinical need, and the only effective treatment is the allogeneic stem cell transplantation [2,39,40]. Unfortunately, just the 20% of PMF patients present the right criteria (less than 75 years of age, proper available donors, absence of additional clinical conditions and other factors) to undergo the stem cells transplant procedure [2,39,40]. Most of the patients are enrolled in clinical trials for new therapy treatments [2,39]. The employment of JAK inhibitors, such as Ruxolitinib (Jakafi, Incyte, Wilmington, DE, USA), even though as a palliative cure, was proved to decrease the spleen size and permits to bypass the splenectomy. Ruxolitinib was approved by FDA (Food and Drug Administration) in 2011 for the PMF [41–43]. The effects of this JAK inhibitor comprehend the reduction of clonal proliferation and the inflammation environment. It is often delivered in association with Hydroxyurea to improve the anemic state of this patients [2,39,44]. Ruxolitinib is not the only JAK inhibitors approved: Fedratinib (Inrebic, Bristol- Myers Squibb, New York, NY, USA)[45] and Pacritinib (Vonjo, CTI Biopharma, WA, USA)[46,47] show also good results in the management of PMF. Currently one other Jak inhibitor treatment is under evaluation: Momelotinib[48] is currently on the III phase clinical trial (NCT02124746) to test its safety and tolerability.

Due to the palliative nature of those treatments and limited number of options after the failure of these first line drugs, the research for more and different therapeutic targets is still growing. At present, a phase II/III study (BOREAS, NCT03662126) is recruiting PMF patients with failure JAK inhibitor therapy to evaluate the efficacy of KRT-232, the inhibitor of MDM2, that is high expressed in circulation malignant CD34+ in PMF patients and acts on the apoptosis signaling of those cells[49]. Other clinical trials are searching for a target in the epigenetic modification: CPI-0610 (Constellation Pharmaceuticals, Cambridge, MA) is currently in a phase III clinical trial in combination with Ruxolitinib or placebo (NCT04603495) in patients that were not been treated with JAK inhibitor before. This drug targets the epigenetic modifiers the bromodomain and extra-terminal domain (BET) protein to decrease the expression of inflammatory molecules, such as NF-kB, or inflammatory cytokine, known to implement the progression of PMF[50]. More cytokines are under clinical trials. In mice models *Gata1$^{low}$*, Reparixin, a small-molecule inhibitor of the interleukin 8 (IL-8) receptors, considerable results in ameliorating the bone marrow fibrosis of the mice by blocking the production of the transforming growth factor – beta 1 (TGF-β1), collagen III and promoting the maturation with the increase of *GATA1* expression [51]. To date, Reparixin is under a phase II study for MPN patients

(NCT05835466). Interesting, the TGF- β 1 signaling pathway was studied as a possible therapeutic target. TGF- β 1 is produced by the PMF megakaryocytes in the bone marrow and in the serum of those patients its levels is elevated respect to the control [52,53]. Furthermore, TGF- β 1 induces a quiescent state in normal stem cells [54,55]. AVID200 (Forbius, Austin, TX, USA) is a TGF-β1/3 trap molecule pre-clinically tested in the PMF murine model, *Gata1$^{low}$* with excellent result (increase in the bone marrow cellularity, decreased of fibrosis and spleen size and number of splenic malignant hematopoietic cells) [56]. AVID200 has been explored in a phase Ib with promising results [56]. Of mention, our laboratory has provided insight on the role of P-Selectin in PMF [28,51] and its inhibitor, Crizanlizumab, is currently under Phase I/II study in combination with Ruxolitinib (ADORE clinical trial, by Novartis)[57].

## Megakaryocytes

The production of platelets is the first and most known function of megakaryocytes. The main site of those large cells is the bone marrow. The process of maturation of those cells from the HSC in the endosteal niche of the bone marrow is denominated megakaryocytopoiesis, while the last stage of maturation and the consequential release of the active platelets is termed as thrombocytopoiesis. Distinctive features of this maturation process are the increase of the megakaryocytes size, the compartmentalization of the cytoplasm and the poly-lobulated nucleus [58,59]. Decades ago, Zucker Franklin firstly described the four stages of the maturation of the megakaryocytes by ultrastructural criteria: Stage 0 or pro-megakaryoblast (< 15μm of diameter), Stage I or pro-megakaryocyte (15 – 50 μm of diameter, high presence of ribosomes in the cytoplasm, well-developed rough endoplasmic reticulum, increase in nuclear size and expression of specific megakaryocytes markers as CD41), Stage II or pro-megakaryocyte (50 - 80 μ m of diameter, appearance of a demarcation membrane system (DMS) and of platelet territories with granules), and Stage III, the mature megakaryocyte (> 80 μ m of diameter, polylobate nucleus and distinctive DMS with platelets territories and the expression of typical megakaryocytic markers, such as CD61 and CD42b) [58,60–62]. Starting from the megakaryoblasts stages, the cell duplicates their DNA without cellular division (a process called *endoreplication*) that lead to the typical nuclear polyploidization of the mature megakaryocytes, with a set of chromosomes up to 64N[62]. Previous study has highlighted how the number of chromosome copy is correlated with the size of the mature cell and the capacity of forming platelets [62,63]. In the pro-megakaryocyte stage, another typical megakaryocyte features appears: the DMS and granulocytes. The DMS is an invagination of the plasma membrane of the cell delimiting areas, named *pro-platelets territories*, with the presence of granules, endosome vesicles containing specific

platelet proteins and growth factor [64]. In particular, the α-granules (about 500 nm in size) are divided in stimulatory granules (with growth factor such as Vascular Endothelial Growth Factor (VEGF) or TGF-β1) and inhibitory granule (with endostatin and thrombospondin). Instead, the dense granules (about 300 nm in size) contain ADP and other molecules implicating in the coagulation process[64].

Intrinsic factors, such as *GATA1* and *GATA2* transcription factors, precisely regulates the maturation of the megakaryocytes. *GATA1* is necessary for the correct maturation of the megakaryocytes, while *GATA2* guides the HSC commitment to the erythroid-megakaryocytic lineage [65,66] . In particular, *GATA1* mutation is proved both by *in vivo* and *in vitro* studies to lead to thrombocytopenia, inhibition of megakaryocytic maturation and altered expression of megakaryocytic-specific genes (such as *PF4*, platelets factor 4, or *CD41*, α chain of glycoprotein IIB)[67–70]. This process is regulated by extrinsic factors too and the most important one is the glycoprotein hormone, TPO[71]. The binding of TPO with its receptor, MPL, activates a sequence of transduction signals (JAK/STAT, MAPK/ERK and PI3K/AKT pathways) regulating the megakaryocytes-specific genes expression[71–73]. Furthermore, in *c-mpl*-deficient mice a reduced number of megakaryocytes and thrombocytopenia was reported [74,75] . TPO influence is present during the whole maturation process and the MPL is always expressed[71] during this process, from the plasma membrane of the progenitor cells to the ones of the platelets. The microenvironment of the bone marrow exerts an important role on the megakaryocytopoiesis regulation. In cells cultured, it was demonstrated that factor as the interleukin 6 (IL-6) [76,77] , from the liver, and the interleukin 3 (IL-3) [77,78] and granulocyte-macrophage colony- stimulating factor (GM-CSF) [78] enhance the effects on the megakaryocytopoiesis of TPO. To exert their primary function, the release of platelets, and complete their maturation, the megakaryocytes need to migrate from the endosteal niche to the perivascular niche. This process is regulated by the interaction CXCR4/CXCL12. The CXCR4 is highly produced by the megakaryocyte progenitors and is progressively downregulated during the maturation, losing the bonding to the CXCL12 produced by the endosteal niche cells and promoting the migration and final maturation of the megakaryocytes[79–82].

In the last years, multiple studies have undercover the heterogenicity of megakaryocytes and those transcriptomic analyses have improved our knowledge on the function and different megakaryocytic subpopulations. In addition to the conventional population of megakaryocyte deputed to the production of platelets (*platelets-poised*), a population that exerts the important function of regulate the HSC niche (*niche-poised*) and a third population with the immuno-expression signature (*immuno-poised*). In physiologic condition, a small percentage of megakaryocyte are present in the

lung, where they exert their function as platelets reservoir [83,84]. Lefrançais [85] described, with RNA-Sequencing (RNA-seq) analysis, the interesting immuno-signature in lung megakaryocytes, opening the road for a more complex and exciting view of their function. With single cell RNA-seq resolution, megakaryocytes from the lung and the bone marrow were compared, discovering antigen- presentation like and/or immune-signature in the lung population [85–88]. This immuno-population of megakaryocytes in the lung unfold the possibility of understanding the mechanism and outcome of pulmonary pathology such as idiopathic pulmonary fibrosis or asthma and lead to new possible therapy targets for those diseases. The *niche-poised* megakaryocytes express an extracellular matrix signature (i.e. collagen genes) and *TGF-β1* responsive genes, indicating their role in the remodeling of the niche's structure[88].

Several studies indicate that during the maturation, probably at the Stage I or at the hematopoietic, stem cells can differentiate towards one of the three megakaryocytic sub-populations and that the microenvironment can influence that outcome [85–88]. Unfortunately, the megakaryocytic sub-populations do not present a distinctive morphology that can be used for identifying them. These expression profile studies were paired with a ploidy analysis that may indicates that a lower ploidy is correlated with the function of the population [88]. *Immuno-poised* megakaryocytes present a lower ploidy (2N-8N), pointing to a more immature stage, while the *platelets-poised* population present the 32N-64N ploidy typical of a mature and active megakaryocyte [88]. Interestingly, the balanced expression of GATA1 and GATA2 (from low to high expression and vice versa, respectively) in the platelets- and *immuno-poised* regulate the maturation of those population, while in the niche poised the expression of those intrinsic factor seems to be reverse[89,90].

### *Role of the Megakaryocytes in the progression of Myelofibrosis*

In the '70, Zucker Franklin was the first to report abnormal megakaryocytes in the bone marrow of PMF patients and to identify those cluster of abnormal megakaryocytes as hallmark for PMF [60]. The PMF megakaryocytes present immature abnormal ultrastructural feature indicating a halt in the progression from the pro-megakaryocyte to the mature megakaryocytes [91]. PMF stem cells are bias towards the megakaryocytic lineage and PMF megakaryocytes are characterized by a defective polyploidization, resulting in a hypo polyploid nuclei, absent or reduced DMS, few atypical granules, high proliferation rate and resistance to the apoptosis signaling [60,92–94]. In accord with the delayed maturation phenotype of PMF megakaryocytes, studies have reported a low expression of the transcription factor *GATA1* in the megakaryocytes from PMF bone marrow and how its restoration can rescue the immature phenotype [95–98]. Both in PMF patients [98] and *Gata1$^{low}$* mice (murine model

for myelofibrosis that express a low level of *Gata1*)[99] the reduced protein levels of GATA1 seems to be connected to a defective ribosome signature.

PMF megakaryocytes drive of the progression of bone marrow fibrosis by secreting a great number of molecules [100,101] . TGF- β 1, Platelet Derived Growth Factor (PDGF), Fibroblast Growth Factor (FGF), VEGF, TPO, PF4, IL-6 and IL-8, Secreted phosphoprotein1 (SPP1), Bone morphogenic proteins (BMPs), Oncostatin M are some of the most interesting megakaryocyte-derived cytokine and growth factors[100].

PMF megakaryocytes express high levels of extracellular matrix component such as fibronectin, type IV collagen and laminin [102] . The production of this components can be direct [102] or indirect. Most of these megakaryocyte-depending factors, such as FGF [101,103], PDGF[104,105], SPP1[106,107] and Oncostatin M[108,109] act on the fibroblast stimulating their activation and the production of collagen and reticulin fibers. PF4 did not directly stimulate the fibroblast but trigger the stromal cells to differentiate in myofibroblast [110] . Even though in PMF patients' high levels of PF4 was not detected [111] , in mice model its ablation ameliorate the bone marrow fibrosis[112].

The stimulation of TGF- β 1 on fibroblast and other cells to produce extracellular matrix is well documented [52,113–116] . TGF- β 1 is primally produced by PMF megakaryocytes and both in PMF patients and murine models is present in high levels [117–120] . Furthermore, defective expression of TGF-β 1 in mice model rescue the TPO-induced fibrosis in the bone marrow of those animals [121] . TGF- β1 creates a malignant feedback loop increasing the production of other factors, for example, VEGF[122,123] or BMP[106,124,125], worsening the clinical situation. To highlight the importance of TGF-β1, this growth factor coordinates the fate of HSC, balancing their quiescent or self-renewal [126] . As mentioned above, for its importance in the augment of fibrosis and progression of PMF, TGF-β1 was used a possible target in a phase Ib clinical trial[56].

The pivotal role of the axis TPO/MPL is enhanced in the megakaryocytes from PMF. The level of MPL expression on the surface of the PMF megakaryocytes membrane is decreased. Moreover, *JAK2*$^{V617F}$ mutation megakaryocytes, with constitutive active JAK2, present an enhanced degradation and reduced the recycling of MPL. This mechanism leads to the increasing of TPO levels, and in a boost of the availability for the HSC that are induced to proliferate [72,99,121].

