PhD *XXXV* cycle

Basic and Applied Biomedical Sciences

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Research project

"Lactate orchestrates tumor microenvironment remodeling and shapes pathogenetic profile in primary myelofibrosis"

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1. Background

1.1 Primary myelofibrosis

Primary myelofibrosis (PMF) or idiopathic myelofibrosis (IF) represents one of the myeloproliferative malignancies known as chronic myeloproliferative neoplasms (MPNs), which represents a heterogeneous family of tumors including chronic myeloid leukaemia (CML), polycythemia vera (PV) and essential thrombocythemia (ET) [1].

MPNs are rare diseases, characterized by a high incidence in 50-75 age range with an onset time increasing with advancing age [2].

The incidence does not depend on the geographic distribution and seems to be more common in males than in females [3-5].

PMF diagnosis, in order to distinguish it from other malignancies, can be challenging. Indeed, the WHO drew up the major diagnostic criteria including 1) bone marrow (BM) histology showing megakaryocytic atypia and marrow fibrosis, 2) a marker of clonal neoplasm or the absence of minor reticulin fibrosis due to another process and 3) clinical features not consistent with another MPN or a myeloid malignancy [6].

PMF is characterized by the clonal proliferation of myeloid cell line in the BM (**Figure 1A**), resulting in fibrotic accumulation leading to marrow failure; its onset is due to the clonal neoplastic transformation of a pluripotent hematopoietic stem cell and the subsequent proliferation of newly formed clones with cancer outbreak [7].

The pathogenetic involvement of a mutant clone is outlined by the hematopoietic monoclonal increase in patients with PMF in myeloid, lymphoid or erythroid lineage

suggesting a stem cell neoplastic outset in which a subpopulation shows a competitive expansion over the others [8].

A wide plethora of mutations is associated with PMF: among them, the most common mutations are related to Janus kinase 2 (JAK2) which is a tyrosine kinase involved in JAK-STAT pathway, crucial in biomechanisms like apoptosis, cell cycle and transduction signaling involving ERK/MAPK pathways [9].

Particularly, approximately the 60% of patients with PMF have the JAK*2V617F* mutation, which results more commonly reported in exon 14 rather than exon 12 [9-11]; JAK2*V617F* is due to the substitution of a phenylalanine with a valine at codon 617 of JAK2 gene included in 9pLOH chromosome region [12].

Furthermore, JAK-STAT results constitutively activated following one or more different mutations and it constitutes one of the biomolecular backbones of genetic mechanisms strictly related to hematopoietic stem niche disruptions [13].

JAK-STAT constitutive activation is substantially due to the JAK triggering of aforementioned downstream signaling cascades including STATs, Ras-MAPK or PI3K-Akt [14-16].

Among the most common mutations, the CALR mutation in correspondence of exon 9, usually occurs in 30% of patients while the thrombopoietin receptor mutation (MPL) mutation has been reported in about 13% of PMF patients [17, 18].

JAK2, MPL and CALR occur in more than 90% of PMF cases and, taken together, represent a very clear example of how different mutations are strictly intercorrelated to obtain the same signaling imbalance: the JAK-STAT hyperactivation (**Figure 1B**).

In particular, CALR is an endoplasmic reticulum (ER) chaperone responsible of calcium storage and the proper protein folding activities.

Moreover, the mutant CALR interacts with MPL which is in turn an important modulator upstream to JAK2 [19, 20].

In physiological conditions MPL is involved in the interaction with thrombopoietin (TPO) through which plays a crucial role in the cell signaling, based on JAK-STAT activation, responsible of megakaryocytes (MKs) regulation as well as hematopoietic stem cells (HSCs) renewal.

Based on these considerations, mutations affecting both CALR and MPL, induce a vicious circle culminating in a strong imbalance of MPL-JAK-STAT axis, which importance was also confirmed by many data showing its involvement in symptoms like fibrosis, anemia or MKs hyperplasia [21, 22].

In addition, among the genetic disorders associated to PMF, several non-JAK2 clonal markers have been selected including epigenetic modifying factors (ASXL1 [23], TET2 [24]), DNA damage repair mechanisms (ATM), signaling regulators (SH2B3, CBL) [25-28].

The occurrence of a mutation or the combination of many of them can affect patient's prognosis representing a predictive factor of the disease course [17, 29].

In the pathological context of PMF, BM results very compromised, showing atypical cell populations and deep functional imbalances involving collagen and fibrotic depositions, thickening and weakening of bones structure, proliferative alterations of several cell lineages inducing leukoerithroblastosis, cytopenia and megakaryocyte hyperplasia [30-32].

Specifically, abnormal immature MKs remain one of the most remarkable features of PMF, occurring in clusters and releasing inflammatory cytokines [33].

Moreover, MKs abnormalities usually result in fibrotic depositions as secondary phenomenon induced by improved TGFβ and MMPs release [34, 35].

Although the comprehension of the PMF pathogenesis is still unclear, it has been established that its severity is mostly attributed to its complex clinical course, notably marked by deep BM changes involving pervasive stroma disruptions inducing fibrosis, neoangiogenesis and osteosclerosis [36].

BM fibrosis represents one of the most crucial pathological expressions of PMF.

Fibrotic depositions are mainly mediated by the progressive accumulation of reticulin fibers, collagen types (I-V) and glycoproteins (fibronectin, tenascin).

All these events are associated to many profibrotic stimuli related both to factors improving extracellular matrix (ECM) accumulation and concurrent factors inhibiting ECM degradation.

In this regard, bone marrow niche is characterized by an enduring inflammatory state which contributes to extramedullary hematopoiesis and consequent typical symptoms as hepato-splenomegaly and lympho-adenomegaly [37].

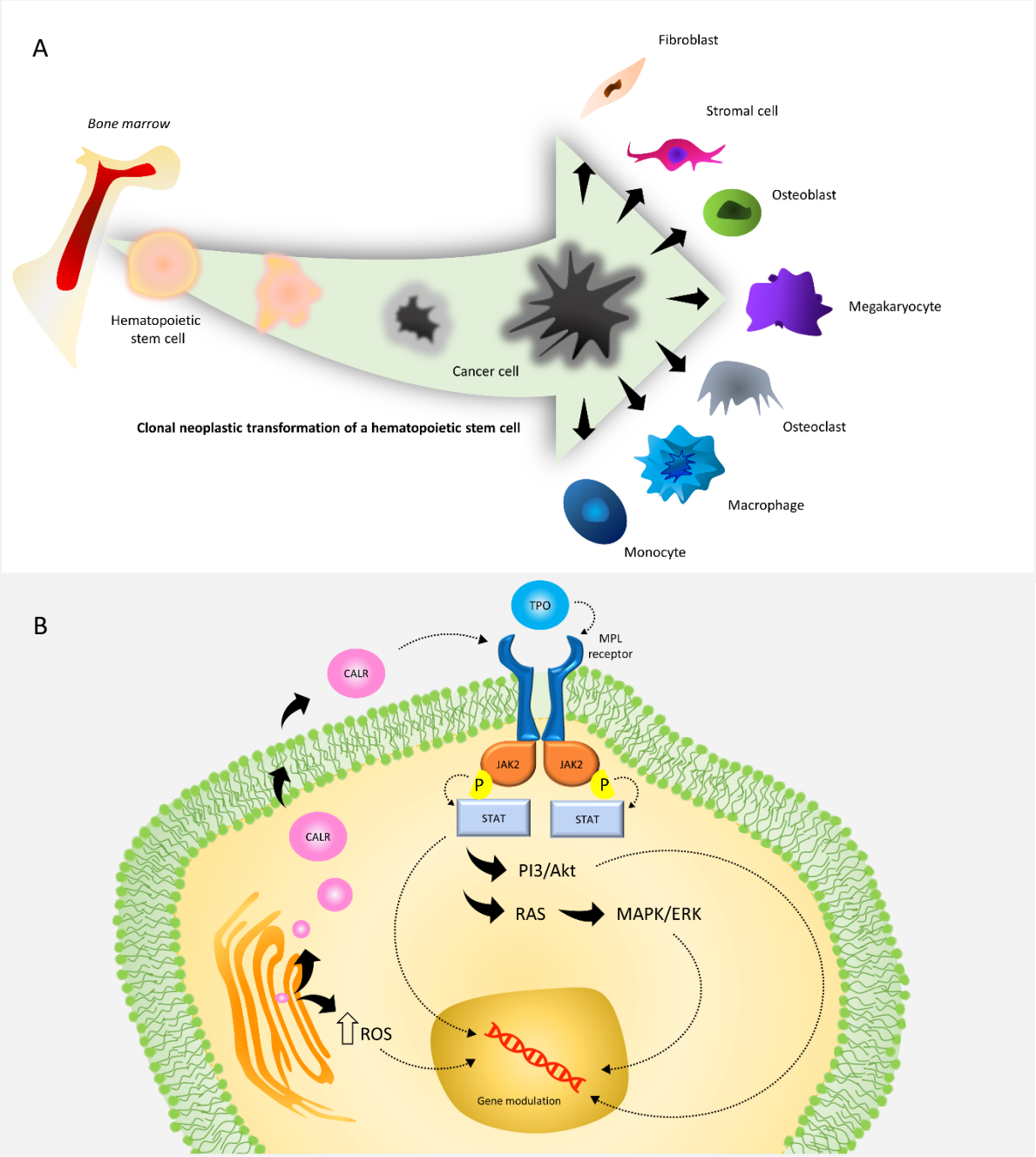


Figure 1 **Primary myelofibrosis**

A. Scheme of PMF neoplastic expansion
The neoplastic clone in the hematopoietic niche can potentially affect one of the many cell subpopulations constituting the BM environment.

B. Biomolecular mechanisms of PMF pathogenesis and the crucial role of JAK-STAT axis
The constitutive activation of JAK STAT axis represents one of the most responsible biochemical alterations affecting onset of PMF shared by the most common mutations associated to the disease (JAK2, MPL, CALR).
In particular, mutated CALR, not retained by endoplasmic reticulum is responsible both for interfacing MPL receptor, eventually mutated too, and to induce the massive accumulation of misfolded protein with consequent increasing in ROS levels. One or the combination of more mutations contribute to activate constitutively JAK-STAT signaling, which produce genetic regulation involving different processes like cell proliferation or apoptosis. JAK STAT transductive pathway acts at different steps of the cascade, influencing cell processes related to the neoplastic expansion of the altered clone in haemopoietic niche.

1.2 Bone marrow: the delicate balance of TME niches

BM represents a heterogenous microenvironment in which physiological homeostasis between several subpopulations is based on the continuous crosstalk among different niches, in close communication throughout environmental signals, biomolecular factors and the vascular network.

The main process strictly related to humoral and cellular regulatory signals is hematopoiesis [38].

In this context, hematopoietic stem cells (HCSs) are defined by their ability to self-renew and to repopulate all blood-cell lineages including both lymphoid and myeloid cells in order to maintain the pool of mature blood cells [39, 40].

HSCs' distribution in BM is closely dependent on their physiological phase of differentiation and activity according to a perfect structural architecture in niches which allow the correct maintenance, self-renewal or mobilization of HSCs [41, 42].

Particularly, within this morphological and interactive architecture, HSCs are able to establish a crosstalk with the surrounding stroma microenvironment, involving different biomolecular signals which produce a very complex network of regulatory factors, through a close interaction with endosteal and vascular niches [36].

Quiescent HSCs are located on the endosteal surface, closely to bone-derived matrix, and their dynamic mobilization for consequent differentiation is tightly regulated by the balance between matrix components and factors released both by osteoblasts (angiopoietin-1, osteopontin, CXC-chemokine ligand 12 (CXCL-12) [43-45]), osteoclasts [46] and perivascular factors [47, 48], both essential for the proper niche function (**Figure 2**).

