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Soutenue le 13/09/2022, par :
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Impact of Tyrosine Kinase Inhibitors and Bone morphogenetic Proteins on persistent Leukemic Stem Cell dormancy in Chronic Myeloid Leukemia patients at remission

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Abstract:

Despite the therapeutic progress of Chronic Myeloid Leukemia (CML) due to the Tyrosine Kinase Inhibitors (TKIs) that targets specifically BCR-ABL kinase, TKI treatment is not yet curative since many CML patients still retain leukemic stem cells (LSCs) and undifferentiated progenitors in their bone marrow (BM) when treatments stop. The human BM is a complex and dynamic microenvironment that provides essential cues to resident cells. A single cell RNA-Seq analysis of BCR-ABL^{+} persistent cells have shown a co-enrichment of BMP signalling, quiescence as well as HSC signatures. In addition, by targeting both BMPR1B and JAK2/STAT3 we showed an efficient differenciation fostering effect on persistent and dormant LSCs hidden in their bone marrow (BM) microenvironment.

As we highlighted a LSC dormancy induction after imatinib treatment, understanding the molecular mechanisms contributing to LSC dormancy in the human BM dynamic ecosystem after CML treatment is likely to give new insights on treatment escape that takes place at remission. To decipher this post treatment pathological mechanistic we have developed a standardized three-dimensional (3D) co-culture model to elucidate pathophysiological mechanisms that control stem cells and take place during hematological and no hematological pathologies. For that, we constructed a biphasic calcium phosphate-based scaffold using human cell lines of mesenchymal stem cells (MSCs) and endothelial cells, thus ensuring a high reproducibility of the medullar niche. By following key steps, we obtained a well-organized bone marrow-like structure with collagen fibers and trabecular bone in which each human cell type could be observed by *in situ* imaging. Furthermore, we demonstrated that viable cells could be retrieved at the end of the 3D culture in order to perform any requested analytical or functional assays. Using this 3D system, we follow how CML treatments contributes to leukemic niche remodelling and monitors LSC quiescence. For this latter purpose, we use a CD34^{+}CD38^{-} LSC model expressing FUCCI system, allowing a dynamic analysis of all cell cycles fractions (G0, G1, early S and G2/M) in response to CML treatment.

Using this strategy, we will decipher the key elements that support LSC dormancy upon CML treatment and define new avenues enabling CML patients' relapse prevention.

List of abbreviations:

Lists of Abbreviations

ABC : ATP-Binding Cassette

ABL : Abelson

AGM : Aorta-Gonad-Mesonephros

ALL : Acute Lymphoblastic Leukemia

AML : Acute Myeloid Leukemia

AP : Acceleration Phase

BAMBI : BMP and Activin Bound Inhibitor

BC : Blast crisis

BCR : Breakpoint Cluster Region

BCR-ABL : Breakpoint cluster region-Abelson

BM : Bone Marrow

BMP : Bone Morphogenetic Protein

BMPR : Bone Morphogenetic Protein Receptor

CAR : CXCL12-abundant reticular

CCyR : Complete Cytogenetic Remission

CD : Cluster of Differentiation

CFC : Colony Forming Cell

CHR : Complete Hematologic Response

CLL : Chronic Lymphocytic Leukemia

CLP : Common Lymphoid Progenitor

CML : Chronic Myelogenous Leukemia

CML : Chronic Myeloid Leukemia

CMP : Common Myeloid Progenitors

CMR : Complete Molecular Response

CP : Chronic Phase

CR : Cytogenetic Remission

CSC : Cancer Stem Cell

DMEM : Dulbecco's Modified Eagle's Medium

DMR : Deep Molecular Response (=MMR)

DNA : Deoxyribonucleic Acid

ECM : Extracellular Matrix

EPO : Erythropoietin

ES : Embryonic Stem Cell

FACS : Fluorescence Activated Cell Sorting

FCS : Fetal Calf Serum

FISH : Fluorescence In Situ Hybridization

FITC : Fluorescein isothiocyanate

FLRG : Follistatin Related Gene

FLT-3L : Fms-related Tyrosine Kinase 3 Ligand

G-CSF : Granulocyte-Colony Stimulating Factor

GDF : Growth and Differentiation Factor

GFP : Green Fluorescent Protein

GM-CSF : Granulocyte Macrophage-Colony Stimulating Factor

GMP : Granulocyte–macrophage Progenitors

HPRT : Hypoxanthine-guanine-Phospho-Ribosyl-Transferase

HR : Hematologic Response

HSC : Hematopoietic Stem Cell

ID : Inhibitor of Differentiation

IFN : Interferon

IL : Interleukin

IMDM : Iscove’s Modified Dulbecco’s Medium

JAK : Janus Kinase

kDa : kilo Dalton

LIC : Leukemia Initiating Cell

LIF : Leukemia Inhibitory Factor

LIN : Lineage

LSC : Leukemic Stem Cell

LT-CIC : Long Term Culture-Initiating Cell

LT-HSC : Long Term-Hematopoietic Stem Cell

MCyR : Major Cytogenetic Response

MMR : Major Molecular Response

MNC : MonoNucleated Cell

MPP : Multipotent Progenitor

MR : Molecular Response

MRD : Minimal Residual Disease

MSC : Mesenchymal Stem Cell

mTOR : mammalian Target Of Rapamycin

NOD/SCID : Non-Obese Diabetic -Severe Combined Immune-Deficient

OCT : Organic Cation Transporte

PBS : Phosphate Buffered Saline

PC : Proportein Convertase

PDGFR : Platelet-Derived Growth Factor Receptor

PE : Phycoerythrin

PE-Cy7 : Phycoerythrin Cyanine 7

Ph : Philadelphia chromosome

qRT-PCR : quantitative Reverse Transcription - Polymerase Chain Reaction

RGM : Repulsive Guidance Molecule

RNA : Ribonucleic acid

ROS : Reactive Oxygen Species

RUNX : Runt Related Transcription Factor

SC : Stem Cell

SCF : Stem Cell Factor

SCT : Stem Cell Transplantation

SHH : Sonic Hedgehog

SKI : Sloan Kettering Virus

SMAD : Son of Mothers Against Decapentaplegic

SMURF : SMAD Ubiquitin Regulatory Factors

SNO: Ski-related novel sequence

SP : Side Population

STAT : Signal Transducer and Activation of Transcription

ST-HSC : Short Term-Hematopoietic Stem Cell

STI-571 : Signal Transduction Inhibitor number 571

TBP : TATA box Binding Protein

TGF-β : Tumor Growth Factor-β

TKI : Tyrosine Kinase Inhibitor

TPO : Thrombopoïetin

YS : Yolk Sac

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1 Introduction:

1.1 The normal hematopoietic system:

1.1.1 Haematopoiesis

Haematopoiesis is a natural lifelong process that starts in the human embryo to ensure entire blood cells production that the body needs. The hematopoietic system remains one of the first complex tissues developed in the mammalian organism genesis[1]. Regarding the mobility of the blood tissue, the obvious complication of the origin tracking tissue pushed the research to be intense in this topic. This process has been studied for over a century showing that even if some differences exist between species, the concept of hematopoietic process is preserved between vertebrates. The research in this branch enables to better understand the haematopoiesis dysregulation processes involved in cancers.

1.1.1.1 Hematopoietic Development:

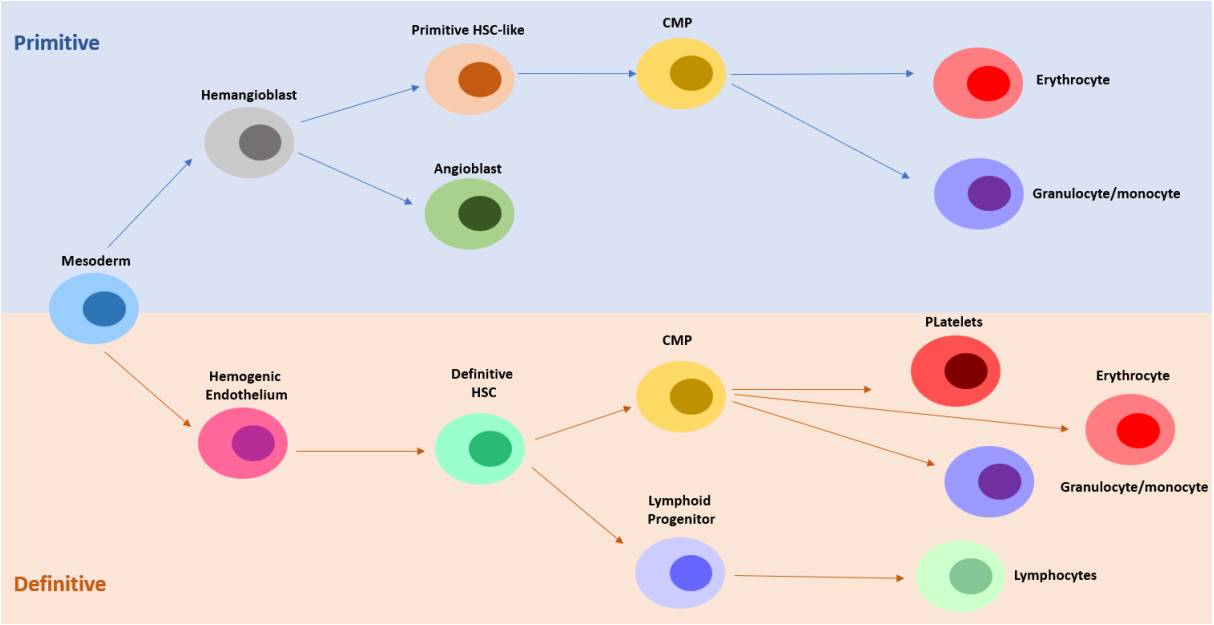

Figure 1: Description of the blood cellular components formation in the primitive and definitive waves. For the transitory primitive wave, the Hemangioblast emerging from the mesoderm will give rise to a primitive HSC-like cell as well as an angioblast. The produced cells from the primitive wave are not pluripotent and do no have the self-renewal feature. Regarding the definitive wave or the definitive hematopoiesis , the Hemogenic Endothelium originates from the mesoderm and gives rise to the adult HSC fitted with self-renewal and differenciation abilities enabling it to form all blood lineages. (*Adapted from Jagannathan-Bogdan et Zon, 2013*)

1.1.1.1.1 Embryonic Development:

Hematopoietic process starts from the embryonic development and is maintained all along the lifetime sustaining an homeostasis by constantly resupplying the body with needed blood cells[2]. Mammalians blood cell development occurs in different sites that are spatially and temporarily separated[3]. Moreover, the mobility of these cells mislead their characteristics and their origins for several years[1]. Nonetheless, in vertebrates, two waves of haematopoiesis, emerging from distinct anatomic sites do exist. The first one, called "primitive wave" and the second one "definitive wave"[2] (Figure 1).