The inflammation plays an important role in the progression of PMF[127]. PMF patients present a high level of IL-6 and in *Pf4*-cre/*JAK2*$^{V617F}$ mice animal model IL-6 increment promotes

erythropoiesis [128–130] . Megakaryocytes expression of IL-6 is promoted by the accumulation of fibronectin, rich in PMF patients [131]. Unfortunately, IL-6 was proved a poor target in the therapy of myeloproliferative neoplasms[128–130]. Of interest, IL-8 plays a significant role in PMF and could be a possible therapeutic target. Megakaryocytes produce IL-8[132] whose level in PMF patients is elevated both in serum and plasma[133]. IL-8 and its receptor (CXCR1 and CXCR2 - CXC chemokine receptor 1and 2 -) are directly implicated in the megakaryocyte proliferation and maturation [134] . LCN-2 plasma levels are elevated and its high production from the megakaryocytes was proved to build the abnormal microenvironment typical of MPN patients[135].

PMF megakaryocytes deeply manipulate the neoplastic microenvironment [136]. The interaction with the fibroblasts is not the only worth mentioning. In this inflammatory background, the mesenchymal stromal cells of the HSC niche are highly disturbed and leads to more clinical alteration[33,127].
The crosstalk with inflammatory cells is of great interest. Macrophages/monocytes were found in great number in the bone marrow of PMF, deepening the inflammatory microenvironment and promoting its progression [137,138] . The strong interaction between PMF megakaryocytes and neutrophils seems to be connected to the outcome of this neoplasm. The process of the passage of neutrophils through the megakaryocytes (the *emperipolesis*) is increased in PMF, which may lead to an increase in the secretion of α granules from those megakaryocytes [91,139,140] . Osteoblasts proliferation is influenced by the secretion of megakaryocytes, leading to a process of osteosclerosis encountered in PMF patients and some murine models[141–143].

Distinctive functional populations of megakaryocytes were explored in the PMF framework. In their remarkable paper, Psaila and coworkers [144] compared HSC from peripheral blood of *JAK2*$^{V617F}$ of PMF patients and matched healthy control. In particular, they investigated the sub-fraction CD38$^{pos}$ and CD38$^{neg}$ of CD34 $^{pos}$ cells expressing the CD41 megakaryocytic marker to identify the megakaryocyte progenitors. After the demonstration that the HSC *JAK2*$^{V617F}$ are skewed toward the megakaryocytic lineage, they identify an aberrant megakaryocytic population with a high inflammatory and fibrotic gene signature respect to the control [144] . The altered production of collagen and other bone marrow-matrix molecules from PMF megakaryocytes [102,145] may suggest a high presence of the *niche-poised* population in the bone marrow of those patients.

## Hematopoietic Stem Cells and their niches in the bone marrow

HSC are the backbone for all the hematopoietic populations. These pluripotential stem cells can maintain their pluripotency with a self-renewal process or differentiate in every functional

hematopoietic cell, from the erythrocytes and megakaryocytes to the immune system populations. Studies demonstrated that HSC are present in the first haematopoiesis (primitive haematopoiesis) at the embryonic stage, in the aorto-gonado-mesonephric region, and then became the only source of new hematopoietic cells in the definitive haematopoiesis in the fetal liver and then in the bone marrow[146,147]. The fate of the HSC, quiescence, self-renewal or differentiation, is finely regulated by intrinsic[148] or extrinsic factors[149]. Intrinsic factors are mostly implicated in the regulation of the cells cycle, such as the retinoblastoma protein (*RB)* family genes, in the quiescent state[150] or in D-cyclins family and *CDK4/6*[151,152]. Extrinsic factors derived from the extracellular matrix or the bone marrow niche can be also influence the fate of the HSC [149]. Interestingly, aged-related alteration of the bone marrow niche population influences the fate of HSC [149]. Aged niche cell population express high levels of pro-inflammatory cytokines and the chronicle exposure to inflammation drives the loss of HSC self-renewal ability and impairs their function [153].

Inflammation in the bone marrow is a known major stimulus to promotes the aged phenotype of the HSC and the myeloid differentiation [154,155]. Furthermore, the role of the mesenchymal stem cells, differentiating into the adipocytic population, increases the secretion of pro-inflammatory cytokine in the bone marrow niche microenvironment and contributes to this senescent phenotype of the HSC [156,157]. The aged HSC increased the risk of malignant transformation, in particular due to the age-related clonal hematopoiesis, the expansion of HSC clones presenting genetic mutation that can lead to the malignant transformation[154].

The bone marrow niche is a unique microenvironment composed by a variegated number of cell populations. The niche support and promotes the hematopoiesis, regulating the state of HSC and their progenies. In particular, two functional anatomical niches in the bone marrow were identified: the endosteal and the vascular niche[158–160]. The endosteal niche, localized in between the endocortical and trabecular surfaces of the bone, maintains the quiescence of the HSC [159–162] while the vascular niche, in the extravascular space between the sinusoids or around the different vessels, promotes the proliferation and the differentiation of those stem cells [159,160,163]. The aging process affects the niche microenvironment [153,164] and, with the increase on the secretion of pro-inflammatory cytokine, may influence the reduction of the regenerative ability of the HSC[165] and plays a role in the progression or outcome of different hematopoietic diseases such as leukemia or myelodysplastic syndromes[166–168].

Deepening the understanding of the complex connection between HSC and the niche and how the aging and inflammation can influence the fate of those important cells present a pivotal role in the

new discovery of the etiology of malignant cells and, consequentially, the onset of hematopoietic syndromes.

## Animal models of Primary Myelofibrosis

Animal models are of vital importance for understanding the mechanism of pathological conditions. Different murine models are used to study PMF[169]. Reproducing the *JAK2*$^{V617F}$ mutation in mice was one of the approaches: transgenic, retroviral overexpression and knock-in mice with this mutation exhibit a PMN phenotype after few months. With the insight on the genetic landscape of PMF and MPN, more mice models with a second mutation paired with *JAK2*$^{V617F}$ were generated. For example, the murine model with *JAK2*$^{V617F}$ mutation paired with the loss of the Lymphocyte Adaptor Protein (*Lnk)*, obtained with the retroviral technique, accelerate the passage from ET to MF [170], indicating the important role of the secondary mutation in the modulation of the MPNs. Transgenic mice without the expression of Enhancer of Zeste 2 Polycomb Repressive Complex 2 (*Ezh2*), cross-inbred with *JAK2*$^{V617F}$, show similar results: the combination of these mutations acerbate the myelofibrotic background[171]. Even though JAK2 is the most common mutation in more than half of PMF patients [2,4], *MPL* and *CALR* mutations occupy an important percentage of those patients (7-10% and 25-30%, respectively) [2,4]. Mice model expressing *MPL*$^{W515L}$, *CALR*$^{del52}$ and *CALR*$^{ins5}$ mutations were proved to be an important asset in recapitulating the myelofibrotic phenotype[172,173].

To study the MPNs the most prominent and known mutations are not the only approaches used. Of note, murine model with TPO over-expression, retrovirus[174] and transgenic models[175], reproduce the fibrosis in the bone marrow and other clinical feature of MF. Mice with trisomy 21 or ablation of *Ab1-1* (Abelson interactor 1) are useful systems to study the MPNs[169].

### *Gata1*$^{low}$ *mice is a model for Myelofibrosis*

As mentioned before, *GATA1* is a pivotal regulator of the differentiation from the megakaryocytic-erythroid progenitors (MEP) to the megakaryocytic lineage [176]. *GATA1* is a zinc finger DNA-binding transcription factor and its locus is on the short arm of the X chromosome (Xp11.23). *GATA1* is composed by 6 exons and present two isoform: the full length and the short isoform (*GATA1s*), missing the N-terminal portion [177]. *GATA1* expression is necessary from the first steps of the megakaryocytic and erythroid differentiation. In the first step from the HSC, with *GATA2*, skew the differentiation towards MEP, but its expression increases in the last phase of the megakaryocyte maturation, downregulating *GATA2*, by interacting with *FOG-1* (Friend Of *Gata*

protein 1)[176,178,179]. A great number of hematologic diseases, such Diamond-Blackfan anemia or acute megakaryoblastic leukemia (AML), are consequence of a dysfunctional *GATA1*. Noteworthy, a low expression of GATA1, due to *RSP14* ribosomopathy, was detected in PMF patients, leading to a defect maturation of the megakaryocytes[97].

The first tryout in 1996 for a transgenic mouse with loss of *Gata1* locus on the X chromosome, resulted in anemic male mice that died during the gestation period. Some of the heterozygous female survived after birth and rescued partially their anemic state, probably due to some residual *Gata1* positive progenitors [180] . Orkin laboratory identified a cis-acting enhancer upstream of the *Gata1* promoter (DNase I hypersensitive region (HS)). In the embryonic stem cells the HS region was replaced this 8kb enhancer with a neomycin cassette [181,182] . The low expression of *Gata1* permitted the survival of those mice. Interesting *Gata1$^{low}$* mice reflect all the clinical aspect of PMF patient. *Gata1$^{low}$* mice present in the bone marrow a higher number of megakaryocytes respect to the control but are thrombocytopenic [183] . *Gata1$^{low}$* megakaryocytes present the typical ultrastructural abnormalities and delay in the maturation of the PMF payients' megakaryocytes [181,183] . This murin model develops anemia at 5 months of age and the fibrosis in the bone marrow around 8 months. At the elderly age (around 12 months) the extramedullary hematopoiesis, with the consequential splenomegaly and high number of megakaryocytes, is present in those animals [181,183] . Furthermore, high levels of TGF-β1 were detected in those mice as well as PMF patients[117,120]. TGF-β1 is not the only molecules that *Gata1$^{low}$*, PDGF, VEGF and osteocalcin were present in high levels [183] . High expression of PDGF-B, mainly secreted by megakaryocytes, and a different signaling pathway was found in *Gata1$^{low}$* mice, identifying another player in bone marrow fibrosis[184]. TPO and its receptor, MPL, are more expressed respect to the control. In the liver and plasma, *Tpo* mRNA and its protein is ~2 times more expressed in the *Gata1$^{low}$* than in the control. Counterintuitively, MPL mRNA levels are increased in *Gata1$^{lwo}$* LSK cells (Lin$^{neg}$Sca1$^{pos}$Kit$^{pos}$), but the protein levels are lower, as shown in PMF patients too[99,185]. With TPO addition, the levels of the MPL protein match the mRNA levels in LSK cells, indicating a hyperactive MPL signaling [99] .Furtehrmore, spleen cells express a higher JAK-STAT signals TPO dependent and the *Gata1$^{low}$* splenomegaly can be reduce through Ruxolitinib treatment [99] . In *Gata1$^{low}$* bone marrow, the deficit in the *RSP14* signature and the development of rough endoplasmic reticulum, with fewer ribosome, decrease the *Gata1* content in megakaryocytes [99] . As mentioned before, in patients, the interaction with immune cells is of great interest [91,139,140] . P-Selectin seems to have a decisive role in this interaction and is abnormally distributed in *Gata1$^{low}$* megakaryocytes, absent in the α granules but present on vesicles on DMS [30]. In PMF patients, P-Selectin is highly expressed in platelets [186] , which can be linked with the

increased emperipolesis observed [91,139,140] . Furthermore, its expression sustains the extramedullary hematopoiesis and its ablation (in mouse model *Gata1*$^{low}$ /*psel*$^{null}$) rescues the thrombotic event [29,187] . In a recent study, RB40.34 (BD Pharmigen, San Diego, CA, USA), a monoclonal antibody against P-Selectin, reduce the bone marrow fibrosis, spleen size and architecture, TGF-β1 and CXCL1 level and restore the *Gata1*$^{low}$ bone marrow hematopoiesis in combination with Ruxolitinib[28].

This slow outcome of the disease mirrors the development of PMF in elder patient, paving the way for a deeper understanding of the progression mechanisms.

## *Literature produced during the PhD course*

### Peer reviewed publications

- "*Resident Self-Tissue of Proinflammatory Cytokines Rather Than Their Systemic Levels Correlates with Development of Myelofibrosis in Gata1$^{low}$ Mice*."

Maria Zingariello, Paola Verachi, Francesca Gobbo, Fabrizio Martelli, Mario Falchi, **Maria Mazzarini** et al. *Biomolecules*, 2022, 12(2):234. doi: 10.3390/biom12020234. PMID: 35204735; PMCID: PMC8961549.