Osteoblastic and vascular compartments are characterized by a heterogenous group of cells such as hematopoietic cells, fibroblasts, osteoblasts and osteoclasts, adipocytes, stromal cells (vascular endothelial-cadherin-positive sinusoidal endothelial cells (SECs)), perivascular cells and mesenchymal stem cells (MSCs) [49]; in such a heterogeneous microenvironment, extracellular matrix (ECM) elements provide both mechanical and functional support [50, 51].

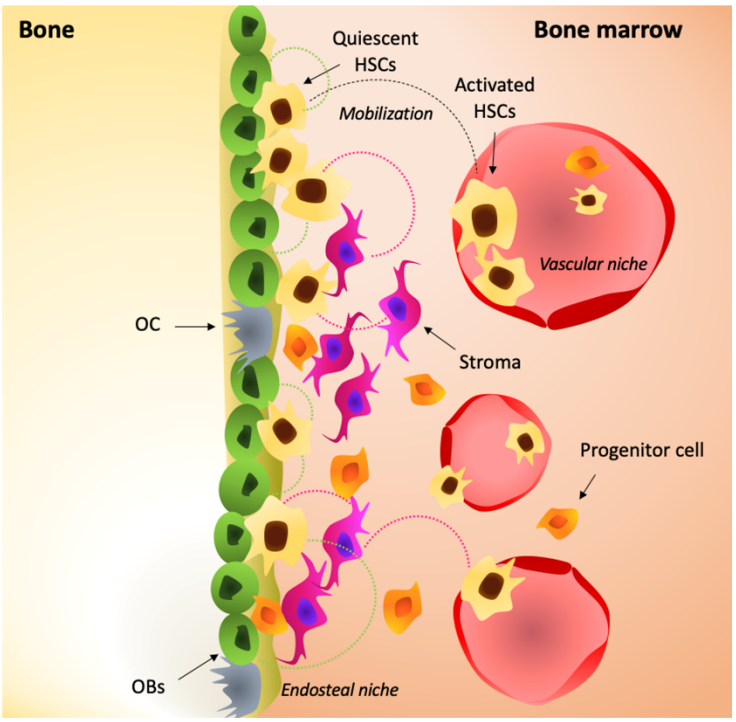


Figure 2 **Bone marrow stem niches**

Bone marrow HSCs are properly distributed in the region between the endosteal surface and the perivascular region depending on their level of activation/quiescence. HSCs mobilization produces a very dynamic environment which strongly depends on the proper setting of bone marrow interactive architecture.

Oppositely to endosteal niche, the vascular one consists in the close interaction between HSCs and highly specialized endothelial cells, known as bone-marrow sinusoidal endothelial cells (BMECs) [52].

Particularly, BMECs usually express surface and adhesion factors like CXCL12, E-selectin or vascular cell adhesion molecule 1 (VCAM1), showing their strong involvement in HSCs mobilization or homing and turnover [47, 53].

As a direct consequence of BMECs interaction, perivascular HSCs are freely exchanged in order to maintain the dynamic homeostasis of the entire hematopoietic environment [40].

In this complex and diversified biological context, PMF results in the inadequate communication between hematopoietic and stromal cells whose concept is perfectly depicted by Dr. Le Bousse-Kerdilès as "bad seeds in bad soil" [36].

This latter concept clarifies the role of the altered stroma "bad soil" that supports the clonal expansion of neoplastic hematopoietic cells "bad seeds" which influence in turn stromal niche, affecting its physiological functionality and producing an intricate vicious circle [54].

Consequently, the role of microenvironment, considered as an extremely sensitive network between different cell compartments, becomes essential to understand the biological mechanisms involved in the pathogenetic development of PMF.

In particular, the functional imbalance of stromal niche, represents the major factor inducing the clinical consequences in PMF, resulting by substantial bone marrow impairment due to multifactorial damage [51].

In this regard, as a consequence of the pathological upsurge, BM environment undergoes morphological and functional changes inducing abnormalities in granulocytes, megakaryocytes, osteoblasts and fibroblasts.

Microenvironmental disruptions couple with increased levels of several inflammatory cytokines, growth factors (b-FGF, VEGF, PDGF) in addition to ECM constituents including fibronectin, reticulin and collagens which all together culminate in the

development of bone marrow damage with a relevant inflammatory and profibrotic backdrop [33].

1.2 TME and the CAFs signature

Since many years, in the context of cancer research, a new paradigm has started to arise, focusing malignant tumor as a pathological condition of general and wide derangement in which neoplastic progression is strongly supported by the entire environment [55].

In this regard, the tumor microenvironment (TME) seems to play a crucial role in regulating most of the mismatches which constitute the dynamic matrix of cancer progression [56].

TME comprises a diversified group of cell types and factors ranging from immune and stromal cells to wider surrounding factors such as hypoxia or ECM setting [57-59].

In particular, it has been discussed that the cross-talk between cancer cells and stroma may result in the emergence of a specific fibroblasts subpopulation known as cancer-associated fibroblasts (CAFs) [60].

Many studies over the years have suggested that CAFs promotion is due to fibroblasts hyperactivation, related to the massive stimulation and tumor reprogramming handled by TME [61] of which mesenchymal stem cells (MSCs) represent an important CAFs source.

It is well known that cancer cell is able to shape the surrounding environment: in fact, it instructs MSCs and stroma in order to constitute a good scaffolding for tumor growth of which CAFs represent one of the most interesting cancer strategies [62].

For this reason, CAFs behave like synthetic engines and reactive part of stromal microenvironment, involved in the production of many tumor factors, supporting tumor environment and metastasis, regulating inflammation, remodelling ECM in order to fuel cancer progression [63-66].

CAFs phenotype seems to be characterized by the expression of different markers like fibroblast activated protein (FAP), fibroblast specific protein 1 (FSP1), α-smooth muscle actin (αSMA) or PDGF receptor-α/β (PDGFRα/β) [67].

CAFs proliferation is usually related to the stimulation by many different components related to a wound insult, including tumor growth factor β (TGF-β), interleukin-6 (IL-6) or platelet-derived growth factor (PDGF) [61, 68].

In this regard, tissue fibrosis and fibrotic accumulation perfectly fit with the concept of chronic insult, both chemical and mechanical, leading to a prolonged stress remodelling response enhancing hyperactivation of fibroblasts [69, 70].

Cancer fibrotic deposition results in the increased accumulation of collagens, fibronectin, laminins and many different ECM constituents with the concurrent recruitment of endothelial cells and immune cells in order to promote angiogenesis and inflammation *in situ* [61]; in this regard, activated fibroblasts are responsible for MMPs release [71].

Furthermore, CAFs proliferation is associated to increased invasiveness in cancer cells supported by enhanced deregulation of fundamental pathways related to Notch and p53 signaling and by mechanical alterations affecting ECM stiffness [72, 73].

In this way, ECM remodelling actively contributes to generation and maintenance of cancer cell niche in order to support cancer progression also promoting immune escape.

Particularly, metabolic CAFs adaptation may regulate the bioavailability of metabolites like arginine and tryptophan, involved in tumor immunity impairment [61, 74].

Immunomodulatory activities of CAFs are usually due to their propension to adopt a dynamic secretory phenotype, releasing cytokines and chemokines which function is strictly related to immunosuppression [75, 76].

In this regard, the literature puts the spotlight on molecules like osteopontin (OPN), upregulated in CAFs, involved in the regulation of alternative activation of monocytes through TGF-β and MCP1, exerting an immune escape role [77].

Moreover, it has been widely demonstrated that “cell-cell” interaction between cancer cell and CAFs triggers a pro-inflammatory profile characterized by the expression of many chemokines including CXCL1, CXCL2, CCL7 which are related to both myeloid and neutrophil recruitment [78].

Among CAFs secretome, factors like CXCL12/SDF1 and CCL5/RANTES or CXCL6/GCP are involved in cancer progression due to their chemotactic potential and activation of leucocytes while MCP-1/CCL2 expression is strictly associated to macrophage recruitment as well as immunosuppressive M2 phenotype polarization [79-81].

In the context of CAF and TME in general, TGF-β signaling is strongly involved both in BM fibrosis and specifically in CAF differentiation [82] following which TGF-β behaves as inducer of the immunosuppressive status.

Particularly, TGF-β overexpression in CAFs is related to natural killer (NK) activation and subsequent Th1 differentiation impairment [83].

Furthermore TGF-β affects dendritic cells (DCs) mobilization as well as cytotoxic T cells (CTLs) influencing T_{reg} proliferation, FOXP3 expression in CD8+ T cells and suppression of CD4+ T cell expansion [84-86].

1.3 Lactate: metabolite and signaling molecule

The role of lactate as waste metabolite and its involvement in energy metabolism has been reported and abundantly discussed for several years by scientific literature [87-89].

Lactate represents one of the main sources of energy involved in many energetic processes including gluconeogenesis and oxidative metabolism [90, 91].

It is well clarified that lactate is the product of pyruvate reduction catalyzed by the enzyme lactate dehydrogenase A (LDHA) both during aerobiosis or anaerobiosis in many different tissues [92].

Particularly, lactate production starts from glucose uptake by the cell through specific transporters (GLUTs); once inside it is sequestered as glucose-6-phosphate and undergoes the glycolytic process by which two molecules of pyruvate are produced.

The first option is for pyruvate to reach mitochondria proceeding through Krebs cycle after conversion to acetyl-CoA: in this case, the metabolic pathway is based on the coupling between glycolysis and OXPHOS.

The second one, usually associated to anaerobiosis, provides the pyruvate reduction to lactate by lactate dehydrogenase (LDH-5), in order to restore the anaerobic glycolysis by NADH oxidation into NAD^+ [93].

In order to mobilize lactate to different cellular district, cells are provided with specific lactate transporters, known as monocarboxylate transporters (MCTs) [94].

These channels transporters are principally localized on the plasma membrane, in which they allow the passage of lactate and pyruvate bidirectionally, depending on the concentration gradient of the substrates [95, 96].

Lactate trafficking is mediated also through mitochondria uptake following which lactate dehydrogenases (LDHs) provide to oxidize it [97, 98].

In addition, the stability and the proper functionality of MCTs require the mechanical interaction with chaperone glycoproteins CD147 or gp170 [99-101].

MCTs are channels encoded by a gene family known as SLC16 (solute carrier 16): it includes 14 members of which MCT1 and MCT4 seem to be the most representative in cancer disease [102].

In fact, several studies show MCTs upregulation in cancer cell lines as well as in human tumor samples [103-107].

Particularly, MCTs overexpression in human cancer is usually related to many of the most relevant pathways usually associated to tumor development: among them, Myc signaling, Wnt pathway, NF-kb and loss of function of p53 represent a considerable group of factors which proportionally correlate with MCTs expression and indirectly with the increased lactate efflux [108-110].

Looking at the importance of cell dynamism in pathological context of cancer, it is clear to understand how MCTs expression perfectly reflects cancer cell metabolic needs in terms of glycolytic rather than oxidative switch or vice versa.

Indeed, focusing on cancer microenvironment, it exists a commensalism and a metabolic symbiosis, supported by MCTs, between oxidative cancer cells, glycolytic cancer cells, and stromal cells [102].

In particular, the hypoxic cancer cell performs anaerobic glycolysis to survive, which implies having high levels of glucose available and producing lactate, mainly exported by MCT4 channels.

Analogously, the oxidative cancer cell is free to switch from a metabolic fuel to another one; particularly, it uses lactate provided by the nearby glycolytic cell, usually imported by

MCT1 and intended for tricarboxylic acids (TCA) cycle improving in turn glucose supplies for glycolytic cells.

In addition, based on the strong dynamic adaptation of microenvironment, oxidative cancer cells can force the host cells to adopt a glycolytic metabolism in order to support themselves as well as cancer growth and invasiveness [102].

1.4 Lactate and TME

The blood concentration of lactate goes from 2mM in physiological conditions to 30-40mM in neoplastic cells and generally in tumor microenvironment (TME) [111].