In vertebrates, the appearance of the first blood cells takes place in the yolk sac (YS) simultaneously with the developing vasculature, it is called the "primitive wave" (Figure 2,3). This blood cells' appearance determines the beginning of human haematopoiesis that takes birth in the extra embryonic YS during the third week of developpement[3] (Figure 3). It involves the hemangioblast that gives birth to non-pluripotent primitive HSC-like cell deprived of self-renewal ability[2]. The latter will give birth to erythrocytes, granulocytes and monocytes[2] (Figure 1). The aim of this transient haematopoiesis is to supply the conceptus with oxygen through the erythrocytes and ensure phagocytosis through monocytes/macrophages as it undergoes fast active growth[4].(The conceptus is the embryo or the fertilized egg with its appendix or associated membranes. It represents the embryonic tissues that will endingly become a part of the final foetus and all the extra embryonic tissues that support foetal developpement[1].).

Considering their physical association and synchronized appearance, it has been demonstrated in birds that both primitive erythrocytes and endothelial cells have a common mesodermal precursor, the hemangioblast [5].

During the early primitive streak of mammalian embryonic development, the earliest group of mesodermal cells migrates from the primitive streak to form the extraembryonic hematopoietic tissues YS and later, the allantois. From this primitive streak emerges the hemangioblast that express Brachury marker and originates from embryonic stem cells (ESCs)[6](Figure 2). In mice, hemangioblast will migrate to the YS where it turns to committed hematopoietic progenitors and endothelial cells[1,2,7] (Figure 1).

A scientific discovery was published in the 70th supposing that the YS cells were the root of the hematopoietic system in adult mammalians. At that time, the literature described the YS cells migration to the bone marrow (BM) where they were believed to stay all along the adult hematopoiesis[8]. Thereafter, several grafting experiences confirmed that the YS is not a source of adult blood cells in vertebrates[9]. Indeed, orthotopic experiences in amphibians grafting of the ventral blood island VBI (yolk sac equivalent) and the dorsal lateral plate DLP(intrabody region) proved that the DLP is the source of adult hematopoietic cells[9].

In the amphibians, molecular marking on the embryos showed that the separation of the VBI & DPL mesoderm occurs very early, at the blastula 32 cell stage[10]. The C3 blastomere gives rise to the DPL (intraembryonic hematopoiesis) while the D4 gives rise to the posterior VBI and the C1 & D1 to the anterior VBI [10]. Discovery of this early stage distinction suggests that the progeny coming from the VBI and the DLP are acting spatially and temporally in a separate distinct way during the embryogenesis[1].

The definitive wave also called definitive haematopoiesis takes place later in embryonic development. The hemogenic endothelium, native from mesoderm will give birth to hematopoietic stem cells (HSCs) that are multipotent and able to generate all blood lineages for adult organism[2] (Figure 2,3).

In mouse model, from the E7.5 to the E10.5, the conceptus generates independently at least five distinct and unrelated classes of hematopoietic cells fitted with progressive increasing & more complex hematopoietic activities[1] (Figure 2). The primitive class comes from the hemangioblast while the pro, meso, meta and definitive classes derive from hemogenic endothelium. The first primitive hematopoietic cell is generated in the YS at E7.5 and acts like a primitive erythroid cell, the pro-definitive hematopoietic cell is developed at E7.5/E8 and appears in the YS, allantois and placenta, it behaves as an erythroid-myeloid progenitor. The Meso definitive hematopoietic cell is developed in E7.5/E8 in the Para-Aortic splanchnopleure (PSp) and operates as an erythroid-myeloid-lymphoid progenitor. Later in the developpement, the Meta-definitive hematopoietic cell appears at E9 in the YS and aorta-gonad-mesonephros (AGM). It functions as a neonatal repopulating HSC and finally the ultimate adult-definitive HSC fitted with a high stem cell potential is generated in the AGM at E10.5 and flows toward

other tissues acting as an adult repopulating HSC able to replenish the body with the whole blood cells [1,11] (Figure 2,3)

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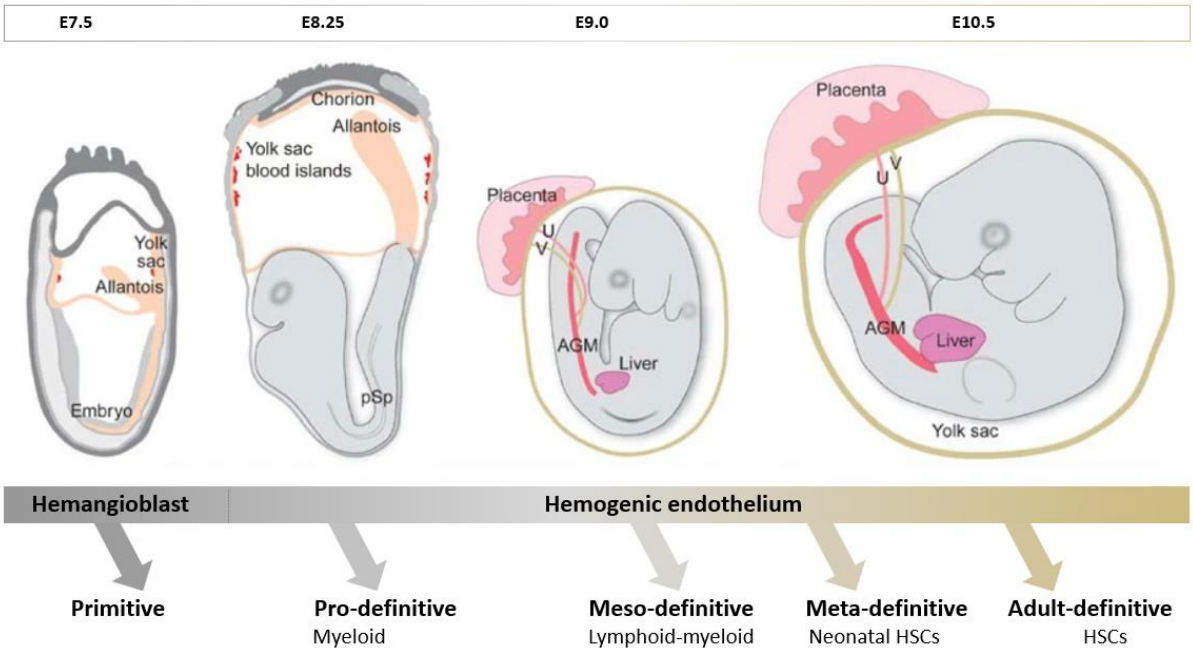


Figure 2: Chronologic description of the Mouse conceptus with the expression of different classes of hematopoietic cells during development.
Several functionally separated hematopoietic cells' classes are generated at different embryonic stages in mouse embryo. The Hemangioblast will give rise to the primitive class while Hemogenic endothelium vascular cells will give rise to the rest. At the conceptus E9 stage, the embryo is comprised in the YS and the present placenta emerged from a fusion between allantois and chorion. Also, in the late E9 hematopoietic progenitors will begin to colonize the conceptus and at E10.5 hematopoietic clusters will loge within AGM dorsal aorta, umbilical, and vitelline arteries where the first HSC will be found. Between E8.25 and E9 the blood circulation is established in mouse model. (Adapted from *Dzierzak et speck, 2008*)

1.1.1.1.2 Adult Hematopoiesis:

The source of adult hematopoietic cells is the intrabody region called dorsal lateral plate [9]. HSC is the root of adult blood cells and replenish the adult body with new blood cells throughout life. In mouse, HSC that is responsible of adult hematopoiesis appears in the E10.5 mouse embryonic day of development[7,11]. Its appearance takes place in the Aorta-Gonad-Mesonephros (AGM), a mesoderm embryonic region that comes from the para-aortic splanchnopleure in mouse and humans[12]. These HSCs able to ensure adult hematopoiesis are

exclusively generated in the AGM region and are located in the ventral aspect of the dorsal aorta [13]. Even if these HSC can be found in other tissues like the liver, they are not generated in the liver *de novo* but this tissue is colonized by these cells at the E9 in mouse[1] and day 23 & 30 of development in human[2,14] (Figure 3).

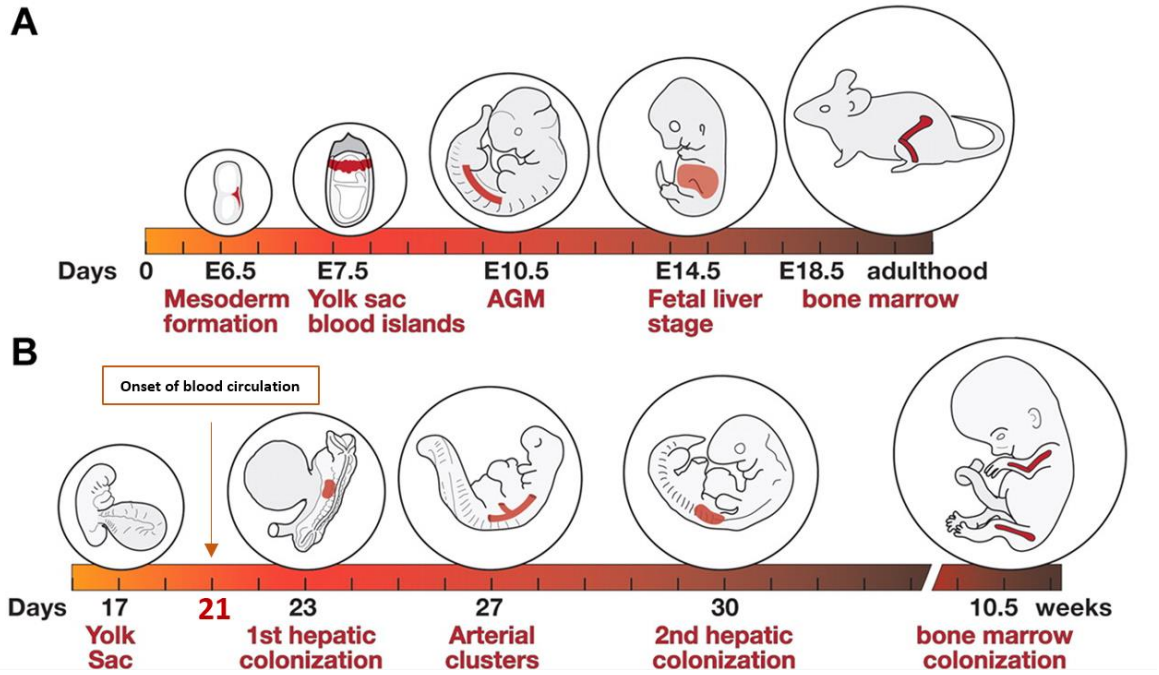


Figure 3: Hematopoiesis location origins in in mouse and human embryos

(A) In Mouse, at E 6,5 the mesoderm emerges during gastrulation. AT E7.5 YS & Blood islands appears. At E9, active blood circulation begins and at E10.5 The AGM, large arteries as well as placenta and liver do emerge. At E14.5 The mouse hematopoiesis takes place in fetal liver. Starting from E18.5, the hematopoiesis site is fixed in the animal bone marrow.

