Heterogeneity of the Bone Marrow Niche in Patients with Myeloproliferative Neoplasms: ActivinA Secretion by Mesenchymal Stromal Cells Correlates with the Degree of Marrow Fibrosis

Benedetta Rambaldi[1,2°], Elisa Diral[1,2†], Samantha Donsante[3], Noemi Di Marzo[1], Federica Mottadelli[1], Lucia Cardinale[1], Erica Dander[1], Giuseppe Isimbaldi[4§], Pietro Pioltelli[2], Andrea Biondi[5], Mara Riminucci[3], Giovanna D'Amico[1], Elena Maria Elli[2], Alice Pievani[1*], Marta Serafini[1*].

[1] Centro Ricerca M. Tettamanti, Department of Pediatrics, University of Milano-Bicocca, Monza, Italy

[2] Department of Hematology, San Gerardo Hospital, University of Milano-Bicocca, Monza, Italy

[3] Department of Molecular Medicine, Sapienza University, Rome, Italy

[4] Department of Pathology, San Gerardo Hospital, University of Milano-Bicocca, Monza, Italy

[5] Department of Pediatrics, Fondazione MBBM/San Gerardo Hospital, Monza, Italy

° Actual affiliation: Department of Medical Oncology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA, US

† Actual affiliation: Hematology Department, ASST Grande Ospedale Metropolitano Niguarda, Milan, Italy

§ Actual affiliation: Department of Pathology, ASST Grande Ospedale Metropolitano Niguarda, Milan, Italy

Corresponding Author:

Dr. Marta Serafini, PhD

Email: serafinim72@gmail.com

16-digit ORCID: 0000-0001-8105-6476

Co-corresponding Author:

Dr. Elena Elli, MD

Email: elena.elli@libero.it

*These authors share senior authorship

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Abstracts (250-250 words):

Mesenchymal stromal cells (MSCs) represent an essential component of the bone marrow (BM) niche and display disease-specific alterations in several myeloid malignancies. The aim of this work was to study possible MSC abnormalities in Philadelphia-negative myeloproliferative neoplasms (MPNs) in relationship to the degree of BM fibrosis. MSCs were isolated from BM of 6 healthy donors (HD) and of 23 MPN patients, classified in 3 groups according to the diagnosis and the grade of BM fibrosis: polycythemia vera and essential thrombocythemia (PV/ET), low fibrosis myelofibrosis (LF-MF) and high fibrosis MF (HF-MF). MSC cultures were successfully established from 21 of 23 MPN patients. MPN derived-MSCs did not exhibit any functional impairment in their adipogenic/osteogenic/chondrogenic differentiation potential and displayed a phenotype similar to HD derived-MSCs but with a decreased expression of CD146. All MPN-MSC lines were negative for the patient-specific hematopoietic clone mutations (JAK2, MPL, CALR). MSCs derived from HF-MF patients displayed a reduced clonogenic potential and a lower growth kinetic compared to MSCs from HD, LF-MF and PV/ET patients. mRNA levels of hematopoiesis regulatory molecules were unaffected in MSCs from HF-MF compared to HD. Finally, *in vitro* ActivinA secretion by MSCs was increased in HF-MF compared to LF-MF patients, in association with a lower hemoglobin value. Increased ActivinA expression on stromal cells and erythroid precursors was also observed in HF-MF BM biopsies. In conclusion, higher grade of BM fibrosis is associated with functional impairment of MSCs and the increased secretion of ActivinA may represent a suitable target for anemia treatment in MF patients.

Keywords (4-6): Myeloproliferative neoplasms, Myelofibrosis, Mesenchymal stromal cells, ActivinA

Declarations

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Conflicts of interest

The authors declare no conflict of interest.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of San Gerardo Hospital-Monza (Date: 31/10/2014; Protocol Code: BM-NICHE).

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent for publication

Not applicable.

Availability of data and materials

All data generated during this study are included in this published article.

Code availability

Not applicable.

Authors' contributions

BR, EME, AP, and MS designed the research. BR, ED, SD, FM, LC, and NDM performed experiments. BR, ED, GI, and EME collected patients' samples and clinical data. BR and AP wrote the manuscript. PP, AB, MR, GDA, EME, and MS revised the manuscript. All coauthors read and accepted the manuscript.