The high lactate concentration seems to be the result of lactate accumulation following the high rate of anaerobic glycolysis as well as the high glucose consumption by cancer cell and its distinguishing metabolic requirements [111].

The accumulation of high levels of lactate and protons in the extracellular environment, in the pathological context of cancer, induces a strong drop of pH identified as lactic acidosis [112], often associated to the relative metabolic switch of surrounding cells in TME [113, 114].

Cancer cell is identified as a highly proliferating cell, in which the substantial metabolic requirements exceed widely its ability to provide nutrients to fuel tumor growth [115].

In this regard, cancer cells need glucose as starting substrate to supply aerobic glycolysis as main metabolic process to generate ATP quickly [116].

Even if it is well established that oxidative phosphorylation would represent a more advantageous metabolic pathway, in terms of ATP molecules per glucose, glycolysis provides energy at a faster rate proportionally to glucose supplies [117].

The glycolytic switch, which involves the high rate of glucose uptake by the cells, represents a specific hallmark performed by tumor cell under normoxic conditions, well known as Warburg effect [118].

In this regard, even if glycolysis seems to be less efficient in terms of ATP production compared to OXPHOS, it represents the most advantageous chance due to its short duration allowing cells to generate many ATP molecules per unit time.

This metabolic choice seems to be related to the boost of anabolic pathways, often upregulated: in fact, glycolytic intermediates work as starting constituents to be hijacked to the main biosynthetic pathway as primary sources of carbon in order to promote macromolecules synthesis to feed and sustain cancer cell survival and proliferation [119, 120].

Consequently, lactate accumulation represents one of the following consequences due to cancer metabolic attitude: in particular, high concentrations of lactate are associated to metastatic progression [121-123] and poor prognosis in a wide variety of tumors [124].

In this context, lactate acts as energetic fuel for adjacent cancer cells that take advantage of OXPHOS [125] and as signaling molecule between cancer and stromal cells [126] in the sophisticated environment which constitutes TME.

Although for many years the literature has minimized the role of lactate, considering it a mere waste product related to cell metabolism [127], in recent decades many articles aim to focus its importance as signaling molecule, identified as a shuttle between oxidative and glycolytic cells and capable to fuel one at the expense of the other [90].

Moreover, the role of lactate has been further extended to signaling molecule involved in a wide plethora of cell modulations including paracrine, autocrine and endocrine effects [128, 129].

In this regard, during the last decade, the scientific literature brought attention to lactate receptor GPR81, also named HCA1 or HCAR1.

GPR81 is a cell-surface Gi type- G protein-coupled receptor highly expressed by adipose tissue and also found in intracellular organelles [130].

This receptor is involved in different metabolic processes, affecting the Gi signaling pathways like cAMP-PKA signaling, through adenylate cyclase (AC) inhibition, or ERK1/2 activation/phosphorylation, due to phospholipase C (PLC), phosphoinositol-3 phosphate kinase (PI3K) or phosphokinase C (PKC) promotion, inducing a cascade of biochemical events culminating with regulation of lipolysis, angiogenesis or osteoblast (OB) differentiation [131-133].

Furthermore, GPR81 behaves as a perfect metabolic sensor, inflammation mediator, but also a cancer promoter [134].

The high extracellular lactate levels in TME, and the subsequent interaction with GPR81, are strongly related to cancer growth, metastasis, angiogenesis and enhanced DNA repair [135-137].

Moreover, many data report the role of lactate in deregulation of immune surveillance system resulting in reduced IFN-γ production by infiltrating T cells, loss of natural killer cells (NKs) activation and tumor growth promotion [138, 139].

In addition, lactate accumulation has been related to improvement of toll-like receptor (TLR) 4-mediated signaling, and loss of proper dendritic cells (DCs) functionality [140]. The strong lactate impact on immune system represents biological evidence induced by the concurrent effect of lactate signaling in concert with TME acidity, which exerts many biological effects on a variety of immune populations [141, 142].

Indeed, low extracellular pH due to tumor acidosis leads to impaired T cells functionality and responsiveness, impaired macrophages polarization as well as wide deregulation of innate immunity populations [143, 144].

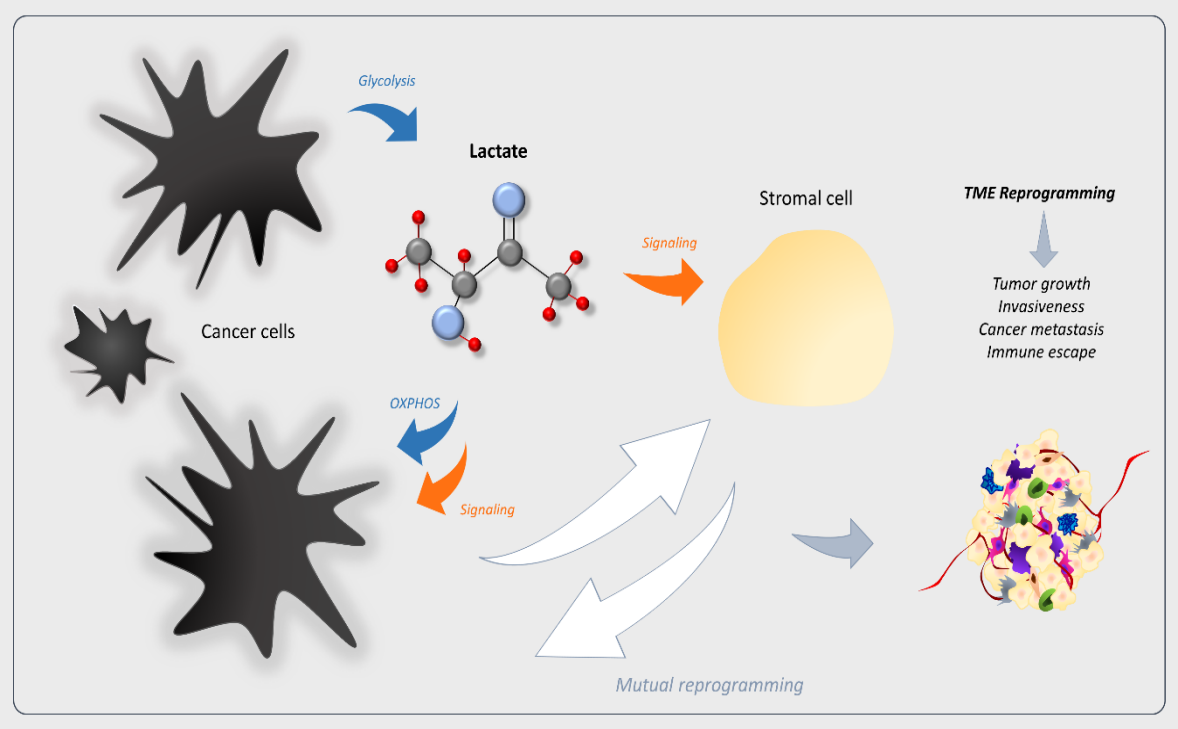


Figure 3 **Role of lactate in TME reprogramming**

Lactate represents an important factor included in metabolic pathway of cells which follow both a glycolytic and an OXPHOS (oxidative phosphorylation) metabolic switch. In this regard, cancer environment represents a very dynamic and flexible model of multiple interaction. In addition to the metabolic involvement, lactate is a signaling molecule which interaction probably affects crosstalk between cancer cells and stromal cells, constituting the core of a very sophisticated mechanism of mutual reprogramming responsible for TME shaping and cancer resistance.

2. Materials and methods

2.1 Cell cultures and treatments

Human bone-marrow stromal cell line HS-5 (ATCC CRL-11882™) was cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) by Gibco, supplemented with 10% of heat-inactivated fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (10,000 U/mL, ThermoFisher Scientific) and maintained at 37°C and 5% CO2. Cell treatments were performed using sodium L-Lactate by Sigma-Aldrich at a final concentration of 20mM, in order to simulate extracellular lactate acidosis condition in cancer.

Moreover, AZD3965 (SelleckChem) was used, as MCT1 channel inhibitor, at a final concentration of 10μM [145].

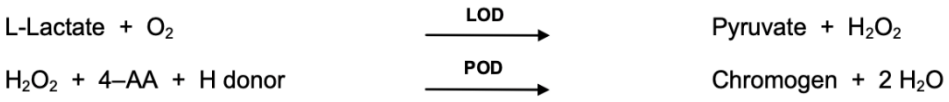
In order to evaluate if lactate functionality depends on its interaction with the hydroxycarboxylic acid receptor 1(HCAR1), the reference selective agonist 3,5-dihydroxybenzoic acid (3,5-DHBA) (Sigma-Aldrich) was used, at a concentration of 150μM [146].

In this regard, 3-hydroxy-butyrate acid (3-OBA) (Sigma-Aldrich) 3mM [146] was selected as HCAR1 signaling inhibitor.

2.2 Lactate quantification

Lactate quantification in sera samples was performed using a colorimetric assay based on a well-known reaction: in particular, lactate is oxidized to pyruvate and hydrogen peroxide by lactate oxidase (LOD). A purple product (quinonimine) is produced by the reaction of

peroxidase (POD), hydrogen peroxide, 4-aminoantipyrine (4-AAP) and a hydrogen donor (TOOS).



A standard curve was created using scalar concentrations of lactate and samples were diluted 1:10 with deionized water. Every mix contained 4-AAP 17mM, TOOS 15mM, POD 0.5U/L and TRIS-HCL pH8 1M.

Background absorbance (blank) was measured at 545nM using a spectrophotometer.

At this point, LOD 2.5mU/L, limiting enzyme, was added to each mix which was incubated for 30 min at room temperature.

The absorbance of each reaction mix, was read at 545nM and the lactate concentration for each sample was obtained by extrapolation of sample absorbance values from the calibration standard curve.

2.3 Osteoblastic differentiation

Human mesenchymal stem cells HS-5 were seeded at a concentration of 2000 cells per well in a 24-multiwell.

After 24 hours, cells were properly treated with Osteoblastic differentiation medium (OB) [147] consisting of 0.2 mM ascorbic acid, 0.1 mM dexamethasone and 10 mM β-glycerophosphate.

Medium was carefully renewed every 48 hours.

The differentiation lasted 10 days.

2.4 Patient specimens and PBMCs isolation

Whole peripheral blood was collected from 30 healthy donors (HC) and 30 patients (PMF) in EDTA vacutainer tubes and diluted 1:1 with Dulbecco's phosphate buffered saline 1X (PBS 1X).

All patients had signed an informed consent approved by the the local ethical committee (Azienda Ospedaliero - Universitaria Policlinico "G. Rodolico-San Marco", n. 54/2022/PO) at Division of Hematology, University of Catania.

Respective sera were obtained by centrifugation of whole blood samples in clot test tubes at 2000 *g* for 10 minutes.

Peripheral blood mononuclear cells (PBMCs) were isolated by healthy donor and PMF buffy coat after separation by density gradient Ficoll-Hypaque (Pharmacia LKB Biotechnology) and cultured in RPMI-1640 medium supplemented with 10% FBS and 1% Penicillin/streptomycin.

2.5 Flow cytometry

Whole blood collected in EDTA vials (50 µL) was stained with monoclonal antibodies (10 µL for each) (Beckman coulter) including: CD11b FITC, CD15 PE, CD14 PC5, HLA-DR-ECD, CD14-FITC, HLA-DR-APC, CD4-APC, CD25-FITC and FOXP3-PE (Beckman Coulter).

Using sequential gating strategy, G-MDSC were identified as $CD11b^{+}CD15^{+}CD14^{-}$ $HLADR^{-}$, M-MDSCs as $CD14^{+}HLA\text{-}DR^{-}$and Treg as $CD4^{+}CD25^{+}FOXP3^{+}$.

Samples were analyzed by using the flowcytometer MACSQuant Analyzer 10, Miltenyi Biotec.