(B) In Human embryo, the hematopoietic development starts in the YS at day 17. Active blood circulation begins at day 21 and the first Hepatic colonization starts at day 23 while the arterial clusters formation takes place later at day 27. Second hepatic colonization is reported to appear at day 30 and finally, BM colonization begins at 10,5 weeks. (*Tavian et Peault, 2005*; *Baron et al., 2012*)

1.1.1.2 Hematopoietic cells:

During hematopoiesis, mammalian Hematopoietic Stem Cells (HSCs) represent multipotent somatic cells fitted with long term self-renewal and differentiation features[2]. HSC remains the crown of the blood cells' hierarchy (Figure 4). Its differentiation into different progenitors then mature cells with an increased commitment and a decreased lineage potential makes it able to generate the entire blood cells. HSCs are endowed and involved in several tasks such as the body protection ensured by white blood cells (WBCs) (eosinophils, basophils, neutrophils), body oxygen supply provided by erythrocytes and bacteria and toxic elements phagocytosis by macrophages[2] (Figure 4).

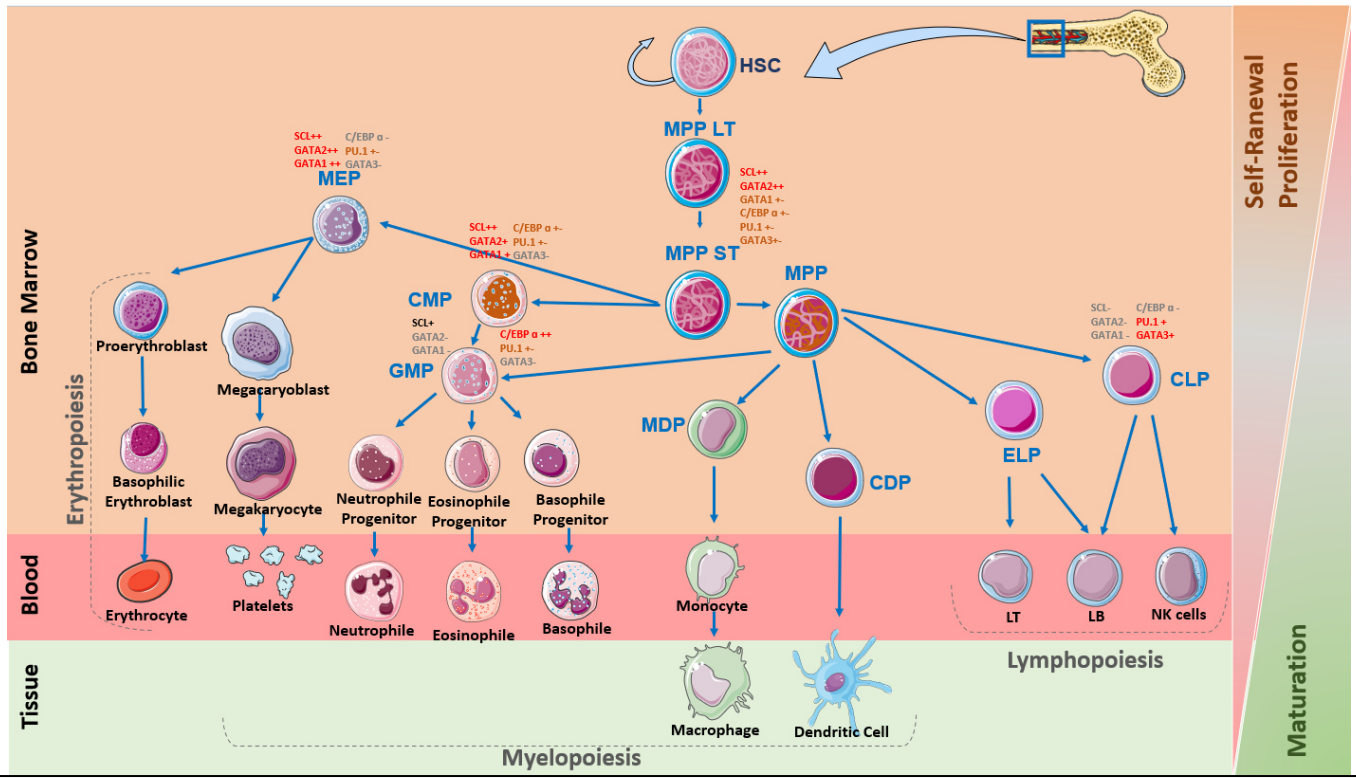


Figure 4: Hematopoiesis:
This process involves an HSC at the top of the blood cells hierarchy. Through proliferation and differenciation processes, HSC will generate the Long Term Multi Potent Progenitors Long Term MPP that will generate Short Term MPP. The latter will give rise to a multitude number of progenitors such as Megakaryocyte Erythroid Progenitor (MEP), Common Myeloid Progenitor (CMP) and Lympho Myeloid Primed Progenitor (MPP). Progenitors will undergo several proliferations decreasing their proliferation ability and increasing differenciation abilities by acquiring specific commitments to become mature cells.

1.1.1.2.1 Progenitors:

The uncommitted HSC is endowed with lifelong self-renewal ability. After integrating specific signals to divide, it may gives birth to a first progenitor and, through several divisions its self-renewal potential decreases while its commitment potential increases. The progenitors are mainly multipotent cells able to undergo differentiations into several cell types. The first progenitor originating from the HSC is called the Long-Term Multipotent Progenitor with intermediate repopulating capacity (LT-MPP). The latter will divide to generate the short term progenitor ST-MPP with short term repopulating ability[15]. Both LT-HSC and ST-HSC do highly express SCL as well as GATA2 genes while C/EBPα, PU.1 GATA1 and GATA3 remains more or less expressed[15]. The ST-MPP can either divide to give birth to two different precursors, the Megakaryocyte Erythroid Progenitor (MEP) and the Common Myeloid Progenitor (CMP) or it can lose its myeloid potential to differentiate into a Lympho Myeloid Primed Progenitor (LMPP or MPP). The CMP myeloid progenitor will express less GATA2 then its origin ST-HSC cell. Nonetheless, it will still highly express SCL and gain in GATA1 expression by losing the GATA3 expression[15]. MPP gives rise to the Common Lymphoid Progenitor (CLP) that will express GATA3 but not SCL, GATA1 or GATA2 genes[15]. MPP can also differentiate into Early Lymphoid Progenitor (ELP), the Granulocyte Macrophage Progenitor (GMP), the Monocyte Dendritic Cell Progenitor (MDP) as well as the Common Dendritic Progenitor (CDP)[15–18]. GMP progenitor will be highly enriched in C/EBPα expression[15] (Figure 4).

1.1.1.2.2 Precursors:

Unlike the progenitors, the precursors are unipotent cells, with a high commitment potential with the ability to differentiate into a specific type of cells. Precursors will give birth to mature blood cells with a limited lifespan and a high commitment potential[2].

The MEP can give either to the Megakaryocyte precursor or the Erythrocyte precursor. The first one will differentiate into Platelets while the second one differentiates into Erythrocytes. The GMP gives birth to Granulocyte progenitor and Monocyte progenitor, the first one gives birth to the three Granulocytes: Basophil, Eosinophil & Neutrophil and the second one gives the Monocyte Macrophage and Osteoclast mature cells. The Dendritic cells' precursor remains either the MDP or the CDP and two different types of dendritic cells do exist, the Monocytoid

Dendritic Cell & the Plasmacytoid Dendritic Cell. Concerning the CLP, it will give rise to Dendritic mature cells, B Cell progenitor or T/NK Cell progenitor. The B Cell progenitor differentiates into Lymphocytes B cells. The T/NK precursor differentiates into lymphocytes T cells or lymphocytes Natural Killer cells [15–18].

1.1.1.2.3 Mature Cells:

Platelets are small discoid mature nucleated cells deriving from megakaryocyte precursor[19]. A recent study showed that platelets production can either happen in the BM or in the lung [20]. During their life cycle, their size deceases this young platelets are larger than the old ones[19,21]. Platelets are known to release small particles in blood circulation and to sample the blood environment. The released particles contain miRNA, mRNA, proteins or enzymes that may play a role in the presentation of the foreign bodies (virus, bacteria,…) to the immune system[19]. As well, it has been shown that platelets express Toll-Like Receptors (TLRs) that are deeply involved in immunity[22]. Finally, platelets display the ability to do autophagy but in an unclear manner[23].

Erythrocytes are red blood cells that confers red colour to blood. These cells originate from a multipotent cell called "hemocytoblast" in the mesenchyme. The fully mature cell is named reticulocyte. Erythrocytes have a flexible shape with a membrane composed of lipids and proteins, their highly distortable shape makes them able to go through capillaries with a diameter narrower than the cell's diameter itself. Erythrocytes contain hemoglobin, a four folded chains of globin, each one binded to a heme that contains an iron ion (Fe^{2+}) and do supply oxygen for the entire body tissues and absorbs CO_2 in return. The absence of organelles such as nucleus, mitochondria or microsomes make them unable of *de novo* synthesis of proteins or lipids and undergo Glycolysis to generate energy [24].

Granulocytes are Basophils, neutrophiles and eosinophils and do derive from GMP progenitor (Figure 4). Basophils exit the BM once they are fully matured except in an inflammation context where the white blood cell will undergo an extramedullary hematopoiesis. Their number increase in case of several diseases like allergy[25], acute or CML [26,27]. Basophil response can be regulated either by IL-3/IgE dependant mechanism or by thymic stromal lymphopoietin (TSLP) dependant mechanisms. The IL-3 mediated activation involves histamine production

and occurs during food allergy, urticaria of allergic rhinitis while TSLP mediated activation leads to IL4 production and occurs during atopic dermatitis, esophagitis and other diseases[28].

Neutrophiles population is a body weapon. Their production takes place in the BM specifically in the venous sinuses and derive from the common myeloid progenitor CMP [29]. To head this commitment, the cytokine 'granulocyte colony stimulating factor G-CSF is mandatory, indeed this cytokine is involved from progenitor differentiation to mature neutrophiles exit from the BM [30,31]. Neutrophils activation can be stimulated either by cytokines, growth factors or pathogens, this activation will push mature neutrophile to progress out of the bone marrow to achieve the infected site[32]. Phagocytosis happens when neutrophils encounters microorganisms, immune cell will encapsule the microorganisms in phagosomes destructing them using NAPDH dependant mechanisms or antibacterial proteins like lysozyme, lactoferrin or cathepsins and neutrophils are the most efficient granulocytes performing phagocytosis [32,33].

Eosinophil needs PU.1, GATA-1, c/EBP (CCAAT-enhancer binding protein) as a mandatory genes for a specific differentiation and granule proteins production[34,35]. Furthermore, a number of cytokines including GM-CSF, IL-3 and more specific IL-5 are needed for eosinophil's maturation from precursors[35]. When microorganism is found, eosinophils perform phagocytosis using their granules that contain galactin 10 and contribute to parasites killing[36].

Monocyte are derived from the common myeloid progenitor and can differentiate either into macrophages or dendritic cells (DCs). A normal monocyte is twice larger than an erythrocyte and its count is comprised between 2 and 8% of WBC, which equals to 200-800 monocytes per blood microliter in healthy adults with a slight larger monocyte number in men compared to women[37]. A disequilibrium in this count can be a monocytosis where monocytes count is too high like in in leukemia & other cancer diseases or monocytopenia when the monocyte counts is too low which is the case after a reaction to a chemotherapy[37]. These powerful body defenders are not abundant in blood circulation but represent a vital role in body protection, they closely patrol for pathogens threats and differentiate when needed[37].