Introduction

Polycythemia vera (PV), essential thrombocythemia (ET) and myelofibrosis (MF) belong to the group of Philadelphia-negative myeloproliferative neoplasms (MPNs), characterized by an abnormal clonal proliferation of one or more hematopoietic cell lineages [1]. Despite the recurrence of similar driver mutations in JAK2, CALR and MPL genes [2], MPNs display a different clinical presentation and outcome. Indeed, differently from PV and ET, bone marrow (BM) failure characterizes the natural history of MF patients, significantly affecting quality of life and life expectancy [3].

The BM niche is composed of a fine network of regulatory signals and many different cell types, including mesenchymal stromal cells (MSCs), that play a pivotal role in maintaining the hematopoietic microenvironment and promoting stem cell homeostasis [4]. In MF, niche alterations such as BM fibrosis, osteosclerosis, neo-angiogenesis, extramedullary hematopoiesis, and abnormal cytokine production are involved in the pathogenesis of the disease progression [5–7]. MSCs contribute to the creation of pathologic microenvironment, sustaining the neoplastic hematopoietic stem cells (HSCs) and compromising normal hematopoiesis and HSC trafficking/homing [6]. Therefore, the expression patterns of adhesion molecules, extracellular matrix elements, growth factors, and chemokines regulating these processes may be distinct in MPN-MSCs compared with their normal counterparts. Even though MSCs isolated from MPN patients do not harbor MPN driver mutations [8], some studies have shown their phenotypic and functional impairment [9–11] and recently demonstrated their crucial role in the development of BM fibrosis [12, 13]. However, how the stage of the disease, expressed by the degree of BM fibrosis, influences MSC functions is not completely defined.

ActivinA is a pleiotropic cytokine belonging to the transforming growth factor (TGF)-β superfamily that is involved in multiple physiological and pathological processes, including inflammation, fibrosis, and regulation of erythropoiesis [14, 15]. Clinical trials based on trap molecules that interfere with the pathological hyperactivation of Activin signaling have been initiated

114 to treat ineffective erythropoiesis, including in MPN patients [16]. However, data on ActivinA
115 dysregulation in MPN BM niche are lacking.
116 The aim of this study was to characterize MSCs from MPN patients (MPN-MSCs), focusing
117 on the impact of the degree of BM fibrosis on MSC functions and their ActivinA production.
118

Methods

Patients

We enrolled 23 patients with MPNs undergoing a BM biopsy for clinical purpose and 6 age-matched healthy donors (HD). In compliance with the Helsinki Declaration and the Ethics Committee of San Gerardo Hospital-Monza approved the study, informed consent was obtained from all individuals. Distinct MPNs were diagnosed according to the 2016 WHO classification [1]. Patients were divided into distinct groups based on the diagnosis of MPNs (PV, ET and MF) and on the degree of BM fibrosis, defined according to the standardized assessment of BM fibrosis of the European Consensus [17]. Primary MF (PMF) and post-PV or post-ET MF were combined. Clinical characteristics were retrospectively collected in order to underline possible differences between groups.

Mesenchymal stromal cells isolation and culture

Patients BM fragments exceeding diagnostic purposes were digested using collagenase solution (3 mg/ml; Sigma-Aldrich, St. Louis, MO, USA), and repeatedly washed with PBS in order to collect mononuclear cells (MNCs). The digested cells were then filtered through a 70-μm nylon filter (Corning Incorporated–Life Science; Durham; USA). In the case of HD, we isolated MNCs with a Ficoll-Paque^{TM} Plus (GE Healthcare, Little Chalfont, Buckinghamshire, UK) density gradient separation from the washout of filter and empty collection bags left after allogeneic BM transplantation. MSCs isolated from trabecular bone by collagenase digestion are known to be virtually identical to their marrow aspirate counterparts [18]. MNCs were cultured at a density of $2x10^5$ cells/cm^2 in complete culture medium: DMEM-Low glucose (1 g/L; Gibco^{TM}, Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS) (Biosera, Ringmer, UK), 50 IU/mL Penicillin and 50 μg/ml Streptomycin (EuroClone, Milan, Italy) and 2 mM