2.6 Immunoblotting

Cells were washed with PBS1X, detached and centrifuged for 5 min at 1200 rpm to collect dry pellet. Proteins extraction was performed using RIPA lysis buffer supplemented with protease inhibitor 100X (Abcam).

Samples were incubated 15 min in ice and centrifuged 12000 *g* for 10 min. A constant quantity of proteins (50μg) was denaturized and loaded for electrophoresis on 4-15% SDS-PAGE gels and then transferred to nitrocellulose membranes.

Membranes were incubated for 1 h with Odyssey Blocking Buffer PBS (LI-COR) and then with primary antibodies overnight at 4°C.

The following primary antibodies were used for immunoblotting: mouse anti-beta actin (1:3000, Cat# ab6276 Abcam, Italy), rabbit anti-Alpha smooth muscle actin (αSMA) (1:1000, Cat# ab124964 Abcam, Italy), rabbit anti-Fibroblast activation protein (FAP) (1:1000, Cat# ab207178, Abcam, Italy), rabbit anti-collagen I (COL1A1) (1:1000, Cat# ab138492, Abcam, Italy), rabbit anti-MCT1/Monocarboxylic acid transporter 1 (1:1000, Cat# ab85021, Abcam, Italy), rabbit anti-SLC16A3/MCT 4 (1:500, Cat# ab234728, Abcam, Italy). Membranes were washed three times with 0.1% tween-20 in PBS1X and then incubated for 1 h with the proper secondary antibody IRDye 800CW Goat anti-mouse (1:5000, Cat# 925-32210, LI-COR Biosciences) or goat anti-rabbit IRDye 680RD (1:10000, Cat# 926-68071, LI-COR Biosciences) [148].

Images were acquired using Odyssey Infrared Imaging Scanner (LI-COR Biosciences, Milan, Italy) and relatives bands were quantified by densitometric analysis using ImageJ software, normalizing every band to βactin optical density.

2.7 Quantitative Real-Time Polymerase Chain reaction (qRT-PCR)

Total RNA was extracted from cells of each treatment group using TRIzol reagent (ThermoFisher scientific), according to recommended protocol, and quantified using UV-vis spectrophotometer NanoDrop1000 (ThermoFisher scientific).

cDNA was reverse-transcribed from 1μg of extracted RNA using High-Capacity cDNA Reverse transcription Kit (Applied Biosystems).

qRT-PCR was performed with PowerUp SYBR Green Master Mix as probe, accordingly to manufacturer's recommended protocol, using QuantStudio 3 Real-Time PCR System (Applied Biosystem).

Expression of human genes in **Table 1** and zebrafish genes in **Table 2** was evaluated.

Each reaction was run in triplicate and the relative expression of each sample mRNA was determined by comparison with the control housekeeping gene β2-microglobulin (B2M) using the $2^{-\Delta\Delta Ct}$ method.

Gene	*Forward*	*Reverse*
B2M	GAGTATGCCTGCCGTGTGAA	TTCATCCAATCCAAATGCGGC
TGFβ	CAAGACCACCCACCTTCTGG	GGGGGTGTCTCAGTATCCCA
MCT1	CTGGAACAAGCAAACGAGGC	AGCTCCAATTACCACTGCCC
MCT4	GCCCTCCTTGGCTCTTACAA	GCAGAAGATCCCTTTGATGTGG
HCAR1	GACCCAATCGCTCCTCTACG	ATGAGAGACCCAGGGAGGTC
ShH	CCAACTCCGATGTGTTCCGT	ATATAACCTTGCCCGCCGC
Smo	GTCGGGCCTCCGGAATG	CTCCACCCGGTCATTCTCAC
SPARC	AACTTTTGGGAGCACGGACT	GTCCCTAGAGCCCCTGAGAA
RUNX	GTAGCCTGGCAGTGTCAGAA	TTTTACCACGCTGCGAAACC
BMP2	AGAATAACTTGCGCACCCCA	GGACCGAATGTCCGTTCCTT
LDHA	GACGTGCATTCCCGATTCCT	AAGGCTGCCATGTTGGAGAT

Table 1 **Human primers**

Gene	*Forward*	*Reverse*
gapdh	ACAGCAACACAGAAGACCGT	GGCAGGTTTCTCAAGACGGA
CD41	TTGTCATTTGGCGCTGTGAG	ACTGGGGACTAAAACTGTATCTTT
mpl	CAACTCCCTACCAGAACGCA	TACTGGCCACAGGTTGTTTGA
tfgb1a	GTCCGAGATGAAGCGCAGTA	TCAAATGAGAGCCAGCGGTT
col1a1a	TTGCTTAGACCTGCGCTTCA	CCAGGGGGATTTTACACGCT
slc16a1a	CAATTGTGGAATGTGGGCCG	CCCACAGCCCATGTAAGTGT
slc163b	AAAGGATGGCACTTCCCCTG	GCCTGTTTGTCCACTAGGCA

Table 2 **Zebrafish primers**

2.8 Immunofluorescence

For immunofluorescence, cells were seeded at a density of 1 x 10^5 cells per well in a 4-well chamber slide system and properly treated for 48 h.

Then cells were washed with PBS1X and fixed with PFA 4% at 4°C for 15 min. Fixed cells were washed, permeabilized with 0.2% TritonX, washed and blocked with 5% normal goat serum (NGS) for 30 min. Slides were incubated with 1% primary antibody and 1% serum overnight at 4°C.

The following primary antibodies were used for immunofluorescence: rabbit anti-collagen I (Cat# ab138492, Abcam, Italy), rabbit anti-Gli1 (Cat# ab217326, Abcam, Italy). Slides were washed and incubated 1h with secondary antibody Goat anti-rabbit IgG, Alexa Fluor 546 (Cat# A-11010, Thermo Fisher Scientific, USA).

Nuclei were counterstained with 4',6-diamidino-2-phenylindole (Dapi, 1:1000, Cat# D1306, Invitrogen) for 5 min at room temperature.

Slides were mounted with mounting medium and digital images were acquired using a Zeiss Axio Imager Z1 epifluorescence microscope (Carl Zeiss AG, Werk Göttingen, Germany), equipped with an AxioCam camera (Zeiss, Jena, Germany).

2.9 Mallory's Trichrome Staining

Slides of cells treated and fixed with PFA 4% were stained using Mallory's trichrome Staining (Cat# 010227, DiaPath, Italy).

The staining was performed to identify connective tissue and collagen, accordingly to manufacturer's recommended protocol. In particular, nuclei and cytoplasm result respectively red and orange (acid fucsin) while the connective tissue and collagen are colored blue (methylene blue fixed by phosphomolybdic acid).

Stained slides were gradually dehydrated, dried and mounted with coverslip and proper mounting medium.

2.10 Alizarin Red S staining

Alizarin Red S staining was performed in order to identify calcium containing osteocyte in differentiated mesenchymal stem cells (Cat# A5533, Sigma-Aldrich, US).

Fixed cells in 24-multiwell were washed with PBS1X and incubated 30 min with 2% Alizarin Red S solution (pH 4.2).

After staining, cells were washed and observed to and digital images were acquired using a Zeiss Axio Imager Z1 epifluorescence microscope (Carl Zeiss AG, Werk Göttingen, Germany), equipped with an AxioCam camera (Zeiss, Jena, Germany).

2.11 Cytokines detection

Cell culture supernatants were collected after 48h from cells and frozen at −80° C until use. Multiplex immunobead assay technology (procartaplex Cytokine/Chemokine Magnetic Bead Panel, THERMO, MA; and Magpix analytical test instrument, Luminex Corp., Austin, TX) was performed on culture medium to determine concentrations of selected cytokines (BMP2, Osteopontin, Calcitonin, Osteoprotegerin). Culture medium from untreated cells and treated were evaluated.

2.12 *In vivo* PMF model: TPO^{High} zebrafish

According to mice models based on the alteration of MKs turnover and proliferation [35, 149, 150], we reproduced an *in vivo* PMF model evaluating a new approach based on

zebrafish treatment with a TPO^{High} mimetic which is a TPO receptor agonist used for the clinical management of immune thrombocytopenia.

Adult zebrafish were maintained at standard conditions and then (number of animals=30) were treated for 10 days (intraperitoneal injection every 48 hours) at a concentration of 100µg/kg, miming a TPO^{High} zebrafish model.

2.13 Immunohistochemistry of kidney bone marrow (KBM) in zebrafish

Zebrafish were culled by anesthetic overdose, fixed in 4% PFA for 2 h to 12 h at 4° C, and their KBM carefully were removed under a stereomicroscope and processed with the primary antibody diluted in PBS containing 5% normal goat serum and 0.1% triton x-100 at 4 °C overnight. The antibodies used are: collagen1 and MCT4, diluted 1:1000 in 5% NGS, 0.5% Triton X100 in PBS overnight. A secondary antibody conjugated with Alexa 633 was used for 12 h at 4 °C. Images were acquired using an inverted Leica TSP8 confocal microscope, after equilibrating samples in 100% glycerol.

Reticulin staining was performed using silver staining according to the manufacturer's instruction.

2.14 Immunohistochemistry of patients biopsies

Sections derived from paraffin-embedded specimens were mounted in slides (4-µm thick) were deparaffinized in xylene and serially hydrated in 100%, 95%, and 80% ethanol.

Tissue slides were quenched in 3% H_2O_2 to block endogenous peroxidase activity, followed by extensive rinsing in double-distilled water and further rinsing for 15 min in 0.01M PBS pH 7.4.

Slides were incubated with an anti-human primary antibody (10μg/ml) for 1 h and then with peroxidase-conjugated secondary antibody for 30 min at room temperature (RT). Sections were washed three times in PBS and antibody binding was revealed using the Sigma fast 3,30-diaminobenzidine tablet set (Sigma). Counterstaining was performed using haematoxylin solution. Anti-human MCT4 (H-90) and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The expression of MCT was quantified using Remmele scoring system (2424168). The score was calculated as previously proposed [151].

2.15 Statistical analysis

Statistical analysis was performed using Prism - GraphPad software. Differences between experimental groups were determined using the Fisher method with statistical significance ($p<0.05$). To compare treatment groups, the null hypothesis was tested by single-factor analysis of variance (ANOVA) for multiple groups. Likewise, the unpaired T-test method was used for two groups. Data are reported as mean ± SD.

3. Results

3.1 Lactate and immunosuppressive subpopulations

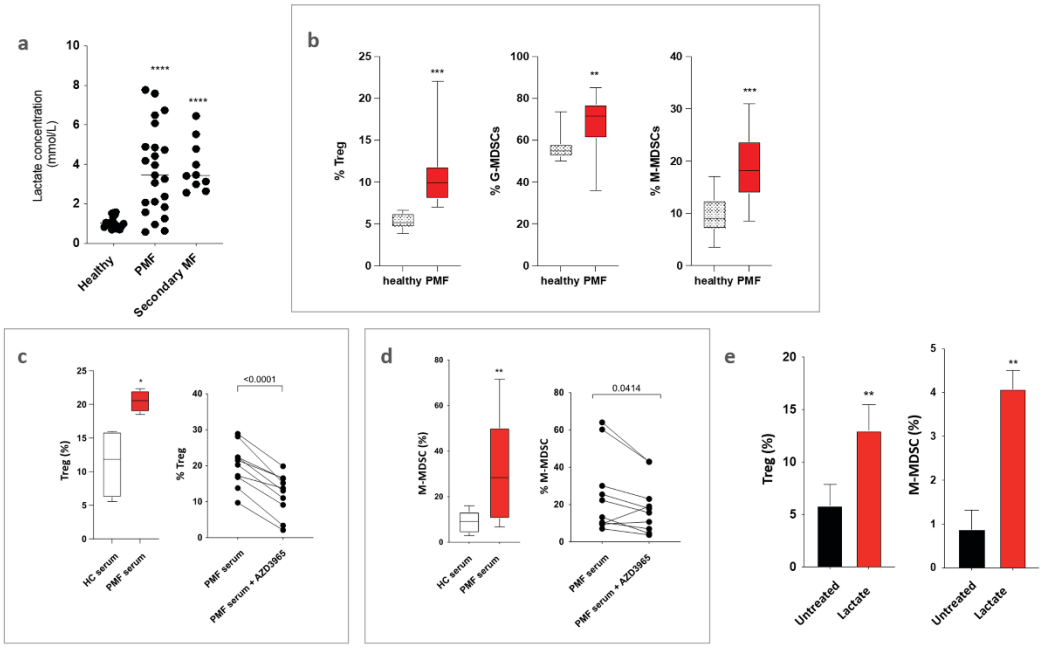


Figure 4 **Immunosuppressive subsets evaluation**

a. Lactate quantification in healthy donors (HC) and myelofibrosis patients; **b.** Percentage of circulating immunosuppressive cells (**%Treg**, **%G-MDSCs**, **%M-MDSCs**) in HC and PMF patients; **c.** Percentage of **Treg** in PBMCs of HC and PMF patients treated with PMF sera with or without AZD3965 inhibitor 10μM; **d.** Percentage **M-MDSCs** in PBMCs of HC and PMF patients treated with PMF sera with or without AZD3965 inhibitor 10μM; **e. Treg** and **M-MDSCs** expansion in healthy PBMCs treated with lactate 20mM.