Macrophages are a key actor in the innate immune system process as they can be APC (Antigen Presenting Cell). It has long been considered that macrophages are exclusively

originating from peripheral monocytes but recent studies deciphered that monocytes mainly replenish and maintain macrophages population but early macrophages are directly differentiating from the YS progenitors[38–40] (Figure 1). These cells act like a phagocyting machine against foreign material like bacteria and viruses but also against dead body cells by engulfing its cell's debris through a *de novo* formed pseudopodia[41]. Macrophages are professional phagocytes and are fully differentiated and specific for some tissues, like Langerhans cells in the skin, Kupffer cells in liver and microglia in central nervous system, alveolar macrophages in lung alveoli and splenic macrophages in spleen[39,41,42].

Dendritic cells remain the major APC in the immune system. After a target intake such as microbial pathogens or apoptotic cells, DC are able to undergo phagocytosis, endocytosis and pinocytosis to internalize its target. After internalization, engulfed targets proteins will be cleaved into peptides and presented via HL1 class 2 molecules to CD4+ T cells in order to link the adaptative immune response[43].

Lymphocytes can either be LT, LB or Natural Killer (NK) and do originate from lymphoid progenitor. NK represent small cells of 6-7µm diameter and enclose perforin and granzymes cytotoxic granules[44,45]. They are involved in innate immunity and classified as innate lymphocytes. These cells are recognized to fight body virally infected cells and controlling early cancer signs and the detection of aberrant cells by NK involved IL-12, 15 and 18 [46,47]. Their ability to kill cancer cells does not require any prior activation as an antigen presentation for example and this is why they were named "Natural" Killers and stay a highlighted path for cancer research therapy[48]. As cancer & infected cells lose their MHC I the NK call will be activated and proceed to cytotoxic enzymes release to lyse the target cell[49].

LB cells express clonally diverse cell surface immunoglobins (Igs) receptors that recognize specifically antigenic epitopes. LB cell receptor (BCR) structure is composed of four peptides of two light chains and two heavy chains embedded in cells transmembrane region. Light chains can be either lambda or kappa and heavy chains can be different isotypes among IgA, IgD, IgG,IgM or IgE. LB cells digest the pathogen and present its antigen at their cell surface through MHC complex. Memory B cells will "memorize" the antigen to help immune system

responding in a quick manner to future invasions by the same pathogen whereas plasma cells will act as sneaks by producing antibodies that will tag pathogens making it a visible target by other immune cells [50].

LT cells coordinate several aspects of the adaptative immunity. They differentiate into more specialized LT cells called Regulatory, helper, memory T cells or cytotoxic T cells[50]. After this differentiation they will migrate through the blood or lymphatic system to achieve peripheral tissues. Peripheral T cells include naïve t cells, memory T cells and regulatory T cells (Treg)[50]. The Treg cells are tasked with the ability to stop immune response once the threat eliminated. Treg cells also stop helper and cytotoxic T cells when it binds accidentally to a non-dangerous cell. Their activation results in an IL-2 production, proliferation and differentiation of effector cells that migrate to the concerned site to proceed to a pathogen clearance Depending on the APC, the naive T cell becomes an effector T cell, either helper, cytotoxic or regulatory T cell. Helper T cells are CD3+ CD4+ , they have a wider range of function compared to cytotoxic LT. We note that activated effectors have a short lifespan but their percentage increase in some leukemias [50,51].

1.1.1.3 Signalling regulation of hematopoiesis:

1.1.1.3.1 Cytokines & Growth Factors:

Cytokines are small molecules that were firstly found in 1950 while scientits were culturing lymphocytes. They discovered that supernatant of cultured cells comprised biologically active soluble substance produced in response to an infection stimulation and responsible of cells' differentiation and proliferation induction. Cytokines are short half-lives molecules produced in very low amounts mainly by macrophages and Th cells in response to an antigen recognition and do represent a wide range of "communication" molecules that share many features with growth factors (GFs) and hormones.

Main differences distinguishing cytokines from GFs and hormones are their expression, production, range of induced effects as well as their target cells. Cytokines can either be upregulated or induced while GFs undergo a constitutive expression and hormones an induced

one. Cytokines are expressed by several cell types with a wide tissue distribution and GFs are also produced by several cell types but with a moderate tissue distribution meanwhile hormones are expressed by specialized well types in glands. Finally, cytokines have autocrine and paracrine effect on several target cells while GFs have a more paracrine/endocrine effect on a broad range of cells and variously hormones do have an endocrine effect on a very limited target cell.

1.1.1.3.1.1 TPO:

Thrombopoietin has a lineage specific role in megakaryocyte production. This cytokine is mainly produced by the liver[52] but a smaller production occurs in spleen, kidney or by stromal cells within the BM[53,54]. It plays a role in megakaryocyte lineage commitment to foster megakaryocyte progenitors proliferation and do increase megakaryocytes' ploidy[55,56]. Megakaryocytes remain the unique hematopoietic cell lineage affected by TPO loss, nonetheless, TPO appears to have a role in HSC quiescence maintenance during adult mice hematopoiesis[57]. In fact, it has been suggested that through β1 integrin activation, TPO preserves immature $CD34^+$ $CD38^-$ HSCs by stromal adhesion interaction[58]. Its downstream effect involves an hematopoietic cytokine receptor called MLP on which the TPO binds, the binding will dimerize MPL allowing phosphorylation of Jak2 molecules that will activate in turn MPL receptor[59,60]. This activated pathway triggers either a downstream MAPK, PI3K or STAT3 and STAT5 signallings[61–63] to induce several biological responses through p21, P27, Cyline D1 or BCL-X espression[64–66]. In case of chemotherapy-induced- thrombocytopenia[67], a lack of MPL^+ cells leads to a low TPO clearance, hence an increase TPO availability supplemented by increased stromal TPO production leading to an increase of SC quiescence[57]. Patients suffering from loss of function mutations in MPL that leads to a megakaryocytic thrombocytopenia (CAMT) do progressively develop BM failure[68,69]. As well, overactivation of Jak2 and active mutations in both MPL and JAK2 has been described in myeloproliferative disorders (MPD)[70–72].

1.1.1.3.1.2 EPO:

Erythropoietin is a peptide hormone produced in human fetal liver during embryogenesis.

EPO's production is an hypoxic process mainly ensured by peritubular fibroblasts in the renal cortex of adult human kidney[73,74]. Nonetheless, other secondary small production sites as spleen, liver, brain or bone marrow (BM) do exist in human adult body[74]. Human erythropoietin is a glycoprotein formed by 166 aa which is approximately a 30.4 kDa molecular weighted protein (UniprotKB P01588). Its 3D structure is made of 4 α-helices, Helix A is connected to Helix D by Cys7 and Cys161, while Helix A and Helix B are connected by Cys29 and Cys33[75]. To initiate a signalling pathway, the EPO will interact with its homodimeric EPO Receptor in human hematopoietic cells. This binding will result in Jak2/STAT5 phosphorylation cascade that activates NF-kB activation and triggers the major erythroid transcription factor GATA-1[76,77]. We note that an EPO-initiated-signalling pathway occurs in non-hematopoietic cells (like heart, brain skeletal or muscle cells) through the heterodimeric receptor EPOR/CD131 that can result in a TGFβ activation[78–81]. EPO has a pleiotropic function as it acts on hematopoietic and non-hematopoietic cells. Its function in deeply involved in BM erythropoiesis activation. Contrary to expectations, very recent study demonstrated that EPO acts directly on BM-HSCs leading to (Common Megakaryocyte erythroid progenitor) MEP's formation[82]. Later in the differentiation process it also acts on the BFU-E and CFU-U erythroid responsive clones then the erythropoiesis continues with an increase of hemoglobin levels in the proerythroblasts (ProEB) and the orthochromatic normoblast (ON) that still contains a nucleus, finally the EPO will be expressed in the reticulocytes and the mature red blood cells[83]. EPO is also responsible of heme biosynthesis within the mitochondria and transferrin-mediated iron transport[84]. EPO does promote bone remodelling by increasing the osteoblastic nut not the osteoclastic activity[85]. Besides being the major hematopoietic growth factor (HGF) responsible of daily massive RBC production through HSC induced-erythropoiesis, Human endothelial and mesenchymal Stem Cells (MSCs) are also EPO targets to induce their angiogenic differenciation[86].

Cytokines' function includes regulation of innate & adaptative immune responses and induction of hematopoietic cells' differentiation. A cytokine can activate another cytokine's production while some cytokines may repress other cytokine's effect or production, plus, the cytokines family knows a certain redundancy to the extent that some Interleukins (ILs) can surrogate the absence of other lacking ILs , for instance IL-3$^{-/-}$ KO mouse had few abnormalities despite IL-3 key role in hematopoiesis[87].

1.1.1.3.1.3 IL-11:

IL-11 is deeply involved in several hematopoietic processes, but its main role is the increase of megakaryocyte production through megakaryocyte precursor. It also has anti-inflammatory features as it inhibits TNFα, IL-1, IL-6, IL-12 and Nitric Oxide (NO) macrophage-mediated production as well as IFNγ Th1-mediated production. It also fosters LB LT and Neutrophils proliferation. Nonetheless, its range of action is not restricted to BM cells, it also acts on fibroblasts in so far as a promoter of collagen deposition. This cytokine is primarily produced by stromal cells within the BM and secondarily by osteoblasts, testes, brain cells and joints. It has a shared gp130 receptor with the IL-6 and the activated downstream signalling pathways involves both STAT1 and STAT3 proteins[50].

1.1.1.3.1.4 IL-12:

IL-12 has a fundamental role in both innate and adaptative immune responses. It dramatically increases IFNγ production which is essential for host defence through macrophages, NK and Th0 stimulations. Indeed, it fosters naïve Th0 and memory $CD4^+$ T cells into IFNγ secreters. It also fosters this IFNγ production through a cooperation with IL-18 by Th1 and NK cells activation. IL-12 does not play a direct role in hematopoietic progenitors' differentiation, but it monitors it indirectly through the induction of GM-CSF production by DC and macrophages and this cytokine will play a key role granulocyte differentiation[50].

1.1.1.3.1.5 IL-3:

IL-3 acts on different hematopoietic cells. It has been demonstrated that this cytokine enhances HSC growth and differentiation. It also stimulates early hematopoietic progenitor for an expansion of matures cells. It has been showed that IL-3 is essential for mast cells and basophils proliferation and immune response against pathogens. It is also a pro-proliferative factor because of its effect on the anti-apoptotic Bcl-2 upregulation. The production of this

cytokine is mainly achieved by activated CD4+ T cells but some specific mast cells with an Fce receptor connected to an IgE can also produce IL-3. Its receptor is expressed in early hematopoietic cells and mostly in myeloid derived cells. It is comprised of IL-3Rα (=CD123) and a βc chain they can be upregulated by several inflammatory cytokines. IL-3 downstream signalling involves Jak2/STAT5 and Ras/MAPK pathways[50].