144 L-glutamine (EuroClone). The growth medium was replaced every 3 days and the cells were
145 trypsinized when culture confluence reached 70%.
146
147 **Flow cytometry analysis**
148 MSC phenotype was analyzed at passage 3, staining cells with phycoerythrin- (PE) or
149 fluorescein isothiocyanate- (FITC) conjugated monoclonal antibodies specific for CD14 (clone
150 61D3; eBioscience, San Diego, CA, USA), CD34 (clone 581; BD Biosciences, Franklin Lakes, NJ,
151 USA), CD45 (clone HI30; BD Biosciences), CD90 (clone 5E10; eBioscience), CD73 (clone AD2;
152 BD Biosciences), CD105 (clone SN6; eBioscence), CD146 (clone P1H12; BD Bioscences), HLA-
153 ABC (HLA-I, clone G46-2.6; BD Biosciences), and HLA-DR (HLA-II, clone G46-6; BD
154 Biosciences). Unstained MSCs were used as negative controls to assess background fluorescence.
155 Analyses were performed using a FACS Canto II instrument with FACS DIVA software (BD
156 Biosciences, San Josè, CA, USA).
157
158 **Mesodermal lineages differentiation**
159 To assess *osteogenic differentiation* capacity, MSCs at passage 3 were seeded at a density of
160 $6x10^4$ cells/cm^2 in 6 well plates in basal medium. After 24 hours, the medium was removed and
161 substituted by Osteogenic Induction Medium, consisting of complete DMEM-Low glucose
162 supplemented with 100 nM dexamethasone (Sigma-Aldrich), 10 mM B-glycerol-phosphate (Sigma-
163 Aldrich), and 50 µM L-ascorbic acid 2-phosphate (Sigma-Aldrich). The osteogenic differentiation
164 was assessed on day 21 by staining cell culture with Alizarin Red Solution (Sigma-Aldrich).
165 For *adipogenic differentiation*, MSCs at passage 3 were seeded at a density of $2x10^5$ cells/cm^2
166 in basal medium. After 24 hours, medium was switched to Adipogenic Induction Medium, consisting
167 of complete DMEM-High glucose (4.5 g/L, Gibco) supplemented with 1 µM dexamethasone (Sigma-
168 Aldrich), 1 µM indomethacin (Sigma-Aldrich), 500 µm 3-isobutyl-1-methylxantine (IBMX; Sigma-
169 Aldrich), and 10 µg/ml human recombinant insulin (Sigma-Aldrich). Differentiation assessment was

performed on day 21, by staining of intracellular lipid droplets with Oil Red O Solution (Sigma-Aldrich).

In order to perform *chondrogenic differentiation*, MSCs at passage 2 or 3 were cultured for 3 weeks using a pellet culture system in 15 ml conical tubes at a density of $3x10^5$ cells/tube in Chondrogenic Differentiation Medium consisting of DMEM-High glucose supplemented with Penicillin-Streptomycin, L-glutamine, ITS™ Premix (BD Biosciences), 1 mM sodium pyruvate (Gibco), 50 μg/ml L-ascorbic acid 2-phosphate (Sigma-Aldrich), 0.1 μM dexamethasone (Sigma-Aldrich), 0.1 mM Non-essential Amino Acid solution (Gibco), and 10 ng/ml Transforming Growth Factor (TGF)-β1 (R&D Systems, Minneapolis, MN, USA). At the end of the culture period, cartilage pellets were fixed with 4% formaldehyde in PBS pH 7.4 and routinely processed for paraffin embedding. Four-micron thick paraffin sections were stained with Hematoxylin and Eosin (H/E).

Mesenchymal stromal cell mutational status

An allele-specific PCR assay was used for the detection of JAK2V617F mutation [19]. A preliminary screening of MPL exon 10 (W515L/K/R/A and S505N) was performed with HRM (High Resolution Melting) analysis and subsequent Sanger sequencing [20]. Evaluation of mutations in exon 9 of the CALR gene was performed by PCR and direct Sanger sequencing [21].

Quantitative RT-PCR (Q-RT-PCR) Analysis

Total RNA was extracted from undifferentiated or differentiated cells using TRIzol™ reagent (Invitrogen™, Thermo Fisher Scientific) according to manufacturer's protocol. 1 μg of RNA was reversely transcribed using the SuperScript II Reverse Transcriptase (Invitrogen™, Thermo Fisher Scientific). The cDNA was amplified for specific targets using TaqMan assays on ABI 7900 Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA). The TaqMan probes used are listed in Supplementary Table 1. As reference, housekeeping gene glyceraldehyde 3-phosphate

dehydrogenase (GAPDH) was used. Gene expression relative to GAPDH was quantified by the $2^{-\Delta\Delta Ct}$ method.