Link to the published paper:

**https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8961549/**

**Brief Summary:**

To explore the possibility of inflammatory cytokines in the serum of PMF as diagnostic markers, we tested their serum levels in our PMF animal model, *Gata1$^{low}$*. We used different techniques to asset the cytokine landscape in *Gata1$^{low}$* (compared to age-matched wild type littermates) before and after the onset of the disease. To compare the levels of the serum in *Gata1$^{low}$* we used Luminex-bead- assay and ELISA analysis and immunohistochemistry. Flow Cytometry gave us insight on the cytokine content and frequency of stromal cells. We even investigated, by confocal microscopy, the localization of GFP-tagged HSC. Even though we didn't notice great difference between *Gata1$^{low}$* and control in the inflammatory-cytokines analyzed both in pre-fibrotic and fibrotic state, we observed elevated levels of LNC2, CXCL1, and TGF-β1 in the fibrotic bone marrow of *Gata1$^{low}$*, especially in malignant megakaryocytes. This alteration on the pro-inflammatory cytokine bone marrow levels, disrupt the niche of HSC cells and a compromising of their localization in *Gata1$^{low}$* has been observed. *Gata1$^{low}$* HSC cells were restricted to areas of femur diaphysis, in areas with microvessels, neo-bones and megakaryocytes, instead of been surrounded by adipocytes in the femur epiphysis as in the control. This study highlights the important of megakaryocytes-derived cytokines into the disruption of the microenvironment for HSC, promoting the myelofibrotic environment. ù

- "*Single cell analysis of the localization of the hematopoietic stem cells within the bone marrow architecture identifies niche-specific proliferation dynamics.*"

**Maria Mazzarini,** Francesca Arciprete, Orietta Picconi, Mauro Valeri, Paola Verachi, Fabrizio Martelli, Anna Rita Migliaccio, Mario Falchi, Maria Zingariello. *Front Med.* 2023, 10:1166758. doi: 10.3389/fmed.2023.1166758. PMID: 37188088; PMCID: PMC10175646.

Link to the published paper:

**https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10175646/**

**Brief Summary:**

The HSC niche plays a pivotal role in the progression of PMF; due to their importance we investigated the interaction of HSC niche with those cells entering into cell cycles with their niche, especially at old age. For this study, we used the *hCD34tTA/Tet-O-H2BGFP* transgenic mice, in which only $CD34^{pos}$ cells (indicating the HSC cells population) expressed *GFP* driven by the TET trans-activator. Doxycycline treatment blocked TET element, and consequentially, GFP expression in HSC. Each division of those cells correspond to the loss of half of their GFP labels (hemi- decrement), giving the possibility of identifying the dynamics of their first 1-4 divisions. To this aim, we first validated user-friendly confocal microscopy methods to determine HSC divisions by hemi- decrement changes in levels of GFP expression. We observed by confocal microscopy a localization of HSC in old mice around vessels. Around arterioles we identify the most fluorescent cells (not dividing cells), indicating a microenvironment that sustain their quiescent and/or self-replication. On the other hand, the cells around the venules dropped or complete lose their GFP content (dividing cells), indicating a microenvironment supporting the differentiation and cells cycle entering. These results reveal that in old mice, HSC cycle is dynamic and biased toward interactions with the niche that instructs them to differentiate.

- *"Shared and distinctive ultrastructural abnormalities expressed by megakaryocytes in bone marrow and spleen from patients with myelofibrosis."*

Maria Zingariello, Vittorio Rosti, Alessandro M. Vannucchi, Paola Guglielmelli, **Maria Mazzarini**, Giovanni Barosi, Maria Luisa Genova, and Anna Rita Migliaccio. Frontiers in Oncology, 2020, 10:584541, doi: 10.3389/fonc.2020.584541. PMID: 33312951; PMCID: PMC7701330.

Link to the published paper:

**https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7701330/**

**Brief Summary:**

To further investigate the abnormalities of malignant megakaryocytes in PMF and their possible role/function in bone marrow and the spleen, the extramedullary site, we used transmission-electron microscopy techniques. We analysed megakaryocytes from 5 bone marrow and 10 spleen sections of PMF patients compared to healthy controls (4 bone marrow and 3 spleen biopsies). Firstly, we observed an increased number of megakaryocytes in PMF patients compared to the controls both in the bone marrow and the spleen. As expected, in the PMF bone marrow the megakaryocytes presented an immature phenotype, blocked at the Stage II of maturation. Those megakaryocytes revealed a thick plasma membrane with abnormal protrusions and a nuclear membrane with large pores, in their cytoplasm a great number of glycosomes, pointed to a defected metabolism with accumulation of insoluble polyglucosan. The PMF spleen megakaryocytes are instead fully developed and matured (defined DMS, platelets territories with platelets) and their plasma and nuclear membranes did not display any abnormalities. However, their mitochondria showed reduced crests, that may be a sign of deficient aerobic energy-metabolism. Those different ultrastructural abnormalities of PMF megakaryocytes from different site, the bone marrow and the spleen, suggests a different metabolism and different sub-populations that are implicated in the different organs.

- "*GATA1-defective immune-megakaryocytes as possible drivers of idiopathic pulmonary fibrosis*." Francesca Gobbo, Maria Zingariello, Paola Verachi, Mario Falchi, Francesca Arciprete, Fabrizio Martelli, Angelo Peli, **Maria Mazzarini,** Jeff Vierstra, Carolyn Mead-Harvey, Amylou C Dueck, Giuseppe Sarli, Stefano Nava, Giacomo Sgalla, Luca Richeldi, Anna Rita Migliaccio. *bioRxiv* [Preprint]. 2023, 19:2023.06.20.542249. doi: 10.1101/2023.06.20.542249. PMID: 37425686; PMCID: PMC10327123.

Link to the published paper:

**https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10327123/**

**Brief Summary:**

To investigate the role of different megakaryocytes population in the fibrotic environment in the lung, we used *Gata1$^{low}$* as a model for Idiopathic pulmonary fibrosis (IPF). We observed a deficit in *GATA1* expression in megakaryocytes from IPF patients' lungs and the same result was observed in *Gata1$^{low}$* mice. Those *immuno-poised* megakaryocytes, in the lung of the animal models, presented a defective RNA expression profile and an increase content of TGF-β1, CXCL1 and P-Selectin by immunohistochemical analysis. Also, the fibrosis in the bone marrow, *Gata1$^{low}$* mice exhibited lung fibrosis with age, and we observed an amelioration of the fibrotic tissue by the ablation of P-Selectin and inhibition of the main players in bone marrow fibrosis, such as TGF-β1 or CXCL1. We provided, not only a novel model for IPF, the *Gata1$^{low}$* mouse, but we highlighted how different megakaryocytic populations are present in different organs and how those population are defective in a PMF/IPF environment.

## Additional peer reviewed publications published during the PhD course

- "*Patients with hypercortisolemic Cushing disease possess a distinct class of hematopoietic progenitor cells leading to erythrocytosis*". Lilian, Varricchio, Geer Eliza B, Martelli Fabrizio, **Mazzarini Maria**, et al. *Haematologica*. 2023, 108(4):1053-1067.

- "*Role of B1 integrin in thrombocytopoiesis*". **Mazzarini Maria**, Verachi Paola, Martelli Fabrizio ,Migliaccio Anna. *Rita Fac Rev.* 2021, 10: 68.

- "*Evolution and new frontiers of histology in bio-medical research*". **Mazzarini Maria**, Falchi Mario, Bani Daniele, Migliaccio Anna Rita. *Microsc Res Tech*. 2021, 84(2):217-237.

- "*hGATA1 Under the Control of a μLCR/β-Globin Promoter Rescues the Erythroid but Not the Megakaryocytic Phenotype Induced by the Gata1low Mutation in Mice."* Martelli Fabrizio, Verachi Paola, Zingariello Maria, **Mazzarini Maria**, et al. *Front Genet*. 2021, 11;12:720552.

## *Discussion*

The basic mechanisms driving the progression of PMF and the onset of clonal malignant HSC are still unclear. Even though, the role of the relationship between megakaryocytes, HSC and their niches has a pivotal importance in this degenerative process of diseases, a deeper insight on this crosstalk is needed. Accordingly to this lack of knowledge, we concentrated our effort to define the influence of megakaryocytes and their secrete molecules on the HSC and their niches and how they modify the myelofibrotic environment, investigating the most relevant megakaryocyte—derived cytokines and their potential as alternative therapeutic targets. In addition, we studied the morphology of megakaryocytes and their modifications in different microenvironments, the localization of HSC in the bone marrow and how megakaryocyte-derived cytokine produced can influence the HSC fate.

To investigate new possible therapeutic targets for PMF, we compared pro-inflammatory cytokines levels in the serum and bone marrow of *Gata1*$^{low}$ and CD1 mice. We identified three pro- inflammatory cytokines (LCN-2, TGF-β1 and CXCL1) implicated in the progression of the disease[188]. According to previous studies demonstrating the aging effect on the HSC behaviour and the composition of their supporting niches[165,189,190], we observed that in elder *Gata1*$^{low}$ mice the pro- inflammatory cytokine burden is less accentuated in serum respect to the bone marrow. Since CD1 murine strain displayed a higher inflammatory background respect to other murine strains [191] , we observed high serum levels of LCN-2, TGF-β1 and CXCL1(the murine equivalent of IL-8) respect to strains such as C57BL6 or DBA2. However, CD1 mice did not develop fibrosis in any organs, indicating that in the *Gata1*$^{low}$ mice the inflammatory background did not trigger the fibrosis and PMF onset. In the bone marrow of elder *Gata1*$^{low}$ mice, the levels of those pro-inflammatory cytokines were remarkably increased respect to the CD1 littermates and to younger *Gata1*$^{low}$ mice. This difference was not as appreciable in the serum. Even though the megakaryocytes are the main population expressing high levels of these cytokines, by immunohistochemical analysis we detected other cell types producing those molecules. This suggested that malignant cells and microenvironment are intertwined: the presence of more cell types producing LCN-2, TGF-β1 and CXCL1 increased their bioavailability, leading to the worsening and progression of PMF.

As mentioned above, megakaryocytes are the main producers of those pro-inflammatory cytokines. The secretion of those molecules is facilitated by the increased emperipolesis and the trans- membrane protein, P-Selectin, plays an essential role in this process [91,139,186,187] . The Verachi paper

demonstrated the importance of P-Selectin as a new possible therapeutic target for PMF: the combination of JAK2 inhibitor, Ruxolitinib, and the P-Selectin monoclonal antibody, RB40.34, significantly decreased the bone marrow levels of TGF-β and CXCL1[28]. At later date, after 54 days, this combination was proved to reduce the bone marrow fibrosis and extramedullary haematopoiesis and to improve the normal and effective haematopoiesis in the bone marrow. Blocking the P-Selectin on the megakaryocytes and the over-activation of JAK2 did not reduce the number of megakaryocytes but improve their maturation stage and GATA1 content.

In literature, the effects of those pro-inflammatory cytokines effect on HSC and their nice is well documented [126,192,193] . In the bone marrow two different HSC niches are described based on their anatomical localization[158,162,163]. The HSC with the most self-renewal potential (long term), and in a quiescent state, are localized in the endothelial niche, while proliferating and differentiating HSC surround the vessels[164]. We studied GFP retention as a tool to observe the dynamics of HSC cycling, in double *huCD34tTA-TetO-H2BGFP* mutant mouse wild type. In *huCD34tTA-TetO-H2BGFP* mice, the histone *H2B* gene is fused with *GFP* gene and the regulatory sequences of human *CD34* regulates their expression. The promoter of those sequences and of *GFP* is under *TET* trans-activator element. When treated with Doxycycline, the *TET* element is inhibited and consequentially inhibits the *GFP* expression itself, and, with no production of GFP protein, in each division GFP content is reduced. This methods of tracking the retention of GFP, is proved to be a powerful tool to identify the niche of HSC related to their cycles [194] . In this study [195] , confocal analysis on those mice highlighted HSC associated with their nice in a pro-inflammatory background such as the one typical of CD1 stain [191] . Unfortunately, nor the flow cytometry or the confocal analysis were able to detect more than four levels of GFP (no more than HSC that divided four times). However, we were able to recognize those four populations and to examine their localization. In aged mice, after a week of Doxycycline treatment, most of the HSC underwent one or three division. The high GFP fluorescent cells distribution in the bone marrow microenvironment before the Doxycycline treatment was near the vessels, but after the pharmacological treatment mostly HSC lost their fluorescence, indicating a high rate of cellular division.

Fascinatingly, around the arterioles the HSC retained the most of the GFP expression, indicating a quiescent state supported by the local niche and the bioavailability of TGF-β similarly high around those structures [196] . Further analyses with the confocal microscopy will assert the location of GFP-retained cells in correlation with age, sex and/or time of treatment with Doxycycline, to fully understand the state of HSC in the bone marrow niches. However, we observed an increase in

cycling division around the venule. The microenvironment surrounding the venule may promote HSC differentiation and their mobilization in the blood flow to migrate in other organs.