The data are expressed as mean SD. Statistical analysis was performed with one-way ANOVA.

*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 versus CTRL (HC or Untreated) group

Data obtained from the first analysis show that lactate expression is significantly increased in sera of patients with both primary and secondary myelofibrosis compared to the sera from healthy controls (**Figure 4a**).

Based on that, the further investigation on immunosuppressive subset shows high levels in percentage of circulating Treg, G-MDSCs and M-MDSCs in PMF patients compared to healthy donors (**Figure 4b**). **Figure 4c** and **Figure 4d** represent respectively Treg and M-MDSCs percentages in PBMCs treated with PMF sera compared to the control.

In particular, PMF sera enhance the immunosuppressive expansion of both cell lines in healthy PBMCs, while the same expansion results decreased to lower values in presence of the MCT1 channel inhibitor AZD3965.

The strong relationship of lactate accumulation and immunosuppressive expansion is further confirmed by data of **Figure 4e**: the treatment of PBMCs with lactate 20mM for 48h produces a relevant increase in both percentages of Treg and M-MDSCs subsets, suggesting definitely that lactate accumulation orchestrates an immunosuppressive profile in PMF.

3.2 Lactate affects MCTs and HCAR1 expression

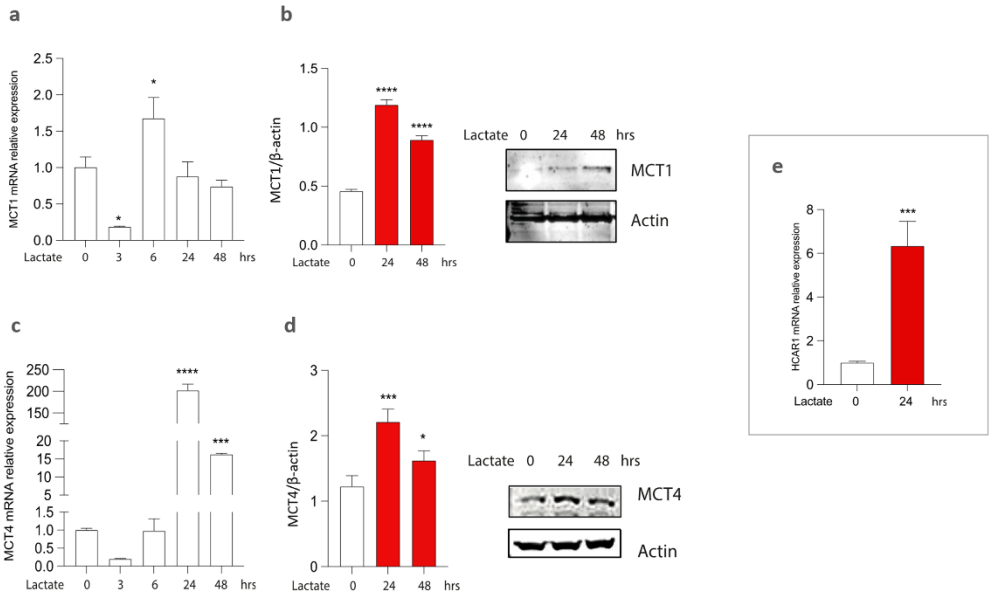


Figure 5 **Lactate channels (MCTs) and lactate receptor HCAR1**

MCTs and HCAR1 evaluation in HS-5 cells treated with lactate 20mM at different time points

a. Time course of **MCT1** gene expression; **b. MCT1** protein expression; **c.** Time course of **MCT4** gene expression; **d. MCT4** protein expression; **e. HCAR1** gene expression;

The data are expressed as mean SD. Statistical analysis was performed with one-way ANOVA.

*P<0.05, **P<0.01, ***P<0.001,****P<0.0001 versus CTRL group.

Gene and protein expression of lactate channels (MCT1 and MCT4) and lactate receptor (HCAR1) have been evaluated.

Particularly, **Figures 5a, 5b, 5c, 5d** show MCT1 and MCT4 modulation reported in time course related to lactate 20mM treatment. The results show an enhanced mRNA expression of MCT1 gene (slc16A1) after 6 hours of treatment with lactate 20mM (**Figure 5a**) and the same enhancement occurs after 24 hours of treatment for MCT4 gene (slc16A3) (**Figure 5c**).

Regarding protein expression, data demonstrate overlapping results for MCT1 and MCT4, showing in both cases a significant increase of protein expression after 24 hours of treatment, which persists although slightly lowering after 48 hours compared to the control (time 0). **Figure e** indicates HCAR1 gene expression which results particularly increased compared to the control after 24 hours of treatment.

3.3 Effects of lactate on fibrosis biomarkers

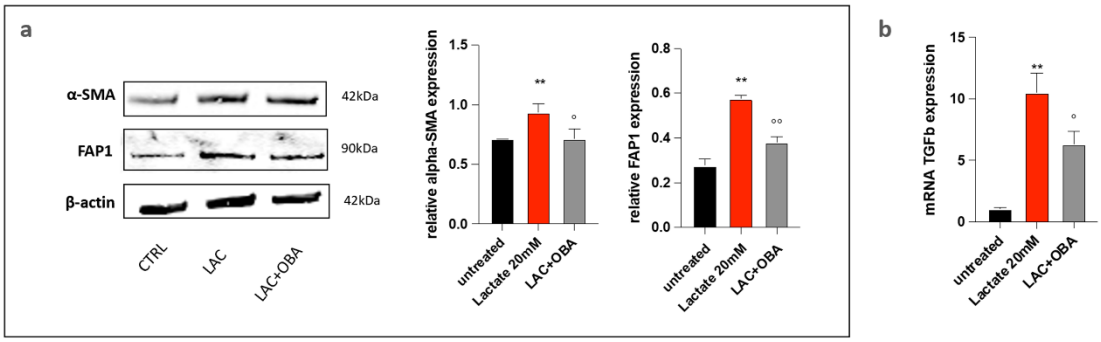


Figure 6 **Lactate modulates fibrosis biomarkers**

Fibrosis biomarkers analysis in HS-5 cells treated with lactate 20mM and co-treated with lactate and 3-OBA antagonist 3mM for 48 hours. **a. αSMA** and **FAP1** protein expression; **b.TGFβ** gene expression

The data are expressed as mean SD. Statistical analysis was performed with one-way ANOVA.

*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 versus untreated group. °P<0.05, °°P<0.01, °°°P<0.001, °°°°P<0.0001 versus Lactate 20mM group.

As shown in **Figure 6a** and **6b**, lactate induces a relevant increase of important fibrosis biomarkers like αSMA, FAP1 (**Figure 6a**) and TGFβ (**Figure 6b**). Lactate effect on this modulation suggests its implication in the pathogenic context of fibrotic accumulation as well as reticulin and fibronectin progressive deposition [152].

Interestingly, the co-treatment with lactate 20mM and HCAR1 antagonist, 3-hydroxybutyric acid (OBA), results in a significant decreased expression of above-mentioned biomarkers, furtherly confirming the role of lactate in profibrotic remodeling.

3.4 Lactate enhances collagen deposition

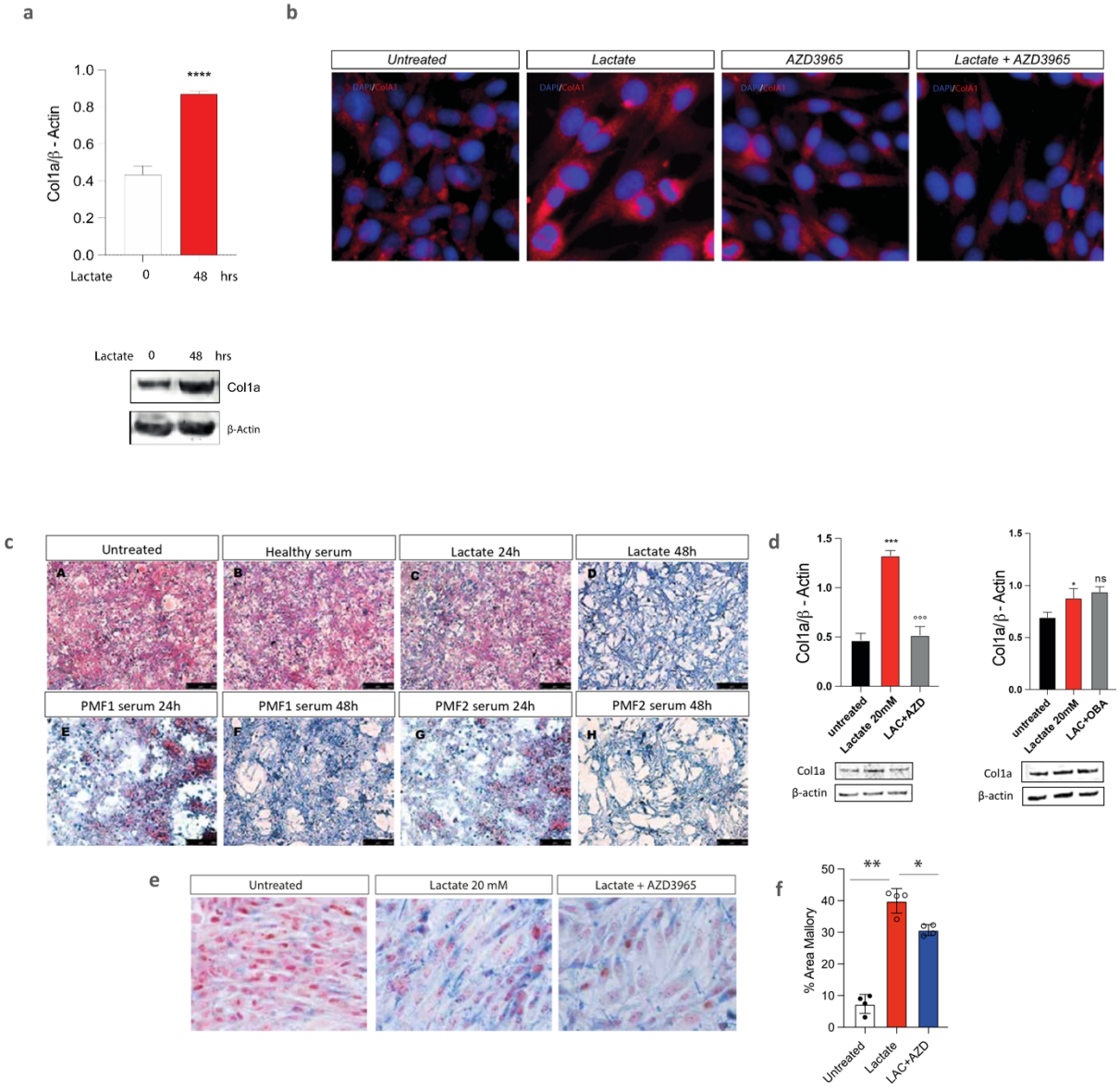


Figure 7 **Role of lactate in collagen deposition**

Treatment of mesenchymal stem cells (HS-5) with lactate 20mM promotes collagen deposition which is partially mediated by MCTs channels.

a. Col1a protein expression (Untreated, lactate 20mM; 48 hours); **b.** Immunofluorescence of **Col1a** (Untreated, lactate 20mM, AZD3965 10µM, lactate + AZD3965; 48 hours); **c.** Mallory's Trichrome staining (Untreated, lactate 20mM, HC and PMF sera; 24 and 48 hours); **d. Col1a** protein expression (Untreated, lactate 20mM, lactate + AZD3965/ lactate + 3-OBA; 48 hours); **e.** Mallory's Trichrome staining (Untreated, lactate 20mM, lactate + AZD3965; 48 hours); **f.** Area quantification in percentage represented in bar graph of Mallory's Trichrome staining.