1.1.1.3.1.6 G-CSF:

As its name outlines, Granulocyte stimulatory factor (G-CSF) growth factor is involved in granulocytes differentiation and survival. Specifically, to initiate a neutrophile production in normal or emergency induced production in case of an inflammatory signals, either from the HSC or from CFU-GM. It's differentiating property made it useful in the clinical therapy where it is used in neutropenic patients or in patients suffering from a poor bone marrow function caused by chemotherapy. This GF is produced within the BM by several cells such as activated LT, endothelial cells, fibroblasts, and some phagocytes. Its receptor needs to dimerize to bind the G-CSF and it is expressed on granulocyte/monocyte precursors. Its downstream signalling appeals a jaK2/STAT3 and Jak1/STAT1 proteins[50].

1.1.1.3.1.7 GM-CSF:

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a GF that promotes haematopoiesis. Its action is to drive hematopoietic commitment of CMP into CFU-GEMM. Under GM-CSF influence CFU-GEMM differentiate into either CFU-GM or CFU-BME. In addition, it has been showed that GM-CSF activates macrophages in induces DCs differentiation. This GF is produced by several cells like activated LT, endothelial cells, macrophages, and stromal cells. Its receptor comprised two chains, a GM-CSF ligand-binding chain a and a non-binding -ligand chain bc that is common for IL-3 and IL-5 receptors. Downstream effects of GM-CSF do involve Jak1/2 followed by STAT5 activation[50].

1.1.1.3.1.8 IL-5:

Interleukin 5 is a homodimer known for its effects on eosinophils. It increases their differentiation, activation as well as their chemotaxis. It increases mature eosinophils' competence to kill pathogens by degranulation. It can also act directly on other cells like mast cells by increasing their histamine release or LB cells by increasing their proliferation and

differentiation. This cytokine is mainly secreted by activated Th2 cells and can also be produced minimally by eosinophils, NK, activated mast cells and B cells. We note that IL-5R is composed by an IL-5a binding chain and a βc non-binding chain that is mostly expressed by eosinophils, mast cells and basophils. Downstream effects of IL-5 pass through Jak2/STAT5 signalling[50].

1.1.1.3.1.9 M-CSF:

This GF is involved in CFU-GM differentiation into macrophages and monocytes. Plus, it has been showed *in vivo* on a mice model that it is involved in osteoclast generation. M-CSF is produced within the BM by fibroblasts, some phagocytes as well as endothelial cells. Its receptor has un intrinsic tyrosine kinase activity and is present on committed monocytes cell membrane[50].

1.1.1.3.1.10IL-6:

Interleukin-6 is the perfect example of a pleiotropic cytokine. It acts in inflammation process as well as adaptative immune response. It is also involved in the endocrine system, in the nervous system and in the hematopoiesis. It fosters neutrophils phagocytosis processes and seems to have a major role in the final steps of B cells' differentiation as well as LB-mediated mucosal Ig1 production. IL-6 showed a supporting LB, LT, megakaryocytes, macrophages, some neurons and osteoclasts maturation and differentiation. As well, it stimulates hepatocytes for fibrinogen production. In response to IL-1 / TNF stimuli, endothelial cells, fibroblasts, and some phagocytes are able to secrete IL-6. Its receptor comprised an IL-6Rα IL-6 binding chain and a gp130 non-binding chain that represent the intracellular signal transducer. IL-6R is mostly present on activated LB cells and less frequently on PB monocytes hepatocytes neutrophils and mature T cells cell surfaces. Once IL-6 is bound to IL-6Rα chain the dimerization of the receptor chain with gp130 will be able to activate intracellular Jak1, Jak2 or Tyk2 proteins through its intracellular Box1/Box2 domain. These Janus kinases will either activate STATs or SHC-Grb2-Sos intracellular domain. Activated STATs includes STAT1,

STAT3 and STAT5 that can either activate NF-IL6 transcription factor (= C/EBPβ), the latter will promote Type I IL-6RE sequence to encode several genes expression like IL-6 gene or activate STAT3 dimer by a nucleic translocation that will target Type II IL-6RE to promote other genes like fibrinogen gene expression. When SHC-Grb2-Sos is recruited, a Ras/MAPK will be engaged to activate some gene expression like c-myc[50].

1.1.1.3.1.11IL-2:

IL-2 is engaged in LT differentiation and has an anti-cancer effect. It increases naïve Th, naïve Tc, γδ T and LB cells activation, proliferation, and differentiation. As well, it activates monocytes and synergize with IL-12 to activate NK proliferation and differentiation into aggressive tumour killing cells (LAK cells) . IL-2 major production is covered by activated T cells after antigen recognition and CD28/B7 interaction. IL-2 receptor has a high affinity for its ligand and is comprised of IL-2Rα (= CD25), IL-2Rβ and IL-2Rγ(=CD132) chains. Nonetheless, in human body, a form of IL-2 receptor comprised of only IL-2Rβ and IL-2Rγ do exist but transduce very low levels of signals resulting from IL-2 binding. Cells expressing IL-2 receptor are B & T cells, monocytes, macrophages and NK cells. After IL-2 binding, Jak1 and Jak3 will be recruited to the intracellular region of both IL-2Rβ and IL-2Rγ. The downstream activation of Src and nuclear translocation of STAT3 & STAT5 will enable cell growth, survival, transcriptional regulation, and effector differentiation[50].

1.1.1.3.1.12SCF:

Stem Cell Factor, also called c-kit (Or CD117) ligand, Steel factor or mast cell growth factor is a cytokine that regulates HSC at different development stages. *In vivo*, SCF maintains early hematopoietic precursors growth and survival. *In vitro*, SCF cytokine is sufficient for HSC survival and self-renewal to generate a pool of undifferentiated SCs. SCF do act on HSC and progenitors but may also synergize with other cytokines and GFs to enhance proliferation and commitment to either lymphoid or myeloid lineages. Indeed, its role is essential for mast cells growth, chemotaxis, adhesion, proliferation, and degranulation. In mice model (SI mutant), it has been showed that a lack of SCF cytokine may be lethal for the embryos and in case it is not, the prenatal model suffers from severe anemia. In humans, a mutation of SCF receptor

called W mutation do exist and gives rise to a clinical disorder called "piebaldism". The latter is not an anemic disorder but causes a pale skin areas and white hair sections on the patient's body. This suggest that SCF is also required for a good melanocyte's survival, proliferation and migration since the foetal development. SCF production is mainly produced by stromal cells in BM, fetal liver and thymus but also by central nervous system as well as gut cells. Two forms of SCF do exist. A secreted free homodimer and a membrane bound form that is present of the secreting cells. This last form is the most important one for hematopoietic cell development but mb-SCF do also maintain HSC quiescence state[88]. Some cytokines may influence SCF production to increase or decrease its effects. TNFα & IL-1 inflammatory cytokines can slightly increase SCF production within the BM while TGFβ can slightly decrease it. SCF receptor is called c-kit or CD117. It has an intracellular tyrosine kinase activity and is highly expressed on pluripotent HSC within the BM, nonetheless it is also found on some CNS & gut cells. SCF ligand binding on c-kit homodimer can activate different downstream responses within the HSC. It can either activate PLC-γ or PI3K PKB/Akt pathway to induce cell survival, or p38 & SAPK/JNK pathways to activate several gene transcription, or through a Ras/MAPK or Jak2/STAT1 enhance SHC proliferation. We note that some cytokines as IL-4 TGFβ or TNF downregulate c-kit receptor expression[50].

1.1.1.3.2 BMPs: Key role cytokines:

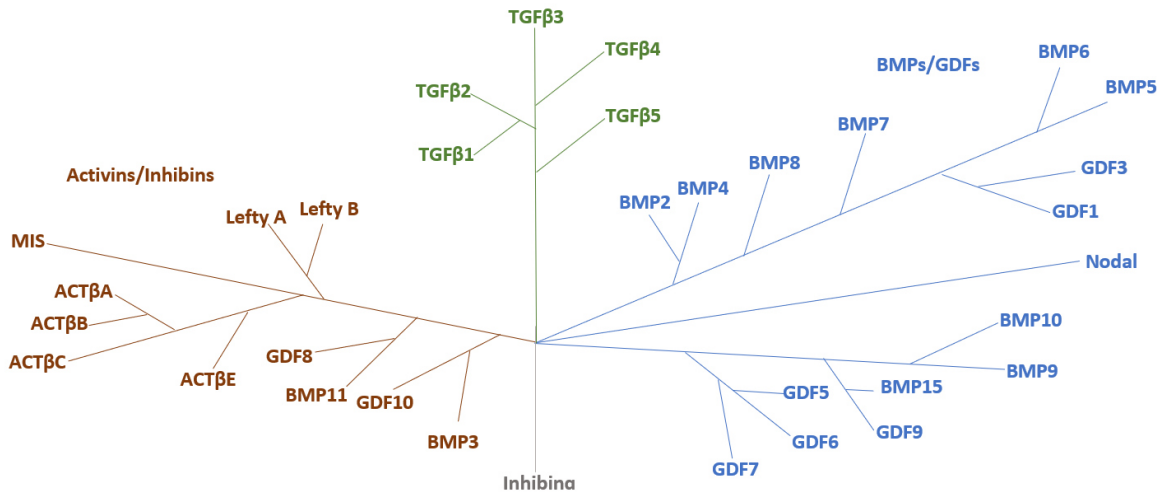


Figure 5: TGFβ Superfamily.

TGF β Superfamily comprises 4 sub-families of cytokines. These sub-families are the TGF β family, BMPs/GDFs Family, Inhibina family and Activins/Inhibins family. (*Adapted from Luo et al., 2019*)

Bone Morphogenetic Proteins (BMPs) discovery dates back to 1965 when Marshall Urist described these small molecules for the first time after demineralized bones implantation in rabbit musles[89]. These molecules had specific ability in bone formation induction[90] and It needed more than two decades to characterize human BMPs for the first time[91]. BMPs are a set of different cytokines involved in many physiological processes. Non exhaustive list of their dysregulation involves BMP2,BMP4 in brain glioma and Alzheimer's disease, BMP2, BMP4 and BMP7 in pulmonary arterial hypertension, BMP2 in muscular dystrophy & osteoporosis and both BMP2 & BMP4 is Chronic Myeloid Leukemia[92,93]. As of today BMPs do belong to the TGFβ superfamilly[94] (Figure 5). The latter includes several cytokine subclasses such as activin/inhibin, TGFβ as well as BMPs/GDFs sub-classes comprising more than fifteen different BMPs[92]. As many research projects were focused on TGFβ superfamily cytokines, many of these molecules were discovered at the same time by different researchers, the reason why a same ligand could have different names. For instance,BMP14 is also GDF15[95]. The numerous set of ligands/ receptors and downstream actors of this superfamily has been extremely conserved through evolution and is involved is several biological normal and cancer processes such as cell adhesion, growth, proliferation, differentiation, migration and apoptosis[94,96].