Colony-forming unit-fibroblast (CFU-F) assay

To assess the number of clonogenic progenitors, cells harvested at passage 0 were seeded at clonal density (1.6 cells/cm^2) and maintained for 14 days in basal medium. To enumerate CFU-F, the cells were fixed with methanol, stained with Giemsa solution (Merck KGaA, Darmstadt, Germany) and scored. The experiment was performed in triplicate for each sample. The clonogenic efficiency was calculated as the number of colonies per 100 initially seeded cells.

Population doubling assay

The population doublings (PD) were calculated for each MSC sample using the following equation: $PD_n = PD_{n-1} + [\log(C1/C0)]/\log 2$, wherein C0: cells number initially seeded and C1: cells number harvested. The PDs of cells from P2 to P9 were determined. Three sets of cultures were repeated for each sample.

ELISA assay for quantification of ActivinA and CXCL12

MSCs were cultured in DMEM-Low glucose 2% FBS for 72 hours. Culture supernatants were harvested and tested for ActivinA and CXCL12 levels using commercially available ELISA kits (R&D Systems), according to the manufacturer's instructions.

ActivinA immunoistochemistry

For ActivinA detection, paraffin-embedded sections from BM biopsies of MPN patients were incubated with a polyclonal goat anti-ActivinA antibody (1:..., AF338, R&D Systems) overnight at +4°C. Detection of binding was performed by…..

The color reaction was developed using 3,3′-diaminobenzidine tetrahydrochloride (DAB, Vector).

Statistical analysis

Data were analysed using GraphPad Prism (GraphPad Software, LA Jolla, CA, USA). Continuous variables were described by median and range values. Hypothesis testing on equality of medians was performed by 2-sided Mann-Whitney test for comparisons between two groups and Kruskal-Wallis analysis of variance for comparisons between more than two groups.

Results

Patient characteristics and MSC isolation

The median age at the time of cell collection was 54 years (range 38-77) for patients and 51 years (range 44-58) for HD (p=0.145). We identified 3 different categories of patients for our analysis: PV/ET, low fibrosis (grade 0-1) MF (LF-MF) and high fibrosis (grade 2-3) MF (HF-MF), respectively formed by 11, 4 and 8 patients. Patient characteristics at the time of the BM biopsy are shown in Table 1. The three groups differed in the median hemoglobin level (p=0.0003), lactic dehydrogenase level (LDH, p=0.03), and spleen size (p=0.03). Specifically, hemoglobin level was significantly lower in HF-MF group compared to both LF-MF (p=0.002) and PV/ET (p<0.0001). LDH concentration in HF-MF was significantly higher than PV/ET (p=0.0125). Spleen size in PV/ET was significantly lower compared to MF irrespectively to the grade of fibrosis (p=0.0356 for HF-MF and p=0.0125 for LF-MF). All these statistical differences between specific groups are in alignment with the typical clinical characteristics of the diseases. All patients were free from cytoreductive therapy at the time of cell collection and none received JAK inhibitors or erythropoiesis-stimulating agents (ESAs). Of note, 3 patients from the HF-MF group and 1 patient from LF-MF group were previously treated with cytoreductive therapy (hydroxyurea).

MPN-MSCs were isolated starting from MNCs collected in the supernatant fraction obtained by collagenase digestion of the BM biopsy. Collagenase pretreatment followed by extensive washing

247 results in release of hematopoietic cells located around the trabecula and of surface-adherent cells
248 and all soft-connective tissue elements from trabecular bone fragment. An inferior number of MNCs
249 was harvested from biopsies of patients with HF-MF (median 0.70 x 10^6cells/mm, range 0.23-2.88)
250 compared to those with LF-MF (median 2.27 x 10^6cells/mm, range 1.75-3.18, p=0.028). No
251 statistical difference was observed between HF-MF and PV/ET (median 1.44 x 10^6cells/mm, range
252 0.40-2.78, p=0.429) and between LF-MF and PV/ET (p=0.131). We were able to obtain MSC lines
253 from all patients except for 1 HF-MF and 1 PV/ET patient.
254
255 ***In vitro* characterization of MPN-MSCs**
256 To determine whether cells isolated from MPN patients were bona fide MSCs, we compared
257 them against MSCs derived from the BM of HD using standardized criteria outlined by the
258 International Society for Cellular Therapy (ISCT) [22]. In appearance, they were all plastic adherent
259 and showed the classic spindle-shaped, elongated morphology. However, some MSCs isolated from
260 MF patients presented a tendency of increased intracytoplasmic inclusions (Fig. 1a). MPN-MSCs
261 expressed high levels of common MSC markers including CD90, CD105, and CD73 and were
262 negative for hematopoietic markers (CD34, CD14, CD45, HLA-DR), similar to HD-MSC (Fig. 1b
263 and data not shown). Of note, the proportion of $CD146^+$ cells, characterized by the capacity to transfer
264 the hematopoietic microenvironment to heterotopic sites upon transplantation [23], was lower in
265 MPN-MSCs (median 46.80%, range 9.50-79.40) than HD (median 75.45%, range 43-91, p=0.011).
266 The same trend was confirmed by the quantification of the CD146 MFI (Fig. 1b).
267 Nineteen MPN-MSC lines were tested for the capacity to differentiate *versus* the adipogenic
268 and osteogenic lineages after 3 weeks of culture with an appropriate induction medium and all
269 samples achieved the specific differentiation, as highlighted with Oil-red-O and Alizarin red positive
270 staining, respectively (Fig. 1c-e). Only one MPN-MSC line failed the osteogenic differentiation. No
271 significant differences were detected in the intensity of differentiation-specific staining in MPN-
272 MSCs in comparison with the staining in HD-MSCs. To better quantify the differentiation efficiency,