The data are expressed as mean SD. Statistical analysis was performed with one-way ANOVA.

*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 versus untreated group. °P<0.05, °°P<0.01, °°°P<0.001, °°°°P<0.0001 versus lactate 20mM group

Data shown in **Figure 7** prove that lactate is involved in collagen accumulation.

In particular, lactate treatment for 48 hours promotes collagen expression on mesenchymal stem cells (**Figure 7 a-f**).

In this regard, collagen expression strongly induced by lactate is restored to values comparable to the control by AZD3965 (**Figure 7 a, 7b, 7d, 7e, 7f**).

Oppositely, collagen promotion seems not to depend on the HCAR1 receptor (OBA), as illustrated in **Figure 7d**.

Furthermore, treatment of mesenchymal stem cells with different PMF patients sera (PMF1 and PMF2) for 48 hours show a significant collagen accumulation as shown by the peculiar morphology and blue collagen deposits in **Figure 7c**.

Interestingly, this latter evidence is obtained treating the stromal cell line with lactate 20mM at the same time point (**Figure 7c**), suggesting that profibrotic depositions in PMF environment depend at least in part on lactate-acidosis.

3.5 Lactate role in osteogenic development

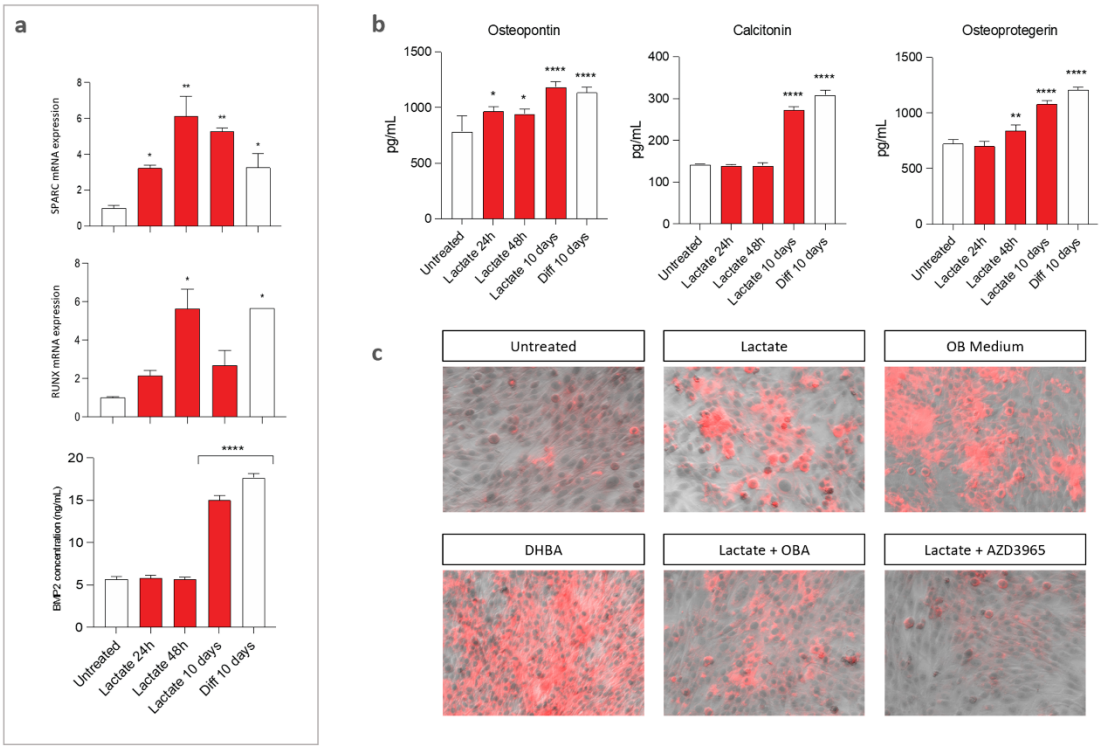


Figure 8 **Lactate induces osteogenic differentiation in mesenchymal stem cells**

a. RUNX and **SPARC** gene expression (first and second panel from top to bottom) **BMP2** protein expression (ng/mL)- MAGPIX analytical test; **b. Osteopontin**, **Calcitonin** and **Osteoprotegerin** - protein expression (pg/mL)- MAGPIX analytical test: (a, b) Cells were treated with OB differentiating medium (0.2 mM ascorbic acid, 0.1 mM dexamethasone and 10 mM β-glycerophosphate) for 10 days. Furthermore, cells were treated with lactate 20mM for 24, 48 hours till a maximum of 10 days; **c.** Alizarin Red Staining: calcium containing osteocytes in differentiated mesenchymal stem cells HS-5. (Untreated; Lactate 20mM; OB differentiation medium; 3,5 DHBA 150μM; Lactate + OBA antagonist (3mM); Lactate + AZD3965 10μM) 10 days.

The data are expressed as mean SD. Statistical analysis was performed with one-way ANOVA.

*P<0.05, **P<0.01 and ***P<0.001, ****P<0.0001 versus untreated group (0 h).

Since bone homeostasis represents a crucial event in the pathogenesis of PMF, the role of lactate in this biological mechanism has been evaluated.

Indeed, as shown in **Figure 8a**, lactate treatment, after both 48 hours and 10 days, upregulates two genes: Runt-Related Transcription Factor 1 (RUNX1), particularly

involved in the proper development of hematopoietic niche with annexed stem cells and secreted protein acidic and cysteine rich (SPARC), which is responsible for collagen calcification in bone matrix.

In this way, lactate promotes gene levels of RUNX and SPARC overlapping to those produced by OB medium.

In order to confirm the involvement of lactate in bone formation and osteogenesis, bone morphogenetic protein 2 (BMP2) protein expression was evaluated; in this case, lactate treatment for 10 days strongly promotes upregulation of BMP2 as well as OB treatment at the same time point.

Moreover, **Figure 8b** shows the same trend for important factors involved in bone matrix homeostasis (Osteopontin, Calcitonin, Osteoprotegerin), which result upregulated by lactate treatment for 10 days.

In conclusion, calcium deposits in cells undergoing osteogenic differentiation were evaluated, as shown in **Figure 8c**.

Cells treated with lactate, but in particular with 3,5-DHBA, show an increase in calcium levels, comparable to OB group.

Furthermore, the combos of lactate with OBA (HCAR1 antagonist) and AZD3965 (MCT1 inhibitor) respectively show a strong restoration of Ca^{2+} accumulation, further suggesting that lactate plays an important role in bone remodeling.

3.6 Lactate and Sonic Hedgehog signaling

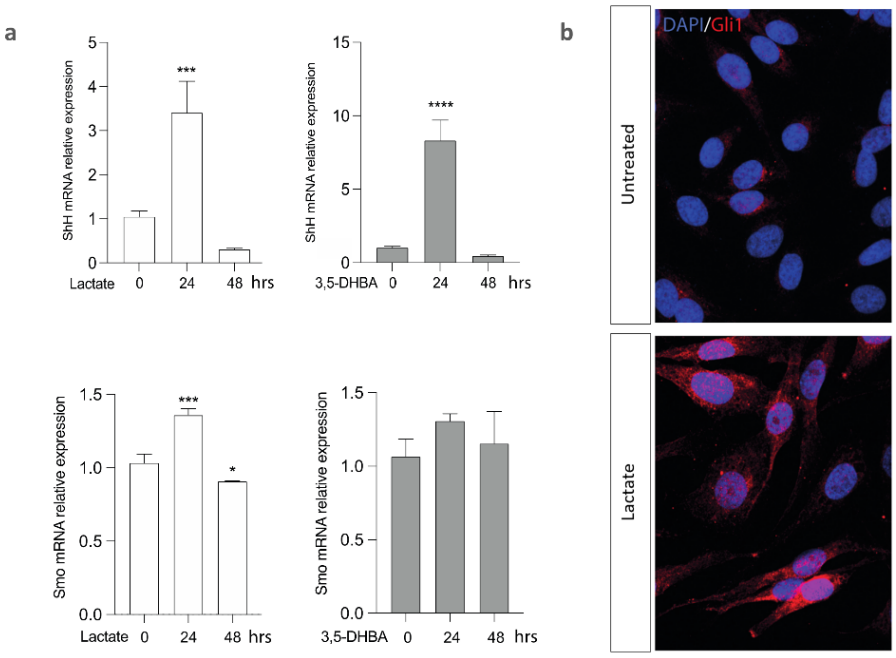


Figure 9 **Lactate stimulates Sonic Hedgehog signaling**

Lactate and HCAR1 agonist (3,5 DHBA) influence Sonic Hedgehog signaling on mesenchymal stem cells (HS-5)

a. Shh gene expression with lactate 20mM (top left panel)**; Shh** gene expression with 3,5-DHBA 150µM (top right panel)**; Smo** gene expression with lactate 20mM (bottom left panel)**; Smo** gene expression with 3,5-DHBA 150µM (bottom right panel); **b.** Immunofluorescence of **Gli1** (Untreated, Lactate 20mM; 48 hours)

The data are expressed as mean SD. Statistical analysis was performed with one-way ANOVA.

*P<0.05, **P<0.01 and ***P<0.001, ****P<0.0001 versus untreated group (0h).

Many scientific evidences suggest the involvement of alterations of Sonic Hedgehog (Shh) signaling in many disorders involving collagen depositions and bone development due to mechanisms like osteoblast differentiation [153, 154].

Based on that, the evaluation of Shh signaling and its modulation in presence of lactate acidosis represented one of our experimental points.

Results in **Figure 9** show that lactate probably plays a role in Sonic Hedgehog signaling in the pathological context of cancer. Effectively, it is shown in **Figure 9a** that lactate treatment induces a significant upregulation of two important factors involved in Sonic Hedgehog signaling (ShH and Smo) already after 24 hours with a following downregulation after 48 hours.

The same effect is obtained treating the cells with a

well known HCAR1 agonist, 3,5-DHBA, except for Smo gene expression, which clearly is not modulated (bottom right panel of **Figure 9a**).

In this regard, immunocytochemical analysis was performed to evaluate the expression of Gli1, transcriptional activator belonging to ShH-dependent Gli proteins family, as well as final mediator of ShH downstream signaling cascade, in response to the treatment with lactate for 48 hours (**Figure 9b**).

As shown in **Figure 9b**, lactate treatment strongly upregulates Gli1 expression, and mediates it through HCAR1 receptor, as suggested by data obtained with the agonist 3,5-DHBA (**Figure 9a**).