To observe the localization of HSC in our PMF murine model, we used a triple mutant murine model: *huCD34tTA-TetO-H2BGFP*. We surprisingly found that high fluorescent GFP in *huCD34tTA-TetO-H2BGFP /Gata1$^{low}$* mice were not present around the numerous adipocytes but in medulla near microvessels structures, bones and megakaryocytes, pointing to the role of malignant megakaryocytes to drive the HSC towards the differentiation and the blood flow where they can migrate to the spleen. Of note, adipocytes secrete supportive-niche growth factors[197] and, with aging, the majority of HSC with the higher self-renewal potential surrounded those cells. Elder *Gata1l$^{ow}$* displayed a great number of adipocytes, accordingly with report of a specific subtype of MF patients with a fatty bone marrow, maybe due to a skewed differentiation of malignant HSC towards the adipocytic lineage[198].

Megakaryocytes influence the HSC localization and their niches, but the microenvironment itself can influence the megakaryocytes phenotype as well. It was already proved the plasticity of megakaryocytes to change their sub-population phenotypes in response to the environment[86,87].

In spleen and in bone marrow of PMF patients (5 and 11 patients respectively), we observed an increased number of megakaryocytes in both organs, well known characteristic of PMF, and, even though different, both populations displayed abnormal ultrastructure [199] . Furthermore, these abnormalities seem to be independent from the type of mutation present (in bone marrow 3 patients have *JAK2* mutations, one *MPL* mutation and one *JAK2*$^{V617F}$; in spleen 4 patients have *JAK2* mutations, one *CALR* mutation, 2 triple negative and 4 *JAK2*$^{V617F}$).

Those TEM data indicated the organ microenvironment as an important factor for the maturation profile. It's known that malignant stem cells from the bone marrow migrate to the spleen and the spleen cells themselves harbour the same mutation[3,200], but megakaryocytes from this extramedullary hematopoietic organ present most cells at the Stage III. The localization of those mature megakaryocytes, with the well development DMS and platelets territories, is near the blood vessels as expected from mature megakaryocytes ready to release platelets [59,61,201] . Instead, in the bone marrow megakaryocytes featured an immature morphology. Furthermore, we observed an accumulation of acid-insoluble polyglucan in numerous cytoplasmic glycosomes, indicating an impaired lipidic metabolism. Considering that lipids are necessary for the formation of DMS[202] , this impaired lipid metabolism of bone marrow megakaryocytes may be another key to understand their

defective mature phenotype. In our paper [199] , expression signature analysis of data from the bone marrow (raw data from [203]) highlighted various glycogen genes differently expressed and implicated in inhered glycogen storage disorders[204–210]. Both the ultrastructural and expression analysis point out to the importance of lipids metabolism in maturation and, therefore, how defect in this pathway may contribute to progression of PMF. Furthermore, *JAK2* mutated animal model for PMF, rescue their disease phenotype by high fatty acid diet supplementation and/or 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase3 (PFKFB3) inhibitor, in combination with Ruxolitinib[211].

On the other hand, the malignant stem cells migrating in the spleen, encountered a healthier environment and were able to produce more mature and active megakaryocytes. Unfortunately, the mutation burden and the inappropriate microenvironment leads to ultrastructural abnormalities even in the spleen. The reduced crest in the mitochondria that we observed in spleen megakaryocytes may derived by the elevated levels of ROS in the organ [212,213] . ROS overexposure may influence the mitochondria abnormalities in those cells [212,213].

In the last years, studies have demonstrated different functional sub-populations of megakaryocytes and that the megakaryocytes can change their sub-population based on the microenvironment [88] . From single cells RNA-sequencing studies, three sub-population were recognized: the *platelets- poised*, expressing genes implicated in the coagulation and platelets formation, the *immuno-poised*, with an immune-response gene signature, and the *niche-poised* megakaryocytes, characterized by genes involved in the regulation of the HSC niche. Previous studies demonstrated that megakaryocytes from the PMF bone marrow express genes of the extracellular matrix[101,102] and HSC supporting factor, such as TGF-β1 [214] , suggesting that the differentiation of the *niche- poised* megakaryocyte is reactivated in PMF.

To identify the various sub-populations in PMF, we investigated the differential expression in the megakaryocytes from the bone marrow and/or the lung of *Gata1$^{low}$* mice and CD1 mice. As expected, CD1 lung megakaryocytes signature was similar to the *immuno-poised* populations, while the bone marrow megakaryocytes to the *platelets-poised* one. In the bone marrow, *Gata1$^{low}$* mice were characterized by a unique signature. Instead, the megakaryocytes from *Gata1$^{low}$* lungs are skewed towards the immuno sub-population, but their signature expression was defective. Furthermore, in those lung megakaryocytes, the defective *Gata1* expression result in immature morphology and contribute to the formation of fibrosis in the lungs. Interestingly, we observed in three IPF lung biopsy a low content of GATA1 respect to the control. Unfortunately, due to the difficulty to obtain and/or performed a lung biopsy, the number of patients was limited. Furthermore, we notice that in

IPF patients the megakaryocytes are localized in the parenchyma near the alveolar epithelium and not in the blood vessels. Of note, inflammation is not the primary cause of fibrosis in some IPF patients, and, thanks to the CD1 inflammatory background that do not induce fibrosis, we propose *Gata1$^{low}$* mice as an alternative murine model for IPF. In other mice models, for example the bleomycin-treated animals, the fibrosis is a direct consequence of inflammation [215] . In the lungs of *Gata1$^{low}$* mice, we observed a high expression of pro-inflammatory cytokines (especially TGF-β, CXCL1) in megakaryocytes and some of those pro-inflammatory cytokines were produced by other cell populations (macrophages, endothelial and epithelial cells, alveolar type II) which increased their bioavailability in the microenvironment. In particular, we noticed the mRNA expression and protein content of the main pro-inflammatory cytokines. While protein content of TGF-β and CXCR2, the receptor for CXCL1, detected by immunohistochemical analysis correlated with an increase in the mRNA level, by contrast, *CXCL1* mRNA is downregulated, probably due to the high levels of CXCR2 that internalize its ligand, but do not permit its degradation, and promote the accumulation of CXCL1 protein in the cytoplasm.

We investigated treatments targeting those pro-inflammatory cytokines, already under study for the bone marrow fibrosis, can affect another organ such as the lung and how those different megakaryocytic populations respond. We observed in *Gata1$^{low}$* mice that the treatment with P- Selectin inhibitor could rescue the fibrotic phenotype in the lungs and reduce number of *Gata1$^{low}$* megakaryocytes and TGF-β and CXCL1 content. While P-Selectin inhibitor was the only treatment that reduce the *Gata1$^{low}$* megakaryocytic number, TGF-β1 inhibition reduced lung deposit of collagens fibers and CXCL1 production. Acting on CXCL1 signaling, we investigated the CXCR1/2 inhibitor and we observed an improvement in the fibrotic tissue with reducing reticulin deposition in lung of *Gata1$^{low}$*. Those results were confirmed even in other IPF model (bleomycin induce mice model [216] ). The presence of fibrosis in *Gata1*$^{low}$ lungs, suggested that the population of immuno- megakaryocytes are abnormal, and probably harboring the same mutation burden as the megakaryocytes in the bone marrow, and the cytokines produced by this population are pathologically dangerous over time.

## *Conclusion*

To date, PMF is still an unmet clinical need. Current pharmacological therapies alleviate the symptoms but do not halt the progression of the neoplasm. The only curative therapy remains allogenic HSC transplantation, but not all the patients are eligible for this procedure. To answer this clinical need, during the PhD fellowship, I tried to undercover the pathogenesis of PMF using animal models in order to identify potential new therapeutic targets. I identified two cytokines, TGF-β1 and CXCL1, that are of great interest in the contest of possible alternative therapies for PMF. The results obtained during this PhD fellowship indicate that these pro-inflammatory cytokines are produced by the abnormal megakaryocytes and demonstrate that they play an active role in the etiology and progression of PMF by showing that their clinical grade inhibitors are effective in rescuing the phenotype of our *Gata1$^{low}$* mouse model. These pre-clinical validations have led to clinical trials for TGF-β1 (AVID200, phase Ib trial that has given promising results [217]) and CXCL1 (Reparixin, recruiting for the phase II of clinical trial), both sponsored by the Icahn School of Medicine at Mount Sinai, New York, NY, USA. However, the etiology and progression of PMF are complex processes which are likely to be sustained by additional megakaryocyte abnormalities still to be identified. These abnormalities may include the altered lipid and glucose metabolism identified by our studies and the possibility that the driving mutations turn the malignant *niche-poised* megakaryocytes into cells that support the malignant instead than the normal HSC. It is very encouraging that the defective metabolism identified by us is currently exploited to study dietary strategies that may improve the effects of the *JAK1/2* inhibitors niche by Dr. Skoda [211] while factors produced by the *niche-poised* megakaryocytes, to be identified as part of additional studies that go beyond the purpose of this PhD fellowship, may represent targets for therapies designed to deplete the malignant HSC.

## *Bibliography*


1. Barbui T, Thiele J, Gisslinger H, et al. The 2016 WHO classification and diagnostic criteria for myeloproliferative neoplasms: document summary and in-depth discussion. *Blood Cancer J*. 2018;8(2):15. doi:10.1038/s41408-018-0054-y
2. Tefferi A. Primary myelofibrosis: 2023 update on diagnosis, risk-stratification, and management. *Am J Hematol*. 2023;98(5):801-821. doi:10.1002/ajh.26857
3. Wang X, Prakash S, Lu M, et al. Spleens of myelofibrosis patients contain malignant hematopoietic stem cells. *Journal of Clinical Investigation*. 2012;122(11):3888-3899. doi:10.1172/JCI64397
4. Luque Paz D, Kralovics R, Skoda RC. Genetic basis and molecular profiling in myeloproliferative neoplasms. *Blood*. Published online November 8, 2022. doi:10.1182/blood.2022017578
5. Al-Ghamdi YA, Lake J, Bagg A, et al. Triple-Negative Primary Myelofibrosis: A Bone Marrow Pathology Group Study. *Modern Pathology*. 2023;36(3):100016. doi:10.1016/j.modpat.2022.100016
6. Pettit K, Kandarpa M, Robinson D, Wu YM, Chinnaiyan A, Talpaz M. Genomic Landscape and Clinical Features of Triple-Negative Myelofibrosis. *Clin Lymphoma Myeloma Leuk*. 2018;18:S268. doi:10.1016/j.clml.2018.07.187
7. Svidnicki MCCM, Campos PDM, Ferreira Filho MA, et al. Mutations in Triple-Negative Patients with Myeloproliferative Neoplasms. *Blood*. 2019;134(Supplement_1):5395-5395. doi:10.1182/blood-2019-128764
8. Cattaneo D, Croci GA, Bucelli C, et al. Triple-Negative Essential Thrombocythemia: Clinical-Pathological and Molecular Features. A Single-Center Cohort Study. *Front Oncol*. 2021;11. doi:10.3389/fonc.2021.637116
9. Torres DG, Paes J, da Costa AG, et al. JAK2 Variant Signaling: Genetic, Hematologic and Immune Implication in Chronic Myeloproliferative Neoplasms. *Biomolecules*. 2022;12(2):291. doi:10.3390/biom12020291
10. Koschmieder S, Mughal TI, Hasselbalch HC, et al. Myeloproliferative neoplasms and inflammation: whether to target the malignant clone or the inflammatory process or both. *Leukemia*. 2016;30(5):1018-1024. doi:10.1038/leu.2016.12
11. Nangalia J, Green AR. Myeloproliferative neoplasms: from origins to outcomes. *Hematology Am Soc Hematol Educ Program*. 2017;2017(1):470-479. doi:10.1182/asheducation-2017.1.470
12. Di Rosa M, Giallongo C, Romano A, et al. Immunoproteasome Genes Are Modulated in CD34+ JAK2V617F Mutated Cells from Primary Myelofibrosis Patients. *Int J Mol Sci*. 2020;21(8):2926. doi:10.3390/ijms21082926
13. Hasselbalch HC. Perspectives on chronic inflammation in essential thrombocythemia, polycythemia vera, and myelofibrosis: Is chronic inflammation a trigger and driver of clonal evolution and development of accelerated atherosclerosis and second cancer? *Blood*. 2012;119(14):3219-3225. doi:10.1182/blood-2011-11-394775
14. Nilsri N, Jangprasert P, Pawinwongchai J, Israsena N, Rojnuckarin P. Distinct effects of V617F and exon12-mutated JAK2 expressions on erythropoiesis in a human induced pluripotent stem cell (iPSC)-based model. *Sci Rep*. 2021;11(1):5255. doi:10.1038/s41598-021-83895-6
15. How J, Hobbs GS, Mullally A. Mutant calreticulin in myeloproliferative neoplasms. *Blood*. 2019;134(25):2242-2248. doi:10.1182/blood.2019000622
16. Pietra D, Rumi E, Ferretti V V, et al. Differential clinical effects of different mutation subtypes in CALR-mutant myeloproliferative neoplasms. *Leukemia*. 2016;30(2):431-438. doi:10.1038/leu.2015.277
17. Di Buduo CA, Moccia F, Battiston M, et al. The importance of calcium in the regulation of megakaryocyte function. *Haematologica*. 2014;99(4):769-778. doi:10.3324/haematol.2013.096859
18. Marty C, Harini N, Pecquet C, et al. Calr Mutants Retroviral Mouse Models Lead to a Myeloproliferative Neoplasm Mimicking an Essential Thrombocythemia Progressing to a Myelofibrosis. *Blood*. 2014;124(21):157-157. doi:10.1182/blood.V124.21.157.157