1.1.1.3.2.1 Structure:

BMPs are dimeric molecules formed by approximately 120 aa. Aside from BMP8 that comprises 8 Cys residues, all other BMP members do comprise 7 Cys residues. They can be classified in 4 groups depending on their similarities. The first group comprises BMP2 & BMP4 that have 80% of homology (Figure 6). The main difference between these two cytokines remains in the amino terminal region that contains a heparin-binding domain for BMP2 and not for BMP4. The second group includes BMP3 and GDF10 (also called BMP-3B or osteogenin). The third group comprises BMP5, BMP6, BMP7, BMP8a&b cytokines that have 78% of homolog. And finally the fourth group includes GDF5, GDF6 and GDF7 that are cartilage derived morphogenetic proteins 1, 2 and 3[97].

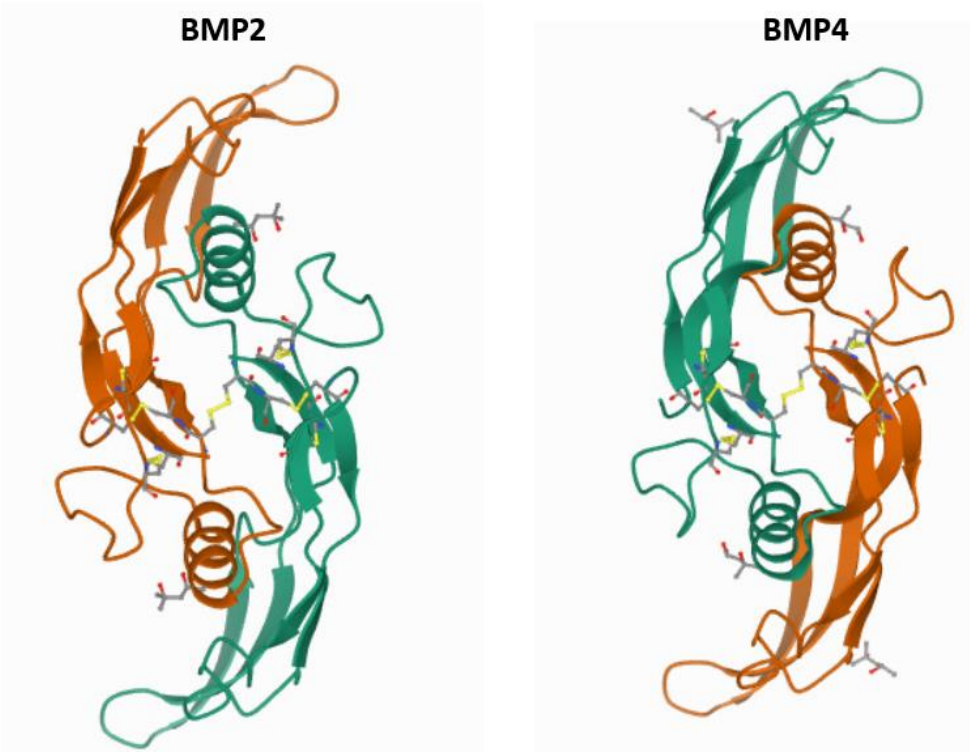


Figure 6: BMP2 & BMP4 homology.

BMP2 and BMP4 do share about 80% of structural similarities and do belong to the same branch of TGFβ superfamily. (The *Guide of pharmacology*)

BMP2 also called BMP2A is a homodimer with a disulphide bond between 78 position of Cys residues. Disulphide bonds are present between each chain position at Cys residues 14 & 79; 43 and 111; 47 & 113. Regarding its similarities with BMP4, the latter is also called BMP2B.

The active BMP4 peptide is a homodimer with a disulphide bond between Cys at position 80

as well as Cys residues with a N-linked glycosylation of asparagine residues at 58 & 73 positions plus disulphide bond formation between Cys residues 16 & 81; 45 & 113 and 49 & 115 (Figure 6) (Guide to pharmacology).

1.1.1.3.2.2 Maturation & production:

BMPs are produced as longer precursors chains sizing between 369 to 513 aa [98]. These long precursors are synthetized within the cells' cytoplasm as dimeric pre-pro-protein complexes containing a peptide signal, a pro-peptide & a C-terminal mature region containing a cystine knot motif. Thereafter, this form undergoes post translational proteolytic processing events[98]. The dimerization & glycosylation of this precursor will happen within the Golgi apparatus[97,99]. To be fully activated, the dimeric precursor needs a cleavage ensured by pro-protein convertases (PC) and serine endo-proteases that will firstly cleave the precursor at S1 then at S2 before mature protein release[97,100](Figure 7).

Within BM compartment, BMPs are mainly supplied by osteoprogenitor cells, osteoblasts, platelets, megakaryocytes as well as stromal cells[101,102].

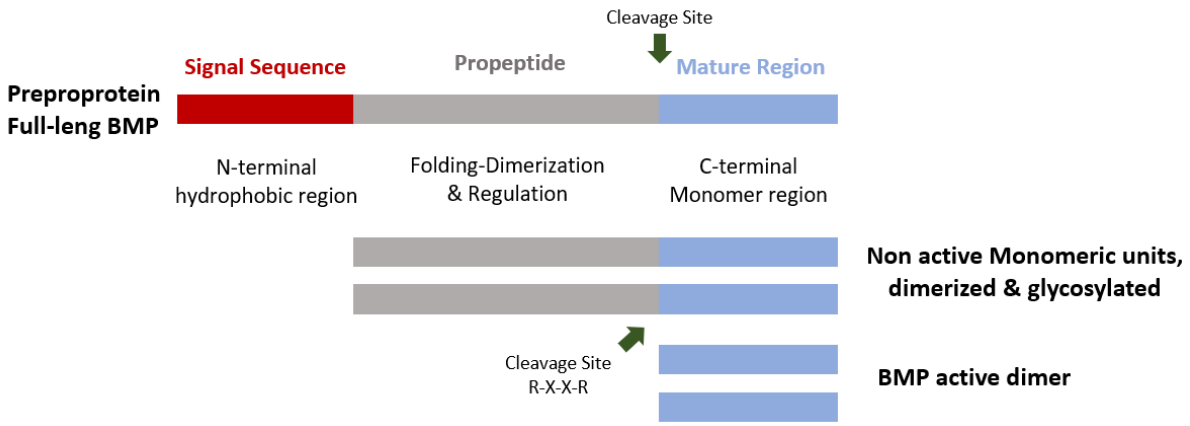


Figure 7: BMPs activation & maturation.

BMPs are synthesized in cells' cytoplasm in a Preproprotein precursor form sizing from 369 to 513 amino acids. Precursors are produced within the cytoplasm as dimeric pro-protein complexes. Convertases & serine endoproteases will act in the cleavage sites (arrows) to release active & mature BMPs monomers and heterodimers in cells' cytoplasm. (*Adapted from Sharma, Sharma 2019*)

1.1.1.3.2.3 Signalling pathway & regulation:

BMPs downstream induced signalling pathway can be either canonical or non-canonical. The canonical pathway consists in BMP ligands recruitment in an homo or hetero dimer forms on heterotrimeric receptors comprising Type I and Type II dimers. Each of these receptors do have a serine threonine kinase activity[103](Figure 8). BMPs can either bind to Type II receptor that will be phosphorylated and will recruit Type I receptor, or directly bind the extracellular part of Type I receptor when Type II receptor is also present[104]. Extracellular BMPs binding to Type I & Type II receptors will induce an intracellular phosphorylation of the receptors Ser/Thr Kinase domain that will in turns phosphorylate intracellular proteins called SMADs[105]. The Phosphorylation of R-SMADs complex comprising SMAD1, SMAD5 and SMAD8 (also called SMAD9) on their carboxy-terminal S-S-X-S motifs will call and phosphorylate another SMAD partner called SMAD4 or Co-Smad. The complex of SMAD1/5/8 & SMAD4 (R-SMADs/Co-SMAD) will translocate into the nucleus to interact either with co-factors, transcription factor or repressors and bind to a consensus DNA sequence named SBE (SMAD Binding Element) to trigger specific gene expression or repression[105,106].

BMP canonical pathway activates some target genes expression such as *Runx* (Runt-related), *ID* (Inhibitor of differentiation) and some *SMADs* genes families[105,107,108] (Figure 8). Aside from this canonical pathway, several studies proved the activation of non-canonical pathways totally or partially by BMPs contribution[70,109–111]. BMP signalling have been described to be directly or indirectly involved in several signalling pathways such as PI3K (Phosphoinositide 3-kinases), ERK (Extracellular signal Regulated Kinases), Cdc42, MAPK & p38 (Mitogen Activated Protein Kinase), JNK (Jun N-terminal Kinase) and Jak2/STAT3 pathways. These non SMADs pathways may cooperate with the canonical SMADs pathway to regulate wide cellular responses[70,111,112].

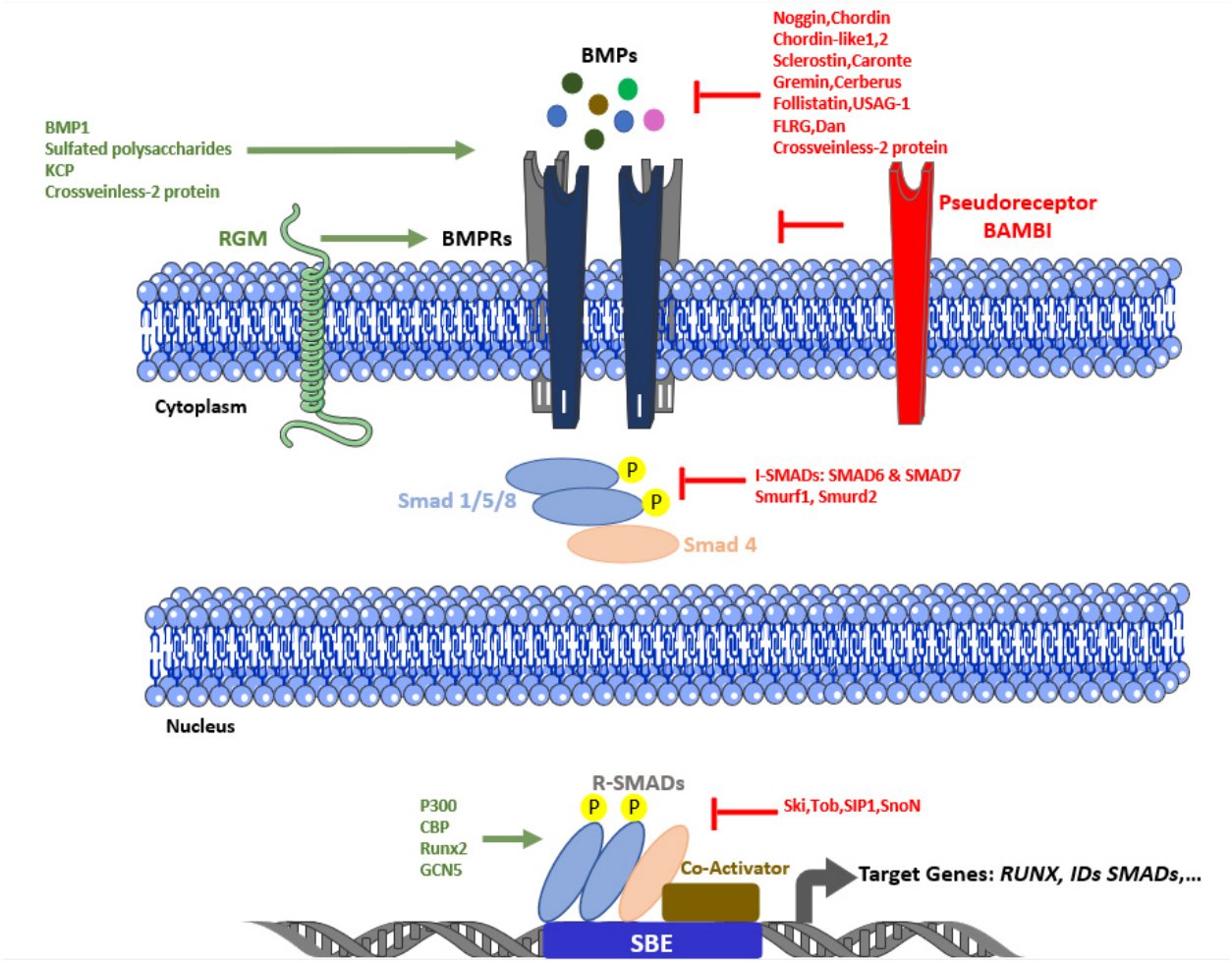


Figure 8: BMPs canonical signalling pathway.