we evaluated the expression of osteogenic and adipogenic pivotal genes, before and after MSCs differentiation induction, by Q-RT-PCR. As expected, we found that *FABP4, LPL,* and *PPARG* expression levels were effectively increased in MSCs after adipogenic differentiation, and *RUNX2, ALPL* after osteogenic differentiation, but no significant differences in induction level were detected between MPN and HD groups (Fig. 1d-f). For *PPARG* we noted a significantly increased up-regulation after adipogenic induction in the MPN- compared to the HD-MSCs (median 2^{-DDCt}: 34.65 *vs*. 8.96 respectively; p=0.033). We also evaluated the expression levels of differentiation master genes in MPN-MSCs under non-differentiating culture conditions and did not find any significant differences compared to control (data not shown). Fourteen samples out of 15 tested were able to differentiate into cartilage after 3 weeks of culture as pellets in chondrogenic conditions, as shown by histomorphology and up-regulation of mRNA levels of chondrogenesis markers *COL2A1, COL10A1, SOX9,* and *ACAN*. No differences were noted between MPN and HD groups (Fig. 1g-h). Overall, these data demonstrate that bona fide MSC culture can be successfully derived from MPN patients.

Finally, all MSC lines were tested for the presence of the concomitant HSC driver mutations (JAK2, CALR, MPL) but none harbored them.

The degree of BM fibrosis is associated with impairment in growth kinetic and clonogenic potential of MPN-MSCs

Next, we assessed whether MPN-MSCs have a similar proliferative potential to HD-MSC and, specifically, whether the type of disease (MF or PV/ET) or the grade of BM fibrosis could impact clonogenic potential and growth rate of these cells. MSCs from all groups (HF-MF, LF-MF, and PV/ET) formed discrete fibroblast colony-forming units and the median number of CFU-F per 10^2 plated cells was 10.70 (range 0-14.50) for HF-MF group, significantly reduced compared to the low fibrosis groups LF-MF and PV/ET (33.35, range: 9.30-59.30; p=0.002) and HD (32.50, 25.30-52.00; p=0.001) (Fig. 2a). Moreover, within the MF group there was a significant difference between HF-

MF and LF-MF (p=0.042). MSCs from HF-MF also displayed a reduced proliferation rate compared to the other groups in a cumulative population-doubling assay. In particular, the median cumulative population-doubling at passage 5 (CPD5) was 5.78 (range: 0.43-8.14) for HF-MF, significantly lower compared to the low fibrosis groups (8.01, range: 5.66-10.26; p=0.006) and HD (8.71, 6.90-9.46; p=0.014) (Fig. 2b). Thus, these data indicated that MPN-MSCs, and especially those isolated from HF-MF patients, presented intrinsic clonogenic and growing defects.

Expression of hematopoietic niche regulatory genes is preserved in MSCs from HF-MF patients

Circulating $CD34^+$ cells are increased in MF patients with BM fibrosis and myeloid metaplasia [24, 25]. To assess the potential role of MSCs in the pathogenesis of the increased HSC trafficking, MSCs from HF-MF patients were analyzed for their basal expression of several cell-bound as well as secreted factors governing the hematopoiesis and their retention within the niche [26]. mRNA levels of hematopoiesis regulatory molecules such as CXCL12, VCAM1, ANGPT1, KITLG, SPP1, and JAG1 were unaffected in MSCs from HF-MF (n=3) compared to HD (n=10) (Fig. 3). In particular, we did not observe any significant difference in basal secreted levels of the stem cell homing chemokine CXCL12 between HF-MF and HD-MSCs (HF-MF: median 680.40 pg/ml, range 602.11-1117.55, n=3 *vs.* HD median 474.90 pg/ml, range 225.53-678.40, n=4, p=0.114).