19. Lundberg P, Karow A, Nienhold R, et al. Clonal evolution and clinical correlates of somatic mutations in myeloproliferative neoplasms. *Blood*. 2014;123(14):2220-2228. doi:10.1182/blood-2013-11-537167
20. Wang Z, Liu W, Wang M, et al. Prognostic value of ASXL1 mutations in patients with primary myelofibrosis and its relationship with clinical features: a meta-analysis. *Ann Hematol*. 2021;100(2):465-479. doi:10.1007/s00277-020-04387-7
21. Lasho TL, Jimma T, Finke CM, et al. SRSF2 mutations in primary myelofibrosis: significant clustering with IDH mutations and independent association with inferior overall and leukemia-free survival. *Blood*. 2012;120(20):4168-4171. doi:10.1182/blood-2012-05-429696
22. Tefferi A, Finke CM, Lasho TL, et al. U2AF1 mutation types in primary myelofibrosis: phenotypic and prognostic distinctions. *Leukemia*. 2018;32(10):2274-2278. doi:10.1038/s41375-018-0078-0
23. Tefferi A. Primary myelofibrosis: 2021 update on diagnosis, risk-stratification and management. *Am J Hematol*. 2021;96(1):145-162. doi:10.1002/ajh.26050
24. Barosi G, Mesa RA, Thiele J, et al. Proposed criteria for the diagnosis of post-polycythemia vera and post-essential thrombocythemia myelofibrosis: a consensus statement from the international working group for myelofibrosis research and treatment. *Leukemia*. 2008;22(2):437-438. doi:10.1038/sj.leu.2404914
25. Zahr AA, Salama ME, Carreau N, et al. Bone marrow fibrosis in myelofibrosis: pathogenesis, prognosis and targeted strategies. *Haematologica*. 2016;101(6):660-671. doi:10.3324/haematol.2015.141283
26. Lekovic D, Gotic M, Perunicic-Jovanovic M, et al. Contribution of comorbidities and grade of bone marrow fibrosis to the prognosis of survival in patients with primary myelofibrosis. *Medical Oncology*. 2014;31(3):869. doi:10.1007/s12032-014-0869-8
27. Lu M, Xia L, Salama ME, Hoffman R. Splenic Micro Environmental Cells from Patients with Myelofibrosis Elaborate a Cascade of Cytokines and Serve As a Niche for Malignant Hematopoiesis. *Blood*. 2016;128(22).
28. Verachi P, Gobbo F, Martelli F, et al. Preclinical studies on the use of a P-selectin-blocking monoclonal antibody to halt progression of myelofibrosis in the Gata1 mouse model. *Exp Hematol*. 2023;117:43-61. doi:10.1016/j.exphem.2022.09.004
29. Zetterberg E, Verrucci M, Martelli F, et al. Abnormal P-selectin localization during megakaryocyte development determines thrombosis in the gata1low model of myelofibrosis. *Platelets*. 2014;25(7):539-547. doi:10.3109/09537104.2013.840720
30. Zingariello M, Fabucci M, Bosco D, et al. Differential localization of P-selectin and von Willebrand factor during megakaryocyte maturation. *Biotechnic & Histochemistry*. 2010;85(3):157-170. doi:10.3109/10520290903149612
31. Barbui T, Carobbio A, Cervantes F, et al. Thrombosis in primary myelofibrosis: incidence and risk factors. *Blood*. 2010;115(4):778-782. doi:10.1182/blood-2009-08-238956
32. Saliba W, Mishchenko E, Cohen S, Rennert G, Preis M. Association between myelofibrosis and thromboembolism: A population-based retrospective cohort study. *Journal of Thrombosis and Haemostasis*. 2020;18(4):916-925. doi:10.1111/jth.14754
33. Leimkühler NB, Schneider RK. Inflammatory bone marrow microenvironment. *Hematology*. 2019;2019(1):294-302. doi:10.1182/hematology.2019000045
34. Pietras EM. Inflammation: a key regulator of hematopoietic stem cell fate in health and disease. *Blood*. 2017;130(15):1693-1698. doi:10.1182/blood-2017-06-780882
35. Baade PD, Ross DM, Anderson LA, Forsyth C, Fritschi L. Changing incidence of myeloproliferative neoplasms in Australia, 2003-2014. *Am J Hematol*. 2019;94(4). doi:10.1002/ajh.25407
36. Hultcrantz M, Ravn Landtblom A, Andréasson B, et al. Incidence of myeloproliferative neoplasms – trends by subgroup and age in a population-based study in Sweden. *J Intern Med*. 2020;287(4):448-454. doi:10.1111/joim.13019

37. Moulard O, Mehta J, Fryzek J, Olivares R, Iqbal U, Mesa RA. Epidemiology of myelofibrosis, essential thrombocythemia, and polycythemia vera in the European Union. *Eur J Haematol*. 2014;92(4):289-297. doi:10.1111/ejh.12256
38. Slot S, Dinmohamed AG, Visser O, te Boekhorst PAW, Zweegman S. Survival in Primary Myelofibrosis: A Population-based Analysis in the Netherlands. *Hemasphere*. 2021;5(7):e595. doi:10.1097/HS9.0000000000000595
39. Passamonti F, Mora B. Myelofibrosis. *Blood*. Published online November 23, 2022. doi:10.1182/blood.2022017423
40. Bacigalupo A, Innocenti I, Rossi E, et al. Allogeneic Hemopoietic Stem Cell Transplantation for Myelofibrosis: 2021. *Front Immunol*. 2021;12. doi:10.3389/fimmu.2021.637512
41. Mascarenhas J, Hoffman R. Ruxolitinib: The First FDA Approved Therapy for the Treatment of Myelofibrosis. *Clinical Cancer Research*. 2012;18(11):3008-3014. doi:10.1158/1078-0432.CCR-11-3145
42. Arana Yi C, Tam CS, Verstovsek S. Efficacy and safety of ruxolitinib in the treatment of patients with myelofibrosis. *Future Oncol*. 2015;11(5):719-733. doi:10.2217/fon.14.272
43. Verstovsek S, Mesa RA, Gotlib J, et al. A double-blind, placebo-controlled trial of ruxolitinib for myelofibrosis. *N Engl J Med*. 2012;366(9):799-807. doi:10.1056/NEJMoa1110557
44. Verstovsek S, Mesa RA, Livingston RA, Hu W, Mascarenhas J. Ten years of treatment with ruxolitinib for myelofibrosis: a review of safety. *J Hematol Oncol*. 2023;16(1):82. doi:10.1186/s13045-023-01471-z
45. Talpaz M, Kiladjian JJ. Fedratinib, a newly approved treatment for patients with myeloproliferative neoplasm-associated myelofibrosis. *Leukemia*. 2021;35(1):1-17. doi:10.1038/s41375-020-0954-2
46. Mascarenhas J, Hoffman R, Talpaz M, et al. Pacritinib vs Best Available Therapy, Including Ruxolitinib, in Patients With Myelofibrosis. *JAMA Oncol*. 2018;4(5):652. doi:10.1001/jamaoncol.2017.5818
47. Mesa RA, Vannucchi AM, Mead A, et al. Pacritinib versus best available therapy for the treatment of myelofibrosis irrespective of baseline cytopenias (PERSIST-1): an international, randomised, phase 3 trial. *Lancet Haematol*. 2017;4(5):e225-e236. doi:10.1016/S2352-3026(17)30027-3
48. Tremblay D, Mesa R. Momelotinib for the treatment of myelofibrosis with anemia. *Future Oncology*. 2022;18(20):2559-2571. doi:10.2217/fon-2022-0276
49. Verstovsek S, Al-Ali HK, Mascarenhas J, et al. BOREAS: a global, phase III study of the MDM2 inhibitor navtemadlin (KRT-232) in relapsed/refractory myelofibrosis. *Future Oncology*. 2022;18(37):4059-4069. doi:10.2217/fon-2022-0901
50. Ferreira Gomes G, Harrison C. Pelabresib (CPI-0610): An Exciting Novel Drug for the Treatment of Myelofibrosis. *Curr Hematol Malig Rep*. 2023;18(4):113-120. doi:10.1007/s11899-023-00696-6
51. Verachi P, Gobbo F, Martelli F, et al. The CXCR1/CXCR2 Inhibitor Reparixin Alters the Development of Myelofibrosis in the Gata1low Mice. *Front Oncol*. 2022;12. doi:10.3389/fonc.2022.853484
52. Ciaffoni F, Cassella E, Varricchio L, Massa M, Barosi G, Migliaccio AR. Activation of non-canonical TGF-beta1 signaling indicates an autoimmune mechanism for bone marrow fibrosis in primary myelofibrosis. *Blood Cells Mol Dis*. 2015;54(3):234-241. doi:10.1016/j.bcmd.2014.12.005
53. Ceglia I, Dueck AC, Masiello F, et al. Preclinical rationale for TGF-beta inhibition as a therapeutic target for the treatment of myelofibrosis. *Exp Hematol*. 2016;44(12):1138-1155 e4. doi:10.1016/j.exphem.2016.08.007
54. Chabanon A, Desterke C, Rodenburger E, et al. A cross-talk between stromal cell-derived factor-1 and transforming growth factor-beta controls the quiescence/cycling switch of CD34(+) progenitors through FoxO3 and mammalian target of rapamycin. *Stem Cells*. 2008;26(12):3150-3161. doi:10.1634/stemcells.2008-0219

55. Wang X, Dong F, Zhang S, et al. TGF-β1 Negatively Regulates the Number and Function of Hematopoietic Stem Cells. *Stem Cell Reports*. 2018;11(1):274-287. doi:10.1016/j.stemcr.2018.05.017
56. Varricchio L, Iancu-Rubin C, Upadhyaya B, et al. TGF-β1 protein trap AVID200 beneficially affects hematopoiesis and bone marrow fibrosis in myelofibrosis. *JCI Insight*. 2021;6(18). doi:10.1172/jci.insight.145651
57. Perkins AC, Burbury K, Lehmann T, et al. Adore: A Randomized, Open-Label, Phase 1/2 Open-Platform Study Evaluating Safety and Efficacy of Novel Ruxolitinib Combinations in Patients with Myelofibrosis. *Blood*. 2020;136(Supplement 1):52-53. doi:10.1182/blood-2020-140408
58. Zucker-Franklin D. *Atlas of Blood Cells: Function and Pathology*. Vol 1. Edi. Ermes. Edi. Ermes; 1988.
59. Machlus KR, Italiano JE. The incredible journey: From megakaryocyte development to platelet formation. *Journal of Cell Biology*. 2013;201(6):785-796. doi:10.1083/jcb.201304054
60. Zucker-Franklin D. Ultrastructural studies of hematopoietic elements in relation to the myelofibrosis-osteosclerosis syndrome, megakaryocytes and platelets (MMM or MOS). *Adv Biosci*. 1975;16(C):127-143.
61. Italiano JE, Shivdasani RA. Megakaryocytes and beyond: The birth of platelets. *Journal of Thrombosis and Haemostasis*. 2003;1(6):1174-1182. doi:10.1046/j.1538-7836.2003.00290.x
62. Vainchenker W, Raslova H. Megakaryocyte polyploidization: role in platelet production. *Platelets*. 2019;00(00):1-10. doi:10.1080/09537104.2019.1667497
63. Mazzi S, Lordier L, Debili N, Raslova H, Vainchenker W. Megakaryocyte and polyploidization. *Exp Hematol*. 2018;57:1-13. doi:10.1016/j.exphem.2017.10.001
64. Italiano JE, Battinelli EM. Selective sorting of alpha-granule proteins. *J Thromb Haemost*. 2009;7 Suppl 1:173-176. doi:10.1111/j.1538-7836.2009.03387.x
65. Ferreira R, Ohneda K, Yamamoto M, Philipsen S. GATA1 Function, a Paradigm for Transcription Factors in Hematopoiesis. *Mol Cell Biol*. 2005;25(4):1215-1227. doi:10.1128/MCB.25.4.1215-1227.2005
66. Dalby A, Ballester-Beltrán J, Lincetto C, et al. Transcription Factor Levels after Forward Programming of Human Pluripotent Stem Cells with GATA1, FLI1, and TAL1 Determine Megakaryocyte versus Erythroid Cell Fate Decision. *Stem Cell Reports*. 2018;11(6):1462-1478. doi:10.1016/j.stemcr.2018.11.001
67. Majewski IJ, Metcalf D, Mielke LA, et al. A mutation in the translation initiation codon of *Gata-1* disrupts megakaryocyte maturation and causes thrombocytopenia. *Proceedings of the National Academy of Sciences*. 2006;103(38):14146-14151. doi:10.1073/pnas.0606439103
68. McNulty M, Crispino JD. Acute Megakaryocytic Leukemia. *Cold Spring Harb Perspect Med*. 2020;10(2):a034884. doi:10.1101/cshperspect.a034884
69. Shimizu R, Engel JD, Yamamoto M. GATA1-related leukaemias. *Nat Rev Cancer*. 2008;8(4):279-287. doi:10.1038/nrc2348
70. Ahmed M, Sternberg A, Hall G, et al. Natural history of GATA1 mutations in Down syndrome. *Blood*. 2004;103(7):2480-2489. doi:10.1182/blood-2003-10-3383
71. Hitchcock IS, Kaushansky K. Thrombopoietin from beginning to end. *Br J Haematol*. 2014;165(2):259-268. doi:10.1111/bjh.12772
72. Yoshihara H, Arai F, Hosokawa K, et al. Thrombopoietin/MPL Signaling Regulates Hematopoietic Stem Cell Quiescence and Interaction with the Osteoblastic Niche. *Cell Stem Cell*. 2007;1(6):685-697. doi:10.1016/j.stem.2007.10.020
73. Varghese LN, Defour JP, Pecquet C, Constantinescu SN. The thrombopoietin receptor: Structural basis of traffic and activation by ligand, mutations, agonists, and mutated calreticulin. *Front Endocrinol (Lausanne)*. 2017;8(MAR):59. doi:10.3389/fendo.2017.00059
74. Gurney A, Carver-Moore K, de Sauvage F, Moore M. Thrombocytopenia in c-mpl-deficient mice. *Science (1979)*. 1994;265(5177):1445-1447. doi:10.1126/science.8073287