3.7 *In vivo* model of TPO^{High} zebrafish

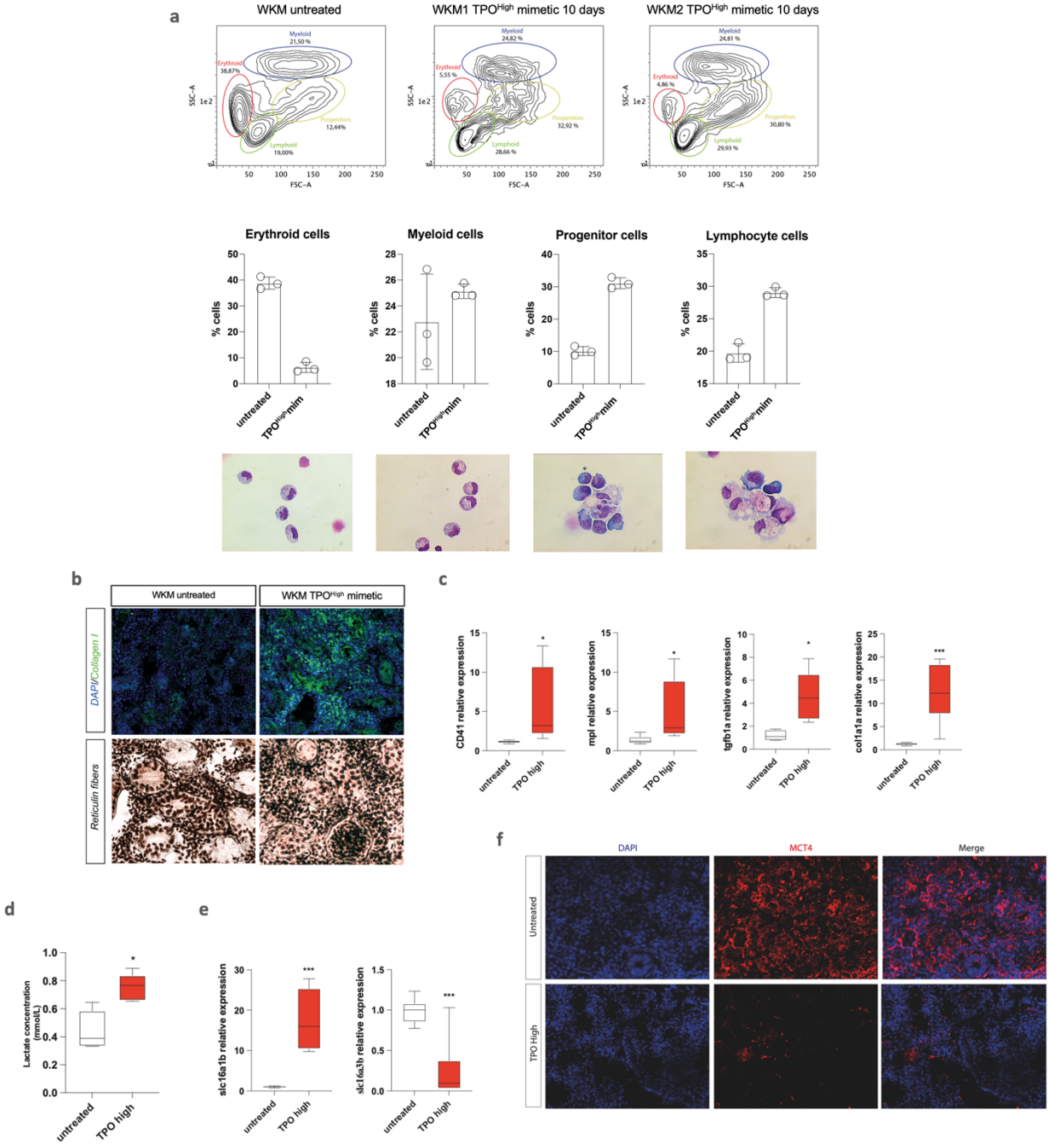


Figure 10 ***In vivo*** **model and cell subsets of TPO^{High} zebrafish**

a. Flow cytometry profiling of WKM in TPO^{High} zebrafish; **b.** Immunofluorescence of **Collagen I** (top panels); Silver staining of **reticulin fibers** (bottom panels); **c. CD41**, **mpl**, **tgfb1a**, **col1a1a** gene expression; **d.** Lactate quantification in TPO^{High} zebrafish WKM; **e. MCT1** and **MCT4** gene expression in TPO^{High} zebrafish WKM.

The data are expressed as mean SD. Statistical analysis was performed with one-way ANOVA.

*P<0.05, **P<0.01 and ***P<0.001, ****P<0.0001 versus untreated.

Data obtained from whole kidney marrow (WKM) analysis in TPO^{High} zebrafish show cell subsets levels typical of PMF, suggesting that the *in vivo* PMF model with TPO^{High} mimetic properly works.

In particular, data obtained by cytometry confirm the increase of myeloid and progenitor cells in the hematopoietic profile of the *in vivo* model (**Figure 10a**)

In addition to cell subpopulations, collagen and reticulin levels have been investigated.

In this regard, **Figure 10b** shows an increase in TPO^{High} zebrafish compared to the untreated, coherently with the PMF pathological outcome that was extensively discussed above.

Furthermore, **Figure 10c** represents a sequence of several well-known biomarkers related to some of the most peculiar biological aspects of PMF pathogenesis, including megakaryocytes dysplasia/hyperplasia and fibrosis: all the markers evaluated show an increased gene expression in TPO^{High} group compared to the untreated.

Figure 10d shows the result obtained by lactate quantification on WKM, confirming increased levels of lactate in TPO^{High} zebrafish compared to the untreated.

Finally, the analysis of MCTs shows an increase of MCT1 expression in TPO^{High} WKM (**Figure 10e**) and at the same time a significant decrease of MCT4 (**Figure 10e**), furtherly confirmed by data obtained by immunofluorescence (**Figure 10f**).

3.8 Preliminary data of PMF patients

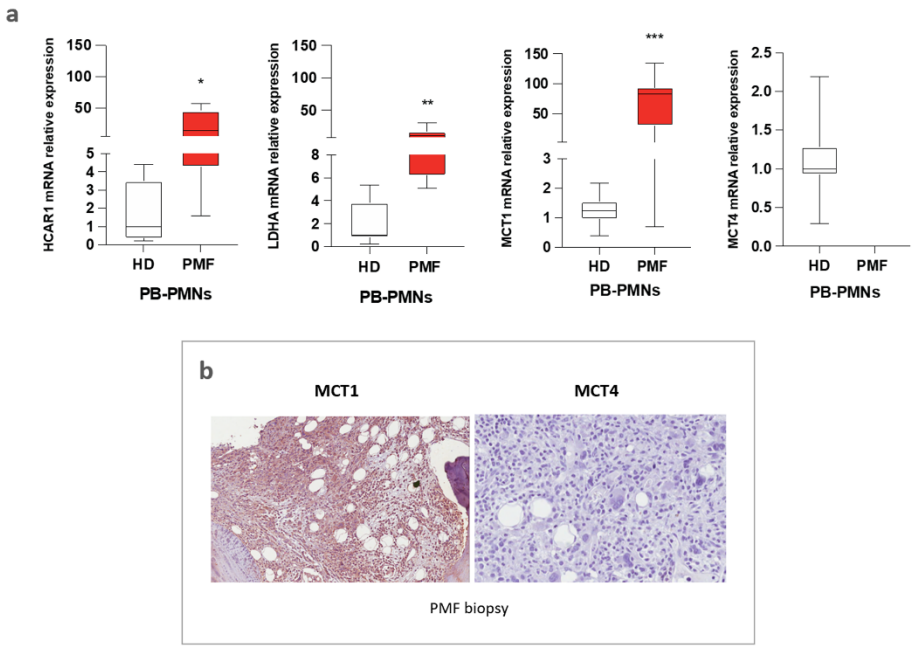


Figure 11 **Preliminary data of patients biological samples**

a. HCAR1, **LDHA**, **MCT1**, **MCT4** gene expression in PB-PMNs isolated from peripheral blood of healthy donors (HD) and patients (PMF); **b.** Immunohistochemical section of PMF bone marrow biopsy.
The data are expressed as mean SD. Statistical analysis was performed with one-way ANOVA.

Figure 11a shows the differential expression of some of the most representative factors involved in lactate metabolism/trafficking in peripheral blood PBMCs of PMF patients compared to health donors (HD).

In particular, gene expression of HCAR1, lactate dehydrogenase A (LDHA) and MCT1 results increased in PMF cells compared to the controls (HD). Oppositely, MCT4 is interestingly downregulated or practically turned off in PMF patients.

This latter evidence is further confirmed by immunohistochemical staining of PMF bone marrow biopsy (**Figure 11b**), showing the overexpression of MCT1 with the concurrent shutdown of MCT4.

4. Discussion

PMF is a rare myeloproliferative malignancy whose onset is due to the clonal neoplastic proliferation of a pluripotent hematopoietic stem progenitor (HSC) in BM niche (**Figure 1B**) [7].

The mutant clone overexpansion is one of the key-evidences in PMF patients which usually show a monoclonal increase in myeloid, lymphoid or erythroid lineage [8].

PMF results in a strongly compromised BM which shows atypical cell lineages and several functional imbalances producing a constant degenerative inflammatory environment.

One of the most evident consequences is certainly the BM fibrotic tissue buildup mediated by the progressive deposition of collagens and reticulin, mainly sustained by a persistent state of multi-level deregulation of the ECM [155].

All the biological disruptions related to PMF are based on the failure of hematopoiesis, a very intricate and orchestrated system.

Indeed, the correct progression of hematopoietic process needs an appropriate balance within the different BM niches, which obviously lacks following PMF outcome.

The lack of coordination due to the neoplastic expansion, affects the BM microenvironment and in particular stromal cells, which are finely instructed to establish a proper crosstalk with every other cell line (independently health or cancer cell) in order to produce a complex network of signaling, defining the TME.

The major expression of tumor reprogramming handled by TME is represented by the onset of a subpopulation of hyperactivated fibroblasts (CAFs) [60] which actively produce a dysfunctional stromal microenvironment in order to fuel cancer progression [64, 65].

Based on these observations, we focused on the importance of lactate in PMF, as signaling molecule in the spotlight of scientific literature [156, 157].

In order to accomplish our scientific aims, we simulated an *in vitro* model of lactate acidosis treating stromal cells HS-5 with lactate 20mM and we coupled obtained data with the evaluation of biological samples of PMF patients.

This analytical strategy allowed to evaluate the microenvironmental shaping from different points of view in order to build a complete perspective of the different roles played by lactate in PMF.

Ultimately, in order to give a major scientific resonance to our evidences, an *in vivo* model of TPO^{High} zebrafish was performed and properly evaluated, as shown in (**Figure 10**), in which the expression of biomarkers as CD41, mp1, tgfβ (**Figure 10c**), collagen and reticulin (**Figure 10b**) is reported.

Several scientific evidences widely support the role of lactate as oncometabolite in many of the cancer biological implications such as angiogenesis, immune escape and metastasis [158-160].

Our preliminary investigations show an increased lactate concentration on PMF sera as well as in TPO^{High} zebrafish WKM samples (**Figure 4a**, **10d**), demonstrating that it is a common property in PMF disease, according to the lactate acidosis widely debated in many scientific cancer papers [158, 161].

In fact, acidosis of TME, due to the progressive lactate and proton shuttling from cells to extracellular space, is responsible of cancer shaping influencing interaction and signaling among different cell subtypes [162].

In addition, as suggested by Iñigo San-Millán et al., lactate production and accumulation represent one of the purposes of Warburg Effect, creating a continuous loop of glucose uptake and lactate production; this latter is involved in several carcinogenesis steps including cell self-sufficiency further sustained by upregulation of MCT1 and HCAR [158].

In this regard, our *in vitro* data confirm the role of lactate in enhancing MCT1 and MCT4 gene and protein expression (**Figure 5a**, **5b, 5c, 5d**) and HCAR1 gene expression (**Figure 5e**).