Increased production of ActivinA by MSCs from HF-MF patients

ActivinA was shown to modulate both fibrosis and regulate erythropoiesis [15], representing a suitable target for the treatment of anemia in MPN patients. Since ActivinA was secreted by MSCs isolated from BM of both patients and HD [27, 28], we evaluated its concentration into the media of cultured MSCs from MPN patients with different grade of BM fibrosis and HD. We observed an increased production of ActivinA by MSCs from HF-MF patients with a median of 1351.34 pg/ml (range 146.67-3055.90) compared to the LF-MF group with a median of 62.50 pg/ml (range 62.50-130.42, p=0.029). Although not statistically significant, a similar trend was also observed in

comparison to the LF groups (median 130.40 pg/ml, range 62.50-383.80) and HD group (median 99.86 pg/ml, range 62.50-298.10) (Fig. 4a). Furthermore, ActivinA levels were significantly higher in CALR mutated MPN patients (n=3, all HF-MF) compared with those with the JAK2 mutation (n=9, 1 HF-MF, 4 LF-MF and 4 PV/ET) (median 2465 pg/ml, range: 237.3 – 2465 *vs.* median 130.4 pg/ml, range: 62.5 – 383.8 respectively, p=0.0199). To confirm the ActivinA overexpression *in vivo*, we performed immunohistochemistry on BM biopsies from MPN patients with different degree of BM fibrosis. Of note, HF-MF patients have shown a higher ActivinA expression than LF-MF patients on extracellular matrix and on both stromal cells and erythroid precursors (Fig 4b).

Even if a statistically significant negative correlation between ActivinA and hemoglobin levels was not observed, HF-MF patients displayed significantly lower hemoglobin levels compared to both LF-MF (p=0.029) and low fibrosis groups (p=0.003) at the time of BM biopsy (Fig. 4c). These data may represent a link between increased ActivinA production by MSCs and low hemoglobin levels in HF-MF patients.

Discussion

MPNs represent a continuous spectrum of disease that ultimately ends in the development of BM fibrosis and subsequently BM failure. MSCs play a key role in the regulation of the BM niche, and preliminary data showed their involvement in the pathogenesis of BM fibrosis in MPNs [5, 6]. In this work, we confirmed the biological impairment of distinct properties of MSCs isolated from MPN patients, highlighting that these alterations occurred specifically in MSCs derived from HF-MF patients and are stably maintained *ex vivo* in the absence of the neoplastic clone. Furthermore, we reported an increased ActivinA secretion by MSCs from HF-MF patients, pointing out a potential additional new mechanism of ineffective erythropoiesis that frequently occurs in patients with myelofibrosis.

Bona fide MSC cultures were successfully established from the BM of ~90% of MPN patients, irrespective of disease/fibrosis subgroup. MPN-MSCs display a similar morphology and phenotype compared to those isolated from HD, with the exception of the decreased expression of CD146. Melanoma cell adhesion molecule (MCAM)/CD146 is expressed by a subpopulation of BM human stromal cells that have an active role in the establishment of the HSC niche [23]. Although a similar lower CD146 expression on *in vitro* cultured MPN-MSCs was previously reported [9], an increase of $CD146^+$ cells was found by immunohistochemical analysis in BM of PMF patients, in accordance with the extent of fibrosis and microvascular density [29]. Therefore, the CD146 expression on cultured MSCs may not necessarily reflect the *in vivo* situation on primary MSCs in the BM. Moreover, the expression of CD146 on primary MSCs is downregulated under hypoxic conditions and can vary depending on their *in situ* localization, with decreasing CD146 expression in the endosteal compared to the perivascular niche [30].

MPN-MSCs do not exhibit any functional impairment in their adipogenic/osteogenic/chondrogenic differentiation potential. To the contrary, Martinaud et al reported an increased osteogenic differentiation capacity of MSCs isolated from PMF patients [11],

while Avanzini et al reported a lower osteogenic ability in a cohort of MPNs [9]. The heterogeneity of patient population may account for the differing observations across the studies.