75. Alexander WS, Roberts AW, Nicola NA, Li R, Metcalf D. Deficiencies in progenitor cells of multiple hematopoietic lineages and defective megakaryocytopoiesis in mice lacking the thrombopoietic receptor c-Mpl. *Blood*. 1996;87(6):2162-2170.
76. Wong GG, Witek-Giannotti JS, Temple PA, et al. Stimulation of murine hemopoietic colony formation by human IL-6. *The Journal of Immunology*. 1988;140(9):3040-3044.
77. Behrens K, Alexander WS. Cytokine control of megakaryopoiesis. *Growth Factors*. 2018;36(3-4):89-103. doi:10.1080/08977194.2018.1498487
78. Migliaccio G, Migliaccio AR, Adamson JW. In vitro differentiation of human granulocyte/macrophage and erythroid progenitors: comparative analysis of the influence of recombinant human erythropoietin, G-CSF, GM-CSF, and IL-3 in serum-supplemented and serum-deprived cultures. *Blood*. 1988;72(1):248-256.
79. Sugiyama T, Kohara H, Noda M, Nagasawa T. Maintenance of the Hematopoietic Stem Cell Pool by CXCL12-CXCR4 Chemokine Signaling in Bone Marrow Stromal Cell Niches. *Immunity*. 2006;25(6):977-988. doi:10.1016/j.immuni.2006.10.016
80. Kowalska MA, Ratajczak J, Hoxie J, et al. Megakaryocyte precursors, megakaryocytes and platelets express the HIV co-receptor CXCR4 on their surface: Determination of response to stromal- derived factor-1 by megakaryocytes and platelets. *Br J Haematol*. 1999;104(2):220-229. doi:10.1046/j.1365-2141.1999.01169.x
81. Rettig MP, Ansstas G, Dipersio JF. Mobilization of hematopoietic stem and progenitor cells using inhibitors of CXCR4 and VLA-4. *Leukemia*. 2012;26(1):34-53. doi:10.1038/leu.2011.197
82. Pinho S, Frenette PS. Haematopoietic stem cell activity and interactions with the niche. *Nat Rev Mol Cell Biol*. 2019;20(5):303-320. doi:10.1038/s41580-019-0103-9
83. Weyrich AS, Zimmerman GA. Platelets in Lung Biology. *Annu Rev Physiol*. 2013;75(1):569-591. doi:10.1146/annurev-physiol-030212-183752
84. Howell WH, Donahue DD. The production of blood platelets in lungs. *Journal of Experimental Medicine*. 1937;65(2):177-203. doi:10.1084/jem.65.2.177
85. Lefrançais E, Ortiz-Muñoz G, Caudrillier A, et al. The lung is a site of platelet biogenesis and a reservoir for haematopoietic progenitors. *Nature*. 2017;544(7648):105-109. doi:10.1038/nature21706
86. Pariser DN, Hilt ZT, Ture SK, et al. Lung megakaryocytes are immune modulatory cells. *Journal of Clinical Investigation*. 2021;131(1):e1373776. doi:10.1172/JCI137377
87. Yeung AK, Villacorta-Martin C, Hon S, Rock JR, Murphy GJ. Lung megakaryocytes display distinct transcriptional and phenotypic properties. *Blood Adv*. 2020;4(24):6204-6217. doi:10.1182/bloodadvances.2020002843
88. Sun S, Jin C, Si J, et al. Single-Cell Analysis of Ploidy and Transcriptome Reveals Functional and Spatial Divergency in Murine Megakaryopoiesis. *Blood*. Published online June 11, 2021. doi:10.1182/blood.2021010697
89. Wang H, He J, Xu C, et al. Decoding Human Megakaryocyte Development. *Cell Stem Cell*. 2021;28(3):535-549.e8. doi:10.1016/j.stem.2020.11.006
90. Boilard E, Machlus KR. Location is everything when it comes to megakaryocyte function. *J Clin Invest*. 2021;131(1). doi:10.1172/JCI144964
91. Centurione L, Di Baldassarre A, Zingariello M, et al. Increased and pathologic emperipolesis of neutrophils within megakaryocytes associated with marrow fibrosis in GATA-1low mice. *Blood*. 2004;104(12):3573-3580. doi:10.1182/blood-2004-01-0193
92. Ciurea SO, Merchant D, Mahmud N, et al. Pivotal contributions of megakaryocytes to the biology of idiopathic myelofibrosis. *Blood*. 2007;110(3):986-993. doi:10.1182/blood-2006-12-064626
93. Balduini A, Badalucco S, Pugliano MT, et al. In Vitro Megakaryocyte Differentiation and Proplatelet Formation in Ph-Negative Classical Myeloproliferative Neoplasms: Distinct Patterns in the Different Clinical Phenotypes. *PLoS One*. 2011;6(6):e21015. doi:10.1371/journal.pone.0021015

94. Psaila B, Wang G, Rodriguez-Meira A, et al. Single-Cell Analyses Reveal Megakaryocyte-Biased Hematopoiesis in Myelofibrosis and Identify Mutant Clone-Specific Targets. *Mol Cell*. 2020;78(3):477-492.e8. doi:10.1016/j.molcel.2020.04.008
95. Vannucchi AM, Pancrazzi A, Guglielmelli P, et al. Abnormalities of GATA-1 in megakaryocytes from patients with idiopathic myelofibrosis. *Am J Pathol*. 2005;167(3):849-858. doi:10.1016/S0002-9440(10)62056-1
96. Vannucchi AM, Bianchi L, Paoletti F, et al. A pathobiologic pathway linking thrombopoietin, GATA-1, and TGF-β1 in the development of myelofibrosis. *Blood*. 2005;105(9):3493-3501. doi:10.1182/blood-2004-04-1320
97. Gilles L, Arslan AD, Marinaccio C, et al. Downregulation of GATA1 drives impaired hematopoiesis in primary myelofibrosis. *Journal of Clinical Investigation*. 2017;127(4):1316-1320. doi:10.1172/JCI82905
98. Lally J, Boasman K, Brown L, et al. GATA-1: A potential novel biomarker for the differentiation of essential thrombocythemia and myelofibrosis. *Journal of Thrombosis and Haemostasis*. 2019;17(6):896-900. doi:10.1111/jth.14433
99. Zingariello M, Sancillo L, Martelli F, et al. The thrombopoietin/MPL axis is activated in the gata1low mouse model of myelofibrosis and is associated with a defective RPS14 signature. *Blood Cancer J*. 2017;7(6):1-11. doi:10.1038/bcj.2017.51
100. Malara A, Abbonante V, Zingariello M, Migliaccio A, Balduini A. Megakaryocyte contribution to bone marrow fibrosis: Many arrows in the quiver. *Mediterr J Hematol Infect Dis*. 2018;10(1):1-18. doi:10.4084/MJHID.2018.068
101. Malara A, Abbonante V, Di Buduo CA, Tozzi L, Currao M, Balduini A. The secret life of a megakaryocyte: emerging roles in bone marrow homeostasis control. *Cell Mol Life Sci*. 2015;72(8):1517-1536. doi:10.1007/s00018-014-1813-y
102. Malara A, Currao M, Gruppi C, et al. Megakaryocytes Contribute to the Bone Marrow-Matrix Environment by Expressing Fibronectin, Type IV Collagen, and Laminin. *Stem Cells*. 2014;32(4):926-937. doi:10.1002/stem.1626
103. Martyré M -C., Le Bousse-Kerdiles M -C., Romquin N, et al. Elevated levels of basic fibroblast growth factor in megakaryocytes and platelets from patients with idiopathic myelofibrosis. *Br J Haematol*. 1997;97(2):441-448. doi:10.1046/j.1365-2141.1997.292671.x
104. Augello C, Gianelli U, Falcone R, et al. PDGFB hypomethylation is a favourable prognostic biomarker in primary myelofibrosis. *Leuk Res*. 2015;39(2):236-241. doi:10.1016/j.leukres.2014.11.012
105. Papadantonakis N, Matsuura S, Ravid K. Megakaryocyte pathology and bone marrow fibrosis: the lysyl oxidase connection. *Blood*. 2012;120(9):1774-1781. doi:10.1182/blood-2012-02-402594
106. Garimella R, Kacena MA, Tague SE, Wang J, Horowitz MC, Anderson HC. Expression of bone morphogenetic proteins and their receptors in the bone marrow megakaryocytes of GATA-1low mice: A possible role in osteosclerosis. *Journal of Histochemistry and Cytochemistry*. 2007;55(7):745-752. doi:10.1369/jhc.6A7164.2007
107. Ruberti S, Bianchi E, Guglielmelli P, et al. Involvement of MAF/SPP1 axis in the development of bone marrow fibrosis in PMF patients. *Leukemia*. 2018;32(2):438-449. doi:10.1038/leu.2017.220
108. Wallace PM, MacMaster JF, Rillema JR, Peng J, Burstein SA, Shoyab M. Thrombocytopoietic properties of oncostatin M. *Blood*. 1995;86(4):1310-1315.
109. Hoermann G, Cerny-Reiterer S, Herrmann H, et al. Identification of oncostatin M as a JAK2 V617F-dependent amplifier of cytokine production and bone marrow remodeling in myeloproliferative neoplasms. *The FASEB Journal*. 2012;26(2):894-906. doi:10.1096/fj.11-193078
110. Schneider RK, Mullally A, Dugourd A, et al. Gli1 + Mesenchymal Stromal Cells Are a Key Driver of Bone Marrow Fibrosis and an Important Cellular Therapeutic Target. *Cell Stem Cell*. 2017;20(6):785-800.e8. doi:10.1016/j.stem.2017.03.008