BMP canonical signaling pathway activates SMAD1/5/8 that recruits SMAD4 for a nucleus translocation before activating target genes such as *RUNX, IDs,* and *SMADs.* This pathway has numerous potentiators that can activate the pathway at the membrane level of et the nucleus level. As well, a multitude of antagonists do inhibit this pathway downstream signaling at the cell membrane, cytoplasm level or at the nucleus level.
(*Adapted from Katagiri, Watabe, 2016*)

1.1.1.3.2.3.1 BMP repression

BMP signalling pathway is controlled at different levels by numerous and variable soluble or membrane regulators. Several antagonists do interfere to block BMP ligand to its receptor, among them Noggin, Chordin, Chordin-like, Chordin-like2, Tsg, Sclerostin, Caronte, Gremin, Cerberus, Follistatin, USAG-1, FLRG and Dan[105,113,114]. Another antagonist widely described in

this pathway inhibition is BAMBI (BMP and activin membrane-bound inhibitor). The latter is a pseudo receptor that mimics Type I receptors but without any intracellular kinase activity. It is co expressed in early stages of development in parallel with some BMPs[115]. BAMBI will sequestrate BMP ligands avoiding active Type I receptor homomeric complex formation[105,114].

At the intracellular level BMP pathway can be inhibited through different modes of action. For instance, inhibitory SMADs such as SMAD6 & SMAD7 also called "I-SMADs" and sharing 36.7% of N-terminus similarities will block R-SMADs [36,116,117]. SMAD6 has a pivotal role in BMP pathway's negative feedback regulation as it acts on BMP pathway through multiple mechanisms. By acting directly on BMPR kinase activity inhibiting preferentially ALK 3/6 subgroup signaling and less ALK 1/2 BMP Type I receptor subgroup. Indeed, it has been demonstrated that SMAD6 shows a high affinity to specific ALK 3 N-terminal residues such as ARG 238, PHE 264, THR 265 and ALA 269[116]. Regarding SMAD7, it is a more wide antagonist for TGFβ superfamily[117]. it is also able to interact equally with both ALK 2 & ALK 3[116]. Indeed, it forms a stable complex with Type I receptors in order to avoid R-SMAD phosphorylation and can also directly sequester R-SMAD & Co-SMAD hetero-complex [118]. SMAD7 can also act as an adaptor protein either by recruiting the phosphatase GADD34-PP1c to ALK5/TbRI for receptor dephosphorylation[119] or by recruiting WWP1/Tiul1 & Nedd4-2 E3 ligases to promote proteasomal degradation of both Type I receptor as well as R-SMADs & co-SMAD[120]. Other TGFb superfamily repressors are Smurf1 & Smurf2. They represent C2-WW-HECT-Domain E3 ubiquitin ligases[121](Figure 8). Smurf1 & Smurf2 are encoded by distinct genes present respectively on human chromosome 7 & chromosome 17 but do share more than 70% of similarities. Post alternative splicing three Smurf1 isoforms can be generated while one single Smurf2 protein has been identified[122,123] (Figure 9). Smurf1 has been described as a R-SMADs inhibitor as it binds to this cytoplasmic complex avoiding the continuum of the downstream activation[123]. Smurf1 & Smurf2 can also cooperates with SMAD7 to repress BMP signalling

forming an inhibitory complex that will translocate from the nucleus to the cytoplasm in order to bind to Type I receptor leading to its proteasomal degradation[119].

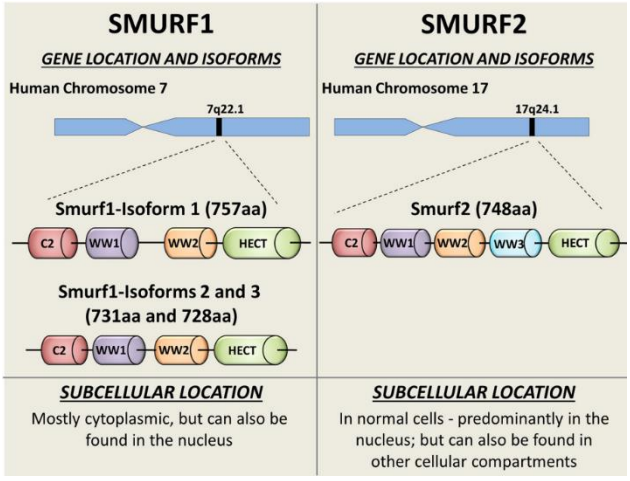


Figure 9: SMURFs, BMP pathway inhibitors.

Schematic representation of *SMURF's* gene locations, proteins isoforms and cells' localisation. Smurf1 & Smurf2 do share a C2-WW-HECT-Domain with E3 ubiquitin ligases feature that inhibits R-SMADs activity. (*Koganti et al., 2018*)

Regarding BMP pathway key role embryogenesis and adult tissue homeostasis, its deregulation at different levels is linked to pathogenesis in several human diseases[124]. As Smurfs and other BMP pathways repressors establish negative feedback on BMP pathway resulting effects. The inhibitors mutation or disability to regulate this pathway will maintain BMP relative actors unrestrained which will result in different malignancies developpement[124]. For instance, a study using *Smurf2 -/-* mice showed that this protein acts as a tumour suppressor as its absence leads to a wide spectrum or tumour development in the concerned animals in a late age stage that equals to 70 years for a human body. Among these tumours, 30% corresponds to haematological malignancies[125,126].

At the nuclear level, the BMP pathway can still be repressed by co repressors such as c-Ski, Tob, SIP1 pr SnoN that will bind to the R-SMAD and Co-SMAD complex and the SBE region repressing specific gene transcription[127–129].

1.1.1.3.2.3.2 BMPs potentiators:

Besides BMP negative regulation, a wide potentiators can regulate positively BMP canonical

pathway. At the extracellular level, a metalloproteinase (BMP1) belonging to the BMP family has been described more than 25 years ago as a BMP potentiator [130,131](Figure 8). Considering the similarity between mature BMPs and heparin at binding level, several sulfated polysaccharides like heparin, dextran and heparan sulfates have been described as BMP2, BMP4 and BMP7 potentiators in osteoblast differenciation[132]. Additionally, the secreted Kielin/chordin-like protein (KCP) identified from an embryonic kidney as a cystine rich protein with 18 Cys repeats is also described as a BMP signalling enhancer[133]. Finally, Crossveinless-2 protein (also called BMPER in Mice) has been described as containing a cystine rich (CR) and a Willebrand D domains rending it as both enhancer and antagonist for the BMP pathway[134].

At the membrane level, a coreceptor family called glycosylphosphatidylinositol (GPI)-anchored membrane proteins (RGMs) has been reported to form a complex with BMP Type I receptors by binding specific BMPs such as BMP2 & BMP4 but no other BMPs like BMP7 and thus enhances BMP signaling[135].

At the intranuclear level BMP pathway can also be positively regulated by several co-activators such as the histone acetyltransferases P300 & CREB-Binding protein (CBP) or by other co-activators as GCN5 or Runx2 that will enhance SMADs-dependant BMP signalling pathway[136–138].

1.1.1.3.3 Jak/STAT Signalling:

The JAK family (Janus kinases) comprises cytoplasmic kinases that are deeply involved in a large range of cell surface signaling receptors. This kinase family includes JAK1, JAK2, JAK3 as well as TYK2[139,140]. The role of the JAK family is to transmit the signal from different regulators through signal transducers called “STATs” allowing a communication between the extracellular microenvironment and the cell’s nucleus. These cytoplasmic factors are phosphorylated and recruited by the JAKs to translocate in homo or heterodimer forms within the nucleus and bind to DNA sequence controlling thus a wide panel of genes expression [139]. This transcription factor’s family includes seven members, STAT1, 2, 3, 4, 5A, 5B and 6 [141]. JAK/STAT signaling is essential in tissue development and homeostasis as it regulates stem cell maintenance, proliferation, and differentiation in hematopoiesis process. Indeed, different

hematopoietic cytokines can activate JAK2 that will in turn, activate STAT5 to regulate HSC survival, proliferation and self-renewal to maintain a pool of HSCs within the human BM[142].

In hematopoiesis, the Stem Cell Factor's receptor is able to activate Jak kinases through its tyrosine kinase activity, leading to hematopoietic cell proliferation and differentiation[139]. The Jak/ STAT pathway remains central and widely requested by the cytokine receptor superfamily including those critical for hematopoiesis development and maintenance like G-CSF, TPO , EPO , IFNs, and ILs [139].

Moreover, both JAK1 & JAK2 are critical for HSC homeostasis maintenance as *in vivo* JAK1 deletion dramatically reduces HSC renewal ability and changes lymphoid & myeloid differentiation[143]. We also note that JAK2 seems to be involved in erythropoiesis process as an *in vivo* knock out leads to a lethal phenotype on embryos due to the lack of red blood cells[144]. A conditional JAK2 knock out leads to an apoptosis increase and quiescence loss of Lin^{-} $Sca\text{-}1^{+}$ $c\text{-}Kit^{+}$ HSCs which confirming its key role in HSC homeostasis maintenance[145]. JAK3 has also been described as being critical for innate lymphoid development [146].

Concerning the STATs transcription factors, STAT1 has been described as a key actor in megakaryopoiesis induction through GATA1 in Mice[147]. As well, STAT3 showed an involvement at different levels in normal hematopoiesis development. The activation of STAT3 by upstream extrinsic stimulation promotes HSC's self-renewal for HSC pool regeneration[148]. In addition, STAT3 activation is mandatory for IFNβ primary pro-B cells induced apoptosis[149]. Finally, Jak/STAT5 plays also a key role in HSC self-renewal, it fosters specifically HSC expansion partially or totally through its target gene HIF2alpha [150], it also promotes multilineage commitment as STAT5A & STAT5B deficient mice have impaired hematopoietic functions at several blood lineages[151]. In the other side, a high activity of STAT5A generates an induced erythropoiesis and a lack of myelopoiesis in immature CD34+ cells while intermediate activity of STAT5A shows a proliferative advantage of CD34+ cells[152].