MCT1 is mainly responsible of lactate uptake by stromal cells [102], and its upregulation, due to lactate exposure, demonstrates a possible role in modulation of environment in order to make the stromal niche more responsive to lactate itself.

Furthermore, it has been shown that MCT1 function in stroma should contribute to metastasis and invasiveness since lactate influx is involved in angiogenesis and bone resorption affecting respectively endothelial cells [163-165] and osteoclasts [166].

Moreover, as proved in **Figure 5e**, HCAR1 gene expression results strongly increased following lactate treatment for 24 hours in the stromal cell line: the same trend was reported in PBMCs of PMF samples compared to health donors (HD) (**Figure 11a**).

The lactate receptor HCAR1 is involved in numerous metabolic pathways including signaling cascades related to lipolysis, angiogenesis or cell differentiation [131-133].

In addition, the interaction of lactate and HCAR1 in cancer, has been recently focused by scientific literature, which confirmed the importance of both factors in metastasis, angiogenesis and tumor progression [135-137].

In this regard, some papers reported a correlation between HCAR1 and MCTs.

In fact, HCAR1 stimulation seems to support the transcriptional induction of MCT1 and MCT4, thereby promoting lactate uptake [167].

Interestingly, our *in vivo* data show that MCT1 (slc16a1b) is upregulated in WKM zebrafish as well as in PMF biopsy while at the same time MCT4 (slc16a3) is particularly downregulated or almost absent (**Figure 10e, 10f, 11**).

MCT4 is the membrane channel responsible of lactate and pyruvate output affecting acid pH of TME: MCT4 downregulation associated to the simultaneous MCT1 increase represents a

dramatic cell modulation suggesting that lactate concentrates inside the cell affecting the proper functionality.

Particularly, this imbalance promotes lactate intracellular accumulation which probably culminates in lactate utilization by mitochondrial reticulum which in turn remarks a conceivable oxidative metabolic switch [168].

This biological overview results further supported by lactate dehydrogenase A (LDHA) expression which results increased in PMF compared to the control (**Figure 11a**), justifying the higher lactate intracellular utilization.

Furthermore, LDHA expression in cancer encourages tumor immune escape [138], enabling glucose consumption as well as converting pyruvate to lactate and finally supporting cancer stem like phenotype and tumor growth [169, 170].

The last decade attended a growing interest in TME and cancer immune response, with particular attention on immune escape due to microenvironment remodeling [171].

Indeed, our data confirm that, in the pathological context of PMF, the expression of myeloid suppressive subpopulations is crucial (**Figure 1b,1c, 1d**) as shown by the significant increase of both myeloid and granulocyte MDSCs as well as of Treg subsets.

In addition, several scientific groups reported the importance of lactate in the regulation of immune subsets in order to support the progressive dysfunction of the proper immune network [159].

Particularly, a paper demonstrates that lactate accumulation produced by CAFs regulates polarization of infiltrating CD4+ T cells, promoting Treg proliferation instead of anti-tumor T_{H1} cells in a prostate cancer model [172].

Moreover, the shaping role of TME played by lactate is further confirmed by the enhanced M-MDSCs expansion [139], which in turn contributes to T cells effectors inhibition [119].

The adjuvant role toward MDSCs development is widely due to cytokines and factors as TGFβ or LDHA, both responsible of MDSCs differentiation and expansion [139, 173].

The strong correlation between lactate accumulation and immune suppressive sub sets in PMF is widely confirmed by our results, showing that lactate supports immune suppressive expansion in PBMCs cultures (**Figure 1e**).

Besides, this regulation seems to take place, at least partially, through MCT1 influx channel, as confirmed by the treatment with AZD3965 as MCT1 inhibitor (**Figure 1c**, **1d**).

Indeed, data obtained show that MCT1 inhibition leads to a significant decrease of expression levels of both M-MDSCs and Treg (**Figure 1c**, **1d**), suggesting that lactate influx is an important step to the proper cell regulation.

Going into detail, one of the most interesting purposes of this work, was to frame the role of lactate in the pathogenetic context of PMF.

The most discriminating hallmark of PMF is the BM fibrosis, characterized by collagen and reticulin accumulation, due to several profibrotic stimuli both improving extracellular matrix (ECM) accumulation and inhibiting ECM degradation.

Our investigation shows a remarkable correlation between lactate and fibrotic deposition, clarifying how lactate levels influence most of distinctive pathological mechanisms of PMF, orchestrating the microenvironmental shaping.

In fact, as shown in **Figure 6**, lactate treatment on stromal cells stimulates the expression of some important biomarkers, such as TGFβ, αSMA and FAP.

Particularly, these factors perfectly match CAFs secretory profile, showing that lactate accumulation should be involved in regulation of fibroblast recruitment and activation [174].

Furthermore, the induction exerted by lactate is significantly restored to values comparable to the control by the co-treatment with OBA, selected as HCAR1 antagonist.

This result confirms that lactate effect on stroma is mediated by its interaction with the receptor HCAR1, towards which lactate acts as signaling oncometabolite.

The massive release of factors like TGFβ or αSMA and the hyperactivation of fibroblasts in CAFs represent the biological background of a common molecular mechanism known as Epithelial-to-Mesenchymal Transition (EMT).

In fibrotic diseases, EMT occurs in ongoing inflammatory conditions and represents the molecular basis of abnormal ECM depositions like collagens and reticulin [175].

The concrete support of lactate on fibrotic deposition is perfectly represented by **Figure 7**, in which lactate is responsible of enhancement of collagen I in stromal cell culture, mostly after 48 hours.

Particularly, the influence on collagen expression seems to depend on MCTs channels (**Figures 7b**, **7d** and **7e**), as shown by the administration of AZD3965, which partially restores the induction promoted by lactate.

A comparable expected result was obtained by treating cells with PMF patients sera (**Figure 7c**): this evidence shows the clear confirmation of how cancer cells shape the neighbor cells supporting the molecular reprogramming of microenvironment in which lactate seems to play a pivotal role.

Assuming that the microenvironment changes are a recurrent notion widely acknowledged by scientific literature, it is interesting to consider every potential factor involved in order to understand as many mechanisms as possible on which a pathology as PMF is based.

In this regard, among different pathways involved in fibrosis, Sonic Hedgehog signaling (Shh) and Gli proteins have aroused growing interest.

Indeed, Shh not only is a physiological process involved in embryonic development [176, 177], but its dysfunction is involved in cell reprogramming and TGFβ pathway [153, 178] beyond the pathogenesis of many malignancies.

Specifically, Horn et al. investigated the role of Shh pathway in fibroblast activation, demonstrating a direct correlation between Shh activation, collagen release and fibrosis progression *in vivo* [153].

According to these evidences, our data show that lactate stimulates Shh and Smo protein expression and ultimately promotes Gli expression, final effector of Shh pathway (**Figure 9a**, **9b**).

Beyond fibrotic deposition, dysfunctional BM of PMF patients is subjected to a complex combination of various functional alterations, including megakaryocyte (MK) hyperplasia and osteosclerosis [179].

In particular, evidences from knockout mice models showed that overexpression of TPO (TPO^{High}) receptor and GATA1 deficiency (GATA1low), are involved in altered MKs development as well as reduced platelet counts [35, 149, 150].

According to these models, we tried to reproduce an in vivo PMF model evaluating a new approach based on zebrafish treatment with TPO^{High} mimetic (**Figure 10**), a TPO receptor agonist used for the clinical management of immune thrombocytopenia (ITP); the binding to TPO receptor causes activation of Mp1, JAK2-STAT5, ERKs and AKT downstream signaling pathways leading increased megakaryocyte turnover [180].

MK dysfunction is involved in most of pathogenetic features of PMF, including neoangiogenesis, CAFs recruiting, bone damage like osteosclerosis [155].

Indeed, osteosclerosis is the most common bone alteration of PMF [181, 182] and is mainly produced by the gradual replacement of marrow by collagen and bone trabeculae accomplished by activated myofibroblasts [183].

Over the years, numerous biomolecular hypotheses have been correlated to the altered bone remodeling, including the importance of osteoblasts (OBs)/osteoclasts (OCs) ratio or the axis between transmembrane receptor activator of NF-kB ligand (RANKL) and osteoprotegerin (OPG) [51].

Particularly, hypersecretion of OPG by OBs, stimulated by TGFβ overproduction, and the simultaneous increase of OBs/OCs ratio, result in enhanced angiogenesis and osteoclastogenesis inhibition with bone resorption impairment [184-186].

Pathways like WNT/β-catenin [187], Jagged1/Notch1 [188] and ShH signaling [189] are specifically involved in altered bone remodeling.

Based on these assumptions, our data show that lactate accumulation on BM stromal cells is involved in pro-osteoblastic attitude through which we assume that lactate has a crucial role in bone modifications as increased bone density and abnormal hardening of osteosclerotic progression in PMF.

Accordingly, lactate is responsible of upregulation of important bone transcription factors like RUNX and SPARC (**Figure 8a**)

In particular, the influence of lactate was compared to the osteoblastic differentiated cell line (Diff. 10 days), selected as positive control.

The most interesting results have been obtained with ten days of lactate which increased the expression of factors specifically involved in bone morphology and functionality including BMP2, osteopontin (OPN), calcitonin and OPG (**Figures 8a**, **8b**).

In this regard, BMPs belong to the TGF-β superfamily, and their overexpression is associated with increased gene expression of type I collagen, osteocalcin, OPN, VEGF and PDGFα during osteoblastic differentiation [190].

Among them, OPN results particularly upregulated in cancer [191] and is involved in many different processes including inflammation, cell migration and biomineralization, affecting OCs functionality [192].

Meanwhile, OPG enhancement is strongly related to endothelial proliferation as well as to OCs impairment which in turn contributes to the imbalanced overactivity of OBs [185, 186].

In this regard, scientific literature clarified that OBs differentiation is supported by Shh protein [154]; based on that, our data suggest that lactate accumulation should condition osteosclerotic damage involving Shh modulation.

In addition, the involvement of Shh in MKs turnover and angiogenetic development further confirm the crucial role that this signaling cascade should play in the multifactorial pathogenesis of PMF [193, 194].

Moreover, as shown in **Figure 8c**, lactate induces accumulation of osteocyte calcium deposits in mesenchymal stem cells as well as HCAR1 agonist 3,5-DHBA.

Interestingly, results obtained with lactate are comparable to the calcium amount produced by differentiated cells (OB group **Figure 8c**), suggesting that lactate is specifically involved in bone structure remodeling.

Furthermore, lactate induction is particularly reverted to values overlapping the control using the MCT1 inhibitor AZD3965 demonstrating again the importance of MCTs channels in lactate trafficking and its proper compartmentalization.

5. Conclusions

PMF is a rare myeloproliferative malignancy due to the clonal proliferation of myeloid cell line leading to BM failure.

Indeed, BM environment results particularly compromised showing atypical cell populations and deep functional imbalances involving collagen and fibrotic depositions, thickening and weakening of bones structure, proliferative alterations of several cell lineages inducing leukoerythroblastosis, cytopenia and megakaryocyte hyperplasia [30-32]. The intricate hematopoietic niche architecture is the biological explanation of how an environmental disruption can seriously affect the proper functionality of entire physiological systems.

In this regard, the crosstalk between cancer and stroma represents a crucial piece of the complex pathogenetic progression of PMF in which lactate represents an important key regulator [102, 195].

Particularly, our data confirm that lactate trafficking seems to be specifically involved in cancer dynamics as immune escape and neoangiogenesis, influencing stromal cells activity.

In addition, lactate influences key mechanisms of PMF pathogenesis as fibrosis, bone remodeling and megakaryocytes hyperplasia, as suggested by our investigation on many factors involved in these pathological mechanisms.

Consequently, our results could represent the first step of a new scientific approach useful to investigate diseases like PMF from a different perspective in which it is essential to consider the importance of the proper microenvironment setup.

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