111. Burstein SA, Malpass TW, Yee E, et al. Platelet factor-4 excretion in myeloproliferative disease: implications for the aetiology of myelofibrosis. *Br J Haematol*. 1984;57(3):383-392. doi:10.1111/j.1365-2141.1984.tb02912.x
112. Gleitz HFE, Dugourd AJF, Leimkühler NB, et al. Increased CXCL4 expression in hematopoietic cells links inflammation and progression of bone marrow fibrosis in MPN. *Blood*. 2020;136(18):2051-2064. doi:10.1182/blood.2019004095
113. Akhmetshina A, Palumbo K, Dees C, et al. Activation of canonical Wnt signalling is required for TGF-β-mediated fibrosis. *Nat Commun*. 2012;3(1):735. doi:10.1038/ncomms1734
114. Caja L, Dituri F, Mancarella S, et al. TGF-β and the tissue microenvironment: Relevance in fibrosis and cancer. *Int J Mol Sci*. 2018;19(5). doi:10.3390/ijms19051294
115. Margadant C, Sonnenberg A. Integrin-TGF-B crosstalk in fibrosis, cancer and wound healing. *EMBO Rep*. 2010;11(2):97-105. doi:10.1038/embor.2009.276
116. Meng X ming, Nikolic-Paterson DJ, Lan HY. TGF-β: the master regulator of fibrosis. *Nat Rev Nephrol*. 2016;12(6):325-338. doi:10.1038/nrneph.2016.48
117. Campanelli R, Rosti V, Villani L, et al. Evaluation of the bioactive and total transforming growth factor β1 levels in primary myelofibrosis. *Cytokine*. 2011;53(1):100-106. doi:https://doi.org/10.1016/j.cyto.2010.07.427
118. Zingariello M, Ruggeri A, Martelli F, et al. A novel interaction between megakaryocytes and activated fibrocytes increases TGF-β bioavailability in the Gata1(low) mouse model of myelofibrosis. *Am J Blood Res*. 2015;5(2):34-61. http://www.ncbi.nlm.nih.gov/pubmed/27069753
119. Yoon SY, Tefferi A, Li CY. Cellular Distribution of Platelet-Derived Growth Factor, Transforming Growth Factor-β, Basic Fibroblast Growth Factor, and Their Receptors in Normal Bone Marrow. *Acta Haematol*. 2000;104(4):151-157. doi:10.1159/000046507
120. Zingariello M, Martelli F, Ciaffoni F, et al. Characterization of the TGF-b1 signaling abnormalities in the Gata1low mouse model of myelofibrosis. *Blood*. Published online 2013. doi:10.1182/blood-2012-06-439661
121. Chagraoui H. Prominent role of TGF-beta 1 in thrombopoietin-induced myelofibrosis in mice. *Blood*. 2002;100(10):3495-3503. doi:10.1182/blood-2002-04-1133
122. Ponce CC, de Lourdes Lopes Ferrari Chauffaille M, Ihara SSM, Silva MRR. Increased angiogenesis in primary myelofibrosis: latent transforming growth factor-β as a possible angiogenic factor. *Rev Bras Hematol Hemoter*. 2014;36(5):322-328. doi:10.1016/j.bjhh.2014.07.010
123. Yang JG, Wang LL, Ma DC. Effects of vascular endothelial growth factors and their receptors on megakaryocytes and platelets and related diseases. *Br J Haematol*. 2018;180(3):321-334. doi:10.1111/bjh.15000
124. Bock O, Höftmann J, Theophile K, et al. Bone Morphogenetic Proteins Are Overexpressed in the Bone Marrow of Primary Myelofibrosis and Are Apparently Induced by Fibrogenic Cytokines. *Am J Pathol*. 2008;172(4):951-960. doi:10.2353/ajpath.2008.071030
125. Andrieux J, Roche-Lestienne C, Geffroy S, et al. Bone morphogenetic protein antagonist gene NOG is involved in myeloproliferative disease associated with myelofibrosis. *Cancer Genet Cytogenet*. 2007;178(1):11-16. doi:10.1016/j.cancergencyto.2007.06.001
126. Blank U, Karlsson S. TGF-β signaling in the control of hematopoietic stem cells. *Blood*. 2015;125(23):3542-3550. doi:10.1182/blood-2014-12-618090
127. Desterke C, Martinaud C, Ruzehaji N, Le Bousse-Kerdiles MC. Inflammation as a Keystone of Bone Marrow Stroma Alterations in Primary Myelofibrosis. *Mediators Inflamm*. 2015;2015:415024. doi:10.1155/2015/415024
128. Woods B, Chen W, Chiu S, et al. Activation of JAK/STAT Signaling in Megakaryocytes Sustains Myeloproliferation In Vivo. *Clinical Cancer Research*. 2019;25(19):5901-5912. doi:10.1158/1078-0432.CCR-18-4089

129. Čokić VP, Mitrović-Ajtić O, Beleslin-Čokić BB, et al. Proinflammatory Cytokine IL-6 and JAK-STAT Signaling Pathway in Myeloproliferative Neoplasms. *Mediators Inflamm*. 2015;2015:1-13. doi:10.1155/2015/453020
130. Cacemiro M da C, Cominal JG, Tognon R, et al. Philadelphia-negative myeloproliferative neoplasms as disorders marked by cytokine modulation. *Hematol Transfus Cell Ther*. 2018;40(2):120-131. doi:10.1016/j.htct.2017.12.003
131. Malara A, Gruppi C, Abbonante V, et al. EDA fibronectin–TLR4 axis sustains megakaryocyte expansion and inflammation in bone marrow fibrosis. *Journal of Experimental Medicine*. 2019;216(3):587-604. doi:10.1084/jem.20181074
132. Takeuchi K, Higuchi T, Yamashita T, Koike K. Chemokine production by human Megakaryocytes derived from CD34-positive cord blood cells. *Cytokine*. 1999;11(6):424-434. doi:10.1006/cyto.1998.0455
133. Tefferi A, Vaidya R, Caramazza D, Finke C, Lasho T, Pardanani A. Circulating Interleukin (IL)-8, IL-2R, IL-12, and IL-15 Levels Are Independently Prognostic in Primary Myelofibrosis: A Comprehensive Cytokine Profiling Study. *Journal of Clinical Oncology*. 2011;29(10):1356-1363. doi:10.1200/JCO.2010.32.9490
134. Emadi S, Clay D, Desterke C, et al. IL-8 and its CXCR1 and CXCR2 receptors participate in the control of megakaryocytic proliferation, differentiation, and ploidy in myeloid metaplasia with myelofibrosis. *Blood*. 2005;105(2):464-473. doi:10.1182/blood-2003-12-4415
135. Lu M, Xia L, Liu YC, et al. Lipocalin produced by myelofibrosis cells affects the fate of both hematopoietic and marrow microenvironmental cells. *Blood*. 2015;126(8):972-982. doi:10.1182/blood-2014-12-618595
136. Varricchio L, Hoffman R. Megakaryocytes Are Regulators of the Tumor Microenvironment and Malignant Hematopoietic Progenitor Cells in Myelofibrosis. *Front Oncol*. 2022;12. doi:10.3389/fonc.2022.906698
137. Verstovsek S, Manshouri T, Pilling D, et al. Role of neoplastic monocyte-derived fibrocytes in primary myelofibrosis. *Journal of Experimental Medicine*. 2016;213(9):1723-1740. doi:10.1084/jem.20160283
138. Molitor DCA, Boor P, Buness A, et al. Macrophage frequency in the bone marrow correlates with morphologic subtype of myeloproliferative neoplasm. *Ann Hematol*. 2021;100(1):97-104. doi:10.1007/s00277-020-04304-y
139. Cunin P, Bouslama R, Machlus KR, et al. Megakaryocyte emperipolesis mediates membrane transfer from intracytoplasmic neutrophils to platelets. *Elife*. 2019;8. doi:10.7554/eLife.44031
140. Cashell AW, Buss DH. The frequency and significance of megakaryocytic emperipolesis in myeloproliferative and reactive states. *Ann Hematol*. 1992;64(6):273-276. doi:10.1007/BF01695470
141. Galán-Díez M, Cuesta-Domínguez Á, Kousteni S. The Bone Marrow Microenvironment in Health and Myeloid Malignancy. *Cold Spring Harb Perspect Med*. 2018;8(7):a031328. doi:10.1101/cshperspect.a031328
142. Forte D, García-Fernández M, Sánchez-Aguilera A, et al. Bone Marrow Mesenchymal Stem Cells Support Acute Myeloid Leukemia Bioenergetics and Enhance Antioxidant Defense and Escape from Chemotherapy. *Cell Metab*. 2020;32(5):829-843.e9. doi:10.1016/j.cmet.2020.09.001
143. Stavnichuk M, Komarova S V. Megakaryocyte-bone cell interactions: lessons from mouse models of experimental myelofibrosis and related disorders. *American Journal of Physiology-Cell Physiology*. 2022;322(2):C177-C184. doi:10.1152/ajpcell.00328.2021
144. Psaila B, Mead AJ. Single-cell approaches reveal novel cellular pathways for megakaryocyte and erythroid differentiation. *Blood*. 2019;133(13):1427-1435. doi:10.1182/blood-2018-11-835371
145. Abbonante V, Di Buduo CA, Gruppi C, et al. Thrombopoietin/TGF-β1 Loop Regulates Megakaryocyte Extracellular Matrix Component Synthesis. *Stem Cells*. 2016;34(4):1123-1133. doi:10.1002/stem.2285

146. Ivanovs A, Rybtsov S, Ng ES, Stanley EG, Elefanty AG, Medvinsky A. Human haematopoietic stem cell development: from the embryo to the dish. *Development*. 2017;144(13):2323-2337. doi:10.1242/dev.134866
147. Jagannathan-Bogdan M, Zon LI. Hematopoiesis. *Development*. 2013;140(12):2463-2467. doi:10.1242/dev.083147
148. Pietras EM, Warr MR, Passegué E. Cell cycle regulation in hematopoietic stem cells. *Journal of Cell Biology*. 2011;195(5):709-720. doi:10.1083/jcb.201102131
149. Saçma M, Pospiech J, Bogeska R, et al. Haematopoietic stem cells in perisinusoidal niches are protected from ageing. *Nat Cell Biol*. 2019;21(11):1309-1320. doi:10.1038/s41556-019-0418-y
150. Viatour P, Somervaille TC, Venkatasubrahmanyam S, et al. Hematopoietic Stem Cell Quiescence Is Maintained by Compound Contributions of the Retinoblastoma Gene Family. *Cell Stem Cell*. 2008;3(4):416-428. doi:10.1016/j.stem.2008.07.009
151. He S, Roberts PJ, Sorrentino JA, et al. Transient CDK4/6 inhibition protects hematopoietic stem cells from chemotherapy-induced exhaustion. *Sci Transl Med*. 2017;9(387). doi:10.1126/scitranslmed.aal3986
152. Fink JR, LeBien TW. Novel expression of cyclin-dependent kinase inhibitors in human B-cell precursors. *Exp Hematol*. 2001;29(4):490-498. doi:10.1016/S0301-472X(01)00619-1
153. Bousounis P, Bergo V, Trompouki E. Inflammation, Aging and Hematopoiesis: A Complex Relationship. *Cells*. 2021;10(6):1386. doi:10.3390/cells10061386
154. Colom Díaz PA, Mistry JJ, Trowbridge JJ. Hematopoietic stem cell aging and leukemia transformation. *Blood*. 2023;142(6):533-542. doi:10.1182/blood.2022017933
155. Grover A, Sanjuan-Pla A, Thongjuea S, et al. Single-cell RNA sequencing reveals molecular and functional platelet bias of aged haematopoietic stem cells. *Nat Commun*. 2016;7(1):11075. doi:10.1038/ncomms11075
156. Lepperdinger G. Inflammation and mesenchymal stem cell aging. *Curr Opin Immunol*. 2011;23(4):518-524. doi:10.1016/j.coi.2011.05.007
157. Munir H, Ward LSC, Sheriff L, et al. Adipogenic Differentiation of Mesenchymal Stem Cells Alters Their Immunomodulatory Properties in a Tissue-Specific Manner. *Stem Cells*. 2017;35(6):1636-1646. doi:10.1002/stem.2622
158. Tamma R, Ribatti D. Bone Niches, Hematopoietic Stem Cells, and Vessel Formation. *Int J Mol Sci*. 2017;18(1):151. doi:10.3390/ijms18010151
159. Gao X, Xu C, Asada N, Frenette PS. The hematopoietic stem cell niche: From embryo to adult. *Development (Cambridge)*. 2018;145(2). doi:10.1242/dev.139691
160. Baccin C, Al-Sabah J, Velten L, et al. Combined single-cell and spatial transcriptomics reveal the molecular, cellular and spatial bone marrow niche organization. *Nat Cell Biol*. 2020;22(1):38-48. doi:10.1038/s41556-019-0439-6
161. Jeffery EC, Mann TLA, Pool JA, Zhao Z, Morrison SJ. Bone marrow and periosteal skeletal stem/progenitor cells make distinct contributions to bone maintenance and repair. *Cell Stem Cell*. 2022;29(11):1547-1561.e6. doi:10.1016/j.stem.2022.10.002
162. Cordeiro-Spinetti E, Taichman RS, Balduino A. The Bone Marrow Endosteal Niche: How Far from the Surface? *J Cell Biochem*. 2015;116(1):6-11. doi:10.1002/jcb.24952
163. Doan PL, Chute JP. The vascular niche: home for normal and malignant hematopoietic stem cells. *Leukemia*. 2012;26(1):54-62. doi:10.1038/leu.2011.236
164. Kokkaliaris KD, Kunz L, Cabezas-Wallscheid N, et al. Adult blood stem cell localization reflects the abundance of reported bone marrow niche cell types and their combinations. *Blood*. 2020;136(20):2296-2307. doi:10.1182/blood.2020006574
165. Bogeska R, Mikecin AM, Kaschutnig P, et al. Inflammatory exposure drives long-lived impairment of hematopoietic stem cell self-renewal activity and accelerated aging. *Cell Stem Cell*. 2022;29(8):1273-1284.e8. doi:10.1016/j.stem.2022.06.012