MSCs derived from HF-MF patients showed intrinsic differences compared to those from other MPN subgroups and HD, including a lower clonogenic potential and reduced proliferation capacity. In contrast, MSCs derived from LF-MF patients were more similar to the normal counterpart, suggesting that progressive MSC alterations may occur during MPN evolution and influence the disease behavior. Considering the older age of the entire cohort (median age >50 y) and the almost uniform distribution of age between specific disease groups, it is reasonable to believe that these differences in MSC features may be only slightly influenced by specific patient age. Although these functional alterations may be considered a reactive counterpart to the pro-inflammatory cytokines production by the neoplastic clone [31], they persist *in vitro* in the absence of any stimulation by hematopoietic cells. The impaired biology of MSCs obtained from HF-MF patients may bear some important clinical implications. Indeed, our results may explain the remarkable difficulties faced when an allogeneic transplant procedure is performed in MF patients with an advanced grade of marrow fibrosis, particularly in terms of early and late poor marrow function [32].

It was previously reported that MPN-MSCs show altered hematopoiesis-supportive capacity [12]. Therefore, we looked at whether the expression of genes involved in hematopoietic niche regulation was altered in MSCs isolated from HF-MF, but no difference was observed compared to HD. Ramos and colleagues reported overexpression of SPP1 and NF-kB and downregulation of ANGPT1 and THPO [33], but their analysis was limited to patients with PV and ET. Patients affected by HF-MF frequently experience BM failure with concomitant myeloid metaplasia and increased $CD34^+$ circulating cells [24, 25]. CXCL12/CCR4 axis is important for the regulation of HSC homing [34]. Despite an increased plasma level and BM deposition of CXCL12 were described in MF patients [35], our findings reveal no difference in the CXCL12 mRNA levels and protein secretion between MSCs from HF-MF and HD. Abnormal HSC trafficking/homing in MF patients may be due to

reduced expression of CXCR4 on HSCs through promoter hypermethylation and disfunction of CXCR4/CXCL12 axis by enhanced proteolysis [36–38].

ActivinA belongs to the TGF-β superfamily and has been demonstrated to increase in certain inflammatory conditions, such as septicemia, inflammatory bowel disease, and rheumatoid arthritis [39]. We hypothesized that the high inflammatory milieu of MF [31] could promote overproduction of ActivinA by MSCs, similarly to what has been described for TGF- β1 [11]. Although evaluated on a small cohort of patients, we observed an increased secretion of ActivinA by MSCs isolated from HF-MF compared to LF-MF, in association with a significantly lower hemoglobin value. Regarding the specific mutational status, ActivinA levels were significantly higher in CALR mutated compared to JAK2 mutated MPN patients. However, it is difficult to dissect the role of driver mutation from the role of the specific disease. The overexpression of ActivinA in HF-MF patients was further validated *in vivo* in BM biopsies, showing a stronger positivity than LF counterpart. ActivinA has already been implicated in the process associated with fibrosis, specifically in lung, kidney and liver [14], but its actual role in the pathogenesis of BM fibrosis is not yet completely defined. Activation of non-canonical TGF-b1 signaling, mediated by ERK and p38, was reported in BM and spleen samples of patients with MF [40]. Interestingly, ActivinA can induce the same non-canonical pathway [41].

ActivinA may be implicated in the modulation of erythropoiesis through a paracrine control in the BM microenvironment. However, the mechanism by which ActivinA influences erythropoiesis under physiological conditions remains unclear as controversial data related to its functions exist [42, 43]. Notably, increased levels of ActivinA correlate with defective erythropoiesis in patients with thalassemia [44]. Specifically, Activin receptor ligand traps are novel molecules that bind activins and ameliorate anemia in different diseases [16, 45]. Sotatercept (ACE-011), an Activin receptor ligand traps with affinity to ActivinA, can promote erythropoiesis indirectly by binding type II Activin receptor (ActRIIA) ligands produced by stromal cells [46]. Sotatercept was tested in a phase 2 trial in MF patients and showed a promising erythroid response, with good tolerance [47]. With the

limitation of a small sample size, our findings provide biological insight for the use of Activin receptor ligand traps for the treatment of anemia in MF patients. In addition, ActivinA may have a role in the abnormal HSC trafficking/homing in HF-MF, since it has been correlated with impairment of $CD34^+$ cells migration towards CXCL12 gradient [27].