166. Mosteo L, Storer J, Batta K, Searle EJ, Duarte D, Wiseman DH. The Dynamic Interface Between the Bone Marrow Vascular Niche and Hematopoietic Stem Cells in Myeloid Malignancy. *Front Cell Dev Biol*. 2021;9. doi:10.3389/fcell.2021.635189
167. Fisher DAC, Fowles JS, Zhou A, Oh ST. Inflammatory Pathophysiology as a Contributor to Myeloproliferative Neoplasms. *Front Immunol*. 2021;12. doi:10.3389/fimmu.2021.683401
168. Kouroukli O, Symeonidis A, Foukas P, Maragkou MK, Kourea EP. Bone Marrow Immune Microenvironment in Myelodysplastic Syndromes. *Cancers (Basel)*. 2022;14(22):5656. doi:10.3390/cancers14225656
169. Jacquelin S, Kramer F, Mullally A, Lane SW. Murine Models of Myelofibrosis. *Cancers (Basel)*. 2020;12(9):2381. doi:10.3390/cancers12092381
170. Bersenev A, Wu C, Balcerek J, et al. Lnk constrains myeloproliferative diseases in mice. *Journal of Clinical Investigation*. 2010;120(6):2058-2069. doi:10.1172/JCI42032
171. Yang Y, Akada H, Nath D, Hutchison RE, Mohi G. Loss of Ezh2 cooperates with Jak2V617F in the development of myelofibrosis in a mouse model of myeloproliferative neoplasm. *Blood*. 2016;127(26):3410-3423. doi:10.1182/blood-2015-11-679431
172. Pikman Y, Lee BH, Mercher T, et al. MPLW515L Is a Novel Somatic Activating Mutation in Myelofibrosis with Myeloid Metaplasia. *PLoS Med*. 2006;3(7):e270. doi:10.1371/journal.pmed.0030270
173. Marty C, Pecquet C, Nivarthi H, et al. Calreticulin mutants in mice induce an MPL-dependent thrombocytosis with frequent progression to myelofibrosis. *Blood*. 2016;127(10):1317-1324. doi:10.1182/blood-2015-11-679571
174. Decker M, Martinez-Morentin L, Wang G, et al. Leptin-receptor-expressing bone marrow stromal cells are myofibroblasts in primary myelofibrosis. *Nat Cell Biol*. 2017;19(6):677-688. doi:10.1038/ncb3530
175. Kakumitsu H, Kamezaki K, Shimoda K, et al. Transgenic mice overexpressing murine thrombopoietin develop myelofibrosis and osteosclerosis. *Leuk Res*. 2005;29(7):761-769. doi:10.1016/j.leukres.2004.12.009
176. Kuhl C, Atzberger A, Iborra F, Nieswandt B, Porcher C, Vyas P. GATA1-Mediated Megakaryocyte Differentiation and Growth Control Can Be Uncoupled and Mapped to Different Domains in GATA1. *Mol Cell Biol*. 2005;25(19):8592-8606. doi:10.1128/MCB.25.19.8592-8606.2005
177. Crispino JD, Horwitz MS. GATA factor mutations in hematologic disease. *Blood*. 2017;129(15):2103-2110. doi:10.1182/blood-2016-09-687889
178. Weiss MJ, Keller G, Orkin SH. Novel insights into erythroid development revealed through in vitro differentiation of GATA-1 embryonic stem cells. *Genes Dev*. 1994;8(10):1184-1197. doi:10.1101/gad.8.10.1184
179. Doré LC, Crispino JD. Transcription factor networks in erythroid cell and megakaryocyte development. *Blood*. 2011;118(2):231-239. doi:10.1182/blood-2011-04-285981
180. Fujiwara Y, Browne CP, Cunniff K, Goff SC, Orkin SH. Arrested development of embryonic red cell precursors in mouse embryos lacking transcription factor GATA-1. *Proceedings of the National Academy of Sciences*. 1996;93(22):12355-12358. doi:10.1073/pnas.93.22.12355
181. Shivdasani RA, Fujiwara Y, McDevitt MA, Orkin SH. A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development. *EMBO J*. 1997;16(13):3965-3973. doi:10.1093/emboj/16.13.3965
182. Mcdevitt MA, Fujiwara Y, Shivdasani RA, Orkin SH. An upstream, DNase I hypersensitive region of the hematopoietic-expressed transcription factor GATA-1 gene confers developmental specificity in transgenic mice. *Proc Natl Acad Sci U S A*. 1997;94(15):7976-7981. doi:10.1073/pnas.94.15.7976
183. Vannucchi AM, Bianchi L, Cellai C, et al. Development of myelofibrosis in mice genetically impaired for GATA-1 expression (GATA-1low mice). *Blood*. 2002;100(4):1123-1132. doi:10.1182/blood-2002-06-1913

184. Kramer F, Dernedde J, Mezheyeuski A, Tauber R, Micke P, Kappert K. Platelet-derived growth factor receptor β activation and regulation in murine myelofibrosis. *Haematologica*. 2020;105(8):2083-2094. doi:10.3324/haematol.2019.226332
185. Moliterno AR, Hankins WD, Spivak JL. Impaired Expression of the Thrombopoietin Receptor by Platelets from Patients with Polycythemia Vera. *New England Journal of Medicine*. 1998;338(9):572-580. doi:10.1056/NEJM199802263380903
186. Villmow T, Kemkes-Matthes B, Matzdorff AC. Markers of platelet activation and platelet–leukocyte interaction in patients with myeloproliferative syndromes. *Thromb Res*. 2002;108(2-3):139-145. doi:10.1016/S0049-3848(02)00354-7
187. Spangrude GJ, Lewandowski D, Martelli F, et al. P-Selectin Sustains Extramedullary Hematopoiesis in the Gata1low Model of Myelofibrosis. *Stem Cells*. 2016;34(1):67-82. doi:10.1002/stem.2229
188. Zingariello M, Verachi P, Gobbo F, et al. Resident Self-Tissue of Proinflammatory Cytokines Rather than Their Systemic Levels Correlates with Development of Myelofibrosis in Gata1low Mice. *Biomolecules*. 2022;12(2). doi:10.3390/biom12020234
189. Young K, Borikar S, Bell R, Kuffler L, Philip V, Trowbridge JJ. Progressive alterations in multipotent hematopoietic progenitors underlie lymphoid cell loss in aging. *Journal of Experimental Medicine*. 2016;213(11):2259-2267. doi:10.1084/jem.20160168
190. Ho YH, del Toro R, Rivera-Torres J, et al. Remodeling of Bone Marrow Hematopoietic Stem Cell Niches Promotes Myeloid Cell Expansion during Premature or Physiological Aging. *Cell Stem Cell*. 2019;25(3):407-418.e6. doi:10.1016/j.stem.2019.06.007
191. Brayton CF, Treuting PM, Ward JM. Pathobiology of Aging Mice and GEM. *Vet Pathol*. 2012;49(1):85-105. doi:10.1177/0300985811430696
192. Costa D, Principi E, Lazzarini E, et al. LCN2 overexpression in bone enhances the hematopoietic compartment via modulation of the bone marrow microenvironment. *J Cell Physiol*. 2017;232(11):3077-3087. doi:10.1002/jcp.25755
193. Sinclair A, Park L, Shah M, et al. CXCR2 and CXCL4 regulate survival and self-renewal of hematopoietic stem/progenitor cells. *Blood*. 2016;128(3):371-383. doi:10.1182/blood-2015-08-661785
194. Qiu J, Papatsenko D, Niu X, Schaniel C, Moore K. Divisional history and hematopoietic stem cell function during homeostasis. *Stem Cell Reports*. 2014;2(4):473-490. doi:10.1016/j.stemcr.2014.01.016
195. Mazzarini M, Arciprete F, Picconi O, et al. Single cell analysis of the localization of the hematopoietic stem cells within the bone marrow architecture identifies niche-specific proliferation dynamics. *Front Med (Lausanne)*. 2023;10:1166758. doi:10.3389/fmed.2023.1166758
196. Kunisaki Y, Bruns I, Scheiermann C, et al. Arteriolar niches maintain haematopoietic stem cell quiescence. *Nature*. 2013;502(7473):637-643. doi:10.1038/nature12612
197. Guerrero-Juarez CF, Dedhia PH, Jin S, et al. Single-cell analysis reveals fibroblast heterogeneity and myeloid-derived adipocyte progenitors in murine skin wounds. *Nat Commun*. 2019;10(1):650. doi:10.1038/s41467-018-08247-x
198. Polino G, Barosi G, Berzuini A, et al. Fatty bone marrow with severe myeloid hypoplasia in idiopathic myelofibrosis. *Haematologica*. 1986;71(2):117-121.
199. Zingariello M, Rosti V, Vannucchi AM, et al. Shared and Distinctive Ultrastructural Abnormalities Expressed by Megakaryocytes in Bone Marrow and Spleen From Patients With Myelofibrosis. *Front Oncol*. 2020;10(Supplement_1):4209-4209. doi:10.3389/fonc.2020.584541
200. Rosti V, Villani L, Riboni R, et al. Spleen endothelial cells from patients with myelofibrosis harbor the JAK2V617F mutation. *Blood*. 2013;121(2):360-368. doi:10.1182/blood-2012-01-404889
201. Ito Y, Nakamura S, Sugimoto N, et al. Turbulence Activates Platelet Biogenesis to Enable Clinical Scale Ex Vivo Production. *Cell*. 2018;174(3):636-648.e18. doi:10.1016/j.cell.2018.06.011
202. Eckly A, Heijnen H, Pertuy F, et al. Biogenesis of the demarcation membrane system (DMS) in megakaryocytes. *Blood*. 2014;123(6):921-930. doi:10.1182/blood-2013-03-492330

203. Ishikawa G, Fujiwara N, Hirschfield H, et al. Shared and Tissue-Specific Expression Signatures between Bone Marrow from Primary Myelofibrosis and Essential Thrombocythemia. *Exp Hematol*. 2019;79:16-25.e3. doi:10.1016/j.exphem.2019.10.001
204. Fu J, Wang T, Xiao X. A novel PHKA2 mutation in a Chinese child with glycogen storage disease type IXa: a case report and literature review. *BMC Med Genet*. 2019;20(1):56. doi:10.1186/s12881-019-0789-8
205. Burwinkel B, Bakker HD, Herschkovitz E, Moses SW, Shin YS, Kilimann MW. Mutations in the Liver Glycogen Phosphorylase Gene (PYGL) Underlying Glycogenosis Type VI (Hers Disease). *The American Journal of Human Genetics*. 1998;62(4):785-791. doi:10.1086/301790
206. Wilson LH, Cho J, Estrella A, et al. Liver Glycogen Phosphorylase Deficiency Leads to Profibrogenic Phenotype in a Murine Model of Glycogen Storage Disease Type VI. *Hepatol Commun*. 2019;3(11):1544-1555. doi:10.1002/hep4.1426
207. Turnbull J, Tiberia E, Striano P, et al. Lafora disease. *Epileptic Disorders*. 2016;18(S2):38-62. doi:10.1684/epd.2016.0842
208. Beauchamp NJ, Dalton A, Ramaswami U, et al. Glycogen storage disease type IX: High variability in clinical phenotype. *Mol Genet Metab*. 2007;92(1-2):88-99. doi:10.1016/j.ymgme.2007.06.007
209. Rodríguez-Jiménez C, Santos-Simarro F, Campos-Barros Á, et al. A new variant in PHKA2 is associated with glycogen storage disease type IXa. *Mol Genet Metab Rep*. 2017;10:52-55. doi:10.1016/j.ymgmr.2017.01.003
210. Choi R, Park HD, Kang B, et al. PHKA2 mutation spectrum in Korean patients with glycogen storage disease type IX: prevalence of deletion mutations. *BMC Med Genet*. 2016;17(1):33. doi:10.1186/s12881-016-0295-1
211. Rao TN, Hansen N, Hilfiker J, et al. JAK2-mutant hematopoietic cells display metabolic alterations that can be targeted to treat myeloproliferative neoplasms. *Blood*. 2019;134(21):1832-1846. doi:10.1182/blood.2019000162
212. Genova ML, Pich MM, Bernacchia A, et al. The Mitochondrial Production of Reactive Oxygen Species in Relation to Aging and Pathology. *Ann N Y Acad Sci*. 2004;1011(1):86-100. doi:10.1196/annals.1293.010
213. Bjørn ME, Hasselbalch HC. The Role of Reactive Oxygen Species in Myelofibrosis and Related Neoplasms. *Mediators Inflamm*. 2015;2015:1-11. doi:10.1155/2015/648090
214. Giandomenico S Di, Kermani P, Mollé N, et al. Megakaryocyte TGFb1 partitions erythropoiesis into immature progenitor/stem cells and maturing precursors. *Blood*. 2020;136(9):1044-1054. doi:10.1182/blood.2019003276
215. Hay J, Shahzeidi S, Laurent G. Mechanisms of bleomycin-induced lung damage. *Arch Toxicol*. 1991;65(2):81-94. doi:10.1007/BF02034932
216. Cheng IY, Liu CC, Lin JH, et al. Particulate Matter Increases the Severity of Bleomycin-Induced Pulmonary Fibrosis through KC-Mediated Neutrophil Chemotaxis. *Int J Mol Sci*. 2019;21(1):227. doi:10.3390/ijms21010227
217. Varricchio L, Iancu-Rubin C, Upadhyaya B, et al. TGF-β1 protein trap AVID200 beneficially affects hematopoiesis and bone marrow fibrosis in myelofibrosis. *JCI Insight*. 2021;6(18). doi:10.1172/jci.insight.145651