In conclusion, our data indicate that a higher grade of BM fibrosis is associated with an impairment in distinctive biological characteristics of MPN-MSCs, including growth kinetics and clonogenic potential. Although additional studies are needed to confirm this finding in a larger cohort of patients, the increased production of ActivinA by MSCs from HF-MF patients further supports the use of Activin receptor ligand traps for the treatment of anemia in myelofibrosis.

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Figure legend:

Fig. 1 *In vitro* characterization of MPN-MSCs

A) Morphology of MPN- and HD-MSCs assessed at passage 0 of culture. Magnification 20x (top) and 40x (bottom). B) Comparative surface antigenic profiling of MSCs derived from MPN patients (n=20) and HD (n=6) analyzed by flow cytometry. C) Adipogenic differentiation of MPN- and HD-MSCs detected by Oil red O staining (HD n=6; MPN n=19). Magnification 20x. D) Q-RT-PCR analysis of adipogenesis-related genes in differentiated MSCs: fatty acid binding protein 4 (*FABP4*), lipoprotein lipase (*LPL*) and peroxisome proliferator-activated receptor gamma (*PPARG*), (HD n=3; MPN n=7). Data were referred to undifferentiated MSCs (dashed line). E) Osteogenic differentiation of MPN- and HD-MSCs detected by Alizarin Red S staining (HD n=6; MPN n=19). Magnification 20x. F) Q-RT-PCR analysis of osteogenesis-related genes in differentiated MSCs: Runt-Related Transcription Factor 2 (*RUNX2*) and alkaline phosphatase (*ALPL*) (HD n=3; MPN n=6). G) Chondrogenic differentiation of MPN- and HD-MSCs demonstrated by the presence of chondrocytes within lacunae in hematoxylin and eosin stained chondroid pellet sections (HD n=6; MPN n=14). Magnification 4x. H) Q-RT-PCR analysis of chondrogenesis-related genes in differentiated MSCs: type II collagen (*COL2A1*), type X collagen (*COL10A1*), SRY-box containing gene 9 (*SOX9*), and aggrecan (*ACAN*), (HD n=3; MPN n=5). Values are expressed as median and range; MPN-MSCs (grey columns) and HD-MSCs (white columns). * $p<0.05$ by 2-tailed Mann-Whitney test.

Fig. 2 Clonogenic and proliferation capacity of MPN-MSCs based on BM fibrosis

A) HF-MF-, LF-MF-, PV/ET- and HD-MSC clonogenic capacity estimated by colony-forming unit-fibroblast (CFU-F) assay and expressed as number of colonies per 1 x 10^2 initially seeded cells. B) HF-MF-, LF-MF-, PV/ET- and HD-MSC cumulative population doublings at passage five (CPD5). Each box plot shows the median and extends from the lowest to the highest value. * $p<0.05$, ** $p<0.01$ by 2-tailed Mann-Whitney test. High fibrosis (grade 2-3) myelofibrosis (HF-MF), low fibrosis (grade

596 0-1) myelofibrosis (LF-MF), and polycythemia vera/essential thrombocythemia (PV/ET),
597 respectively formed by 7, 4 and 10 patients; 6 healthy donors (HD).
598
599 **Fig. 3 Hematopoietic niche regulatory genes in MSCs from HF-MF patients**
600 C-X-C motif chemokine ligand 12 (*CXCL12*), vascular cell adhesion molecule 1 (*VCAM1*),
601 angiopoietin 1 (*ANGPT1*), KIT Ligand (*KITLG*), osteopontin (*SPP1*), and Jagged1 (*JAG1*) baseline
602 expression in HF-MF- (n=3) and HD-MSCs (n=10) evaluated by Q-RT-PCR. Expression levels for
603 each gene compared with the *GAPDH* housekeeping gene are shown. Each dot represents a single
604 patient and horizontal line represents the median. 2-tailed Mann-Whitney test.
605
606 **Fig. 4 ActivinA secretion from MPN-MSCs based on BM fibrosis and patient hemoglobin level**
607 **distribution**
608 A) After 72 hours of culture, supernatants of MSCs were collected and ActivinA concentration was
609 analyzed by ELISA. Results are displayed for each group: HD (n=5), HF-MF (n=4), LF-MF (n=4)
610 and PV/ET (n=5). B) Immunohistochemical staining of ActivinA in BM sections of MF patients
611 Representative....C) Distribution of the Hb level at the time of BM biopsy for each patient evaluated
612 for MSC ActivinA secretion. Each box plot shows the median and extends from the lowest to the
613 highest value.
614 * $p<0.05$, ** $p<0.01$ by 2-tailed Mann-Whitney test.