1.1.2 Hematopoietic Stem Cell: HSC

1.1.2.1 HSC Identification & Isolation :

HSC represents a tiny fraction of multipotent cells endowed with a specific ability to generate the whole types of blood cells[153]. HSC frequency within the bone marrow is around 0.005 to 0.01% of total nucleated hematopoietic cells[154]. Notwithstanding their tiny percentage within the BM, HSCs do have an utmost role in blood cells homeostasis, and they do persist for lifespan in a steady state to insure their critical function. They might undergo symmetrical or asymmetrical division (Figure 12) either to ensure a pool of HSCs or to replenish the hematopoietic system with comitted progenitors [155].

Different *in vitro* and *in vivo* studies were widely carried out to identify and isolate HSCs.

1.1.2.1.1 In Vitro Studies :

Nowadays, to identify and isolate HSC *in vitro*, the most common method remains the flow cytometry. Thanks to specific cell surface markers, flow cytometry is commonly adopted in either clinical use or research field. Despite various controversial between the scientists, CD34 is the most relevant and used marker to identify human HSC but we note that multiple studies showed that some HSCs are $CD34^-$ or $CD34^{low}$. In fact, in 1997 scientists reported that a human side population (SP) identified by a high Hoechst dye efflux activity is $CD34^-$ Lin^- but is enriched in Long-Term-Culture-Initiated-Capacity (LTC-IC) and do develop $CD34^+$ cells after a stromal-supported cell culture[156]. And interestingly this $CD34^-$ HSC population do exist in other mammal species close such as monkey[156] .

$CD34^+$ cells are estimated to be very rare within the human body. Indeed, it counts around 1 % of nucleated BM cells[157]. Nonetheless this differentiation cluster is not exclusive for HSCs. HSC derived progenitors do express CD34 as well but the more the cell is committed the less CD34 will be expressed. In addition, various other non HSC derived cells do express CD34 at their cell surface such as vascular endothelial cells, multipotent precursors, osteoblast precursor, cord blood progenitors, hair follicle cells and muscle satellite or muscle stem cells

making this CD34 marker far from being exclusive to HSCs[158–162]. Plus, some controversial studies are questioning the expression of CD34 on Mesenchymal Stem Cells (MSCs) within the bone marrow. As their lack of CD34 positivity was observed while culturing it on plastic plates, their real carrying of this differentiation cluster in vivo remains unclear[163].

Regarding the non-exclusivity of CD34 expression on HSCs, the combination of other HSC markers does increase the purity of HSC selection and isolation (Figure 10). The antigen cocktail that is commonly used to isolate HSC includes the lineage antigen (Lin) that is not expressed on differentiated cells[164]. Besides CD34, we also use CD38 (=T10 antigen) and CD45RA given both are expressed on more committed cells[165–167] . Apart from these markers, CD90 (Thy-1) is also added to the cocktail considering its high expression on primitive cells compared to the differentiated ones in addition to CD49f which is the integrin α6 expressed at cell surface of a various number of SCs [167–169]. We note that CD45RA is negative in HSC but positive in a leukemic stem cell (LSC) sub population detected in AML[170].

As the HSC selective markers were differently accepted by researchers over the years, a functional HSC characterisation through *In vitro* functional assays was established to quantify HSCs by long term cultured experiments[171]. The Long-Term Culture-Initiating-Cells (LT-CIC) was designed for mouse *ex vivo* studies at the beginning but over time it becomes adapted for human cells[172,173]. In this *in vitro* assay, primitive hematopoietic cells are cultured on a supportive stromal feeder layer for five weeks and supplemented with specific cytokines supplemented medium depending on the required progenitors. Following these 5 weeks of stromal supported culture, a Colony Forming Cell (CFC) assay will be performed on 2 other weeks. The retrieved cells will be seeded on a semi-solid methylcellulose medium to assess retrieved cells ability to form myeloid colonies allowing a progenitors quantification. The experiment read out will be reported on a graph as a survival fraction (SF) that represents the number of formed colonies divided by 100* number of initial seeded cells. LT-CIC is used to generate mature granulocytes, macrophages, lymphoid or NK cells[174,175]. Nonetheless, the primary use for this assay is to quantify primitive hematopoietic progenitors that are able to initiate and sustain myeloid progenitors' production for several weeks. These progenitors are considered to share the same phenotypic and functional features as *in vivo* repopulating

HSCs[176,177]. .

1.1.2.1.2 In Vivo Studies :

Cell Type	Cell surface marker	Cell Type	Cell surface marker
LT-HSC, Weissman model	Lin⁻ c-kit⁺ Sca1⁺ CD135⁻ Thy1^{low}	HSC	Lin⁻ CD34⁺ CD38⁻ CD90⁺ CD45RA⁻ CD49f⁺
ST-HSC, Weissman model	Lin⁻ c-kit⁺ Sca1⁺ CD135⁺Thy1^{low}	MPP	Lin⁻ CD34⁺ CD38⁻ CD90⁻ CD45RA⁻
MPPs, Jacobsen model	Lin– c-kit+ Sca1+ CD135⁺ CD34⁺	MLP	Lin⁻ CD34⁺ CD38⁻ CD135⁻ CD123⁻ CD45RA⁺
CLP	Lin⁻ C-kit^{low} Sca1⁺ CD127⁺ CD135⁺	CMP	Lin⁻ CD34⁺ CD38⁺ CD10⁻ CD135⁺ CD123low CD45RA⁻
CMP	Lin⁻ c-kit⁺ Sca1⁻ CD127⁻ CD16/32⁻ CD34⁺	GMP	Lin⁻ CD34⁺ CD38⁺ CD10⁻ CD135⁺ CD123^{low} CD45RA⁺
GMP	Lin⁻ c-kit⁺ Sca1⁻ CD127⁻ CD16/32⁺ CD34⁺	MEP	Lin⁻ CD34⁺ CD38⁺ CD10⁻ CD135⁻ CD123⁻ CD45RA⁻
MEP	Lin⁻ c-kit⁺ Sca1⁻ CD127⁻ CD16/32⁻ CD34⁻		

Figure 10: Classification & hierarchy of mouse and human hematopoietic stem cells HSCs and progenitors specific markers.

Table representing specific cell surface markers of stem and progenitor cell in mouse and human BM. LT-HSC long-term hematopoietic stem cells, ST-HSC short-term hematopoietic stem cells MPP multipotent progenitors, CLP common lymphoid progenitor, GMP granulocyte macrophage progenitor, MEP megakaryocyte erythrocyte progenitor, MLP multi lymphoid progenitor. (*Adapted from Kumar, Madabushi, 2018*)

Considering CD34 and Lin as key elements in human HSC detection, CD34 appears to be lost in mice HSC where the major HSC markers remain Sca1⁺ cKit⁺[178]. LT-HSC ST-HSC do express Sca1 and cKit at their cell surgace[179]. However, their expression of CD135 (=FLT3) is different. LT-LSC do not express this tyrosine kinase while ST-LSC do[179]. It may be explained by the fact that LT-HSC stays in a non-differentiated state on a long period meanwhile ST-LSC can undergo rapid commitment so CD135 may increase its response to the surrounding signals[180]. The HSC investigations studies in mice models do evaluate the ability of injected HSC to reconstitute the hematopoietic lineage within the animal. To evaluate the high reconstitution potential of an human HSC it classically needs to use immunodeficient mice as NOD-SCID with a xenogeneic transplant system showing an extremely reduced immune responses against the “non-self” cells[181]. Post irradiation of the NOD-SCID models, their hematopoietic cells will be destroyed, then, the transplanted HSC ability to replenish the model will be assessed. Few months post human HSC transplantation in a immunodeficient mouse model, a cytometry analysis revealed the presence of lymphoid and myeloid cells within the animal bone marrow[182].

Furthermore, another method of lineage tracing was adopted to evaluate the maintenance of long-term hematopoiesis reconstitution in transplanted mice models. The authors monitored a transgene expression in a fraction of HSCs. An inducible genetic labelling revealed that the newly transplanted HSCs do contribute to the adult hematopoiesis generating more than 2/3 of myeloid lineage and platelets. Furthermore, this HSC replenishment behaviour could be increased by an IFN treatment[183]

1.1.2.2 HSC special features:

1.1.2.2.1 HSC Dormancy

HSCs do have a noteworthy regenerative potential regarding their ability to give rise to a huge number of differentiated mature blood cells through a human lifespan. An HSC stays in a dormant state during almost all its life within the BM and undergoes a waking up process depending on the system homeostasis requirement[184,185]. Following a tissue injury or a mature cell request, dormant HSC will undergo an awakening to initiate a cell division giving rise to a daughter immature or committed cell following the body needs. A non-responsive dormant HSC leads to an hematopoiesis failure because of the mature blood cells lack as well as a highly active HSC leads to an exhaustion of the SC pool and a loss of long term stemness potential[186]. It appears that HSC quiescence exists in different depths knowing that the highest HSC dormancy level depicts the most regenerative potential[187]. Quiescent HSC has a very low metabolic activity, however it keeps a weak level of metabolic activity by mainly undergoing low glycolysis to supply its functional needs[188,189] (Figure 11). HSC quiescence is maintained by different processes such as its minimal mitochondria clearance giving that a high number of quiescent HSC mitochondria remain inactive, its large number of lysosomes and autophagy mechanisms[188,189]. Regarding its transcriptional activity, Dormant HSC do express very low c-MYC and cyclin dependant kinases (CDKs)[190–192]. At the contrary, it do express several cyclin kinase inhibitors including p57, p21 or p27 to avoid its cell cycle entry[193–195]. Leading intrinsic and extrinsic players do control HSC quiescence maintenance, hence a lack of these actors as well as their inhibition lead quiescent HSC to a gradual cell cycle re-entry (Figure 11).

1.1.2.2.1.1 Intrinsic factors

A wide panel of numerous intrinsic factors expressed in different cell compartments do maintain HSC in a dormant state such as Reactive oxygen species (ROS), transcription factors such as Growth factor independent 1 (GFI-1), Mixed lineage leukemia (MII), Polycomb Ring Finger protein (BMI1), FoxO, Promyelocytic leukemia protein (PML), Early growth response 1 (Egr1) and Pre-B-cell leukemia homeobox1 (PbX1), SMAD2/3, cytoplasmic proteins such as mTORC1, TP53, protein kinase AKT, cellular Casitas B lineage lymphoma gene (c-Cbl) or nuclear proteins like F-box and WD repeat domain containing 7 (Fbw7), Retinoblastoma protein (pRb), p107,P130,Mi2b, P53, P21, P57,P15 and P27[186,196–199] (Figure 11).

1.1.2.2.1.2 Extrinsic factors controlling HSC quiescence

Aside from intrinsic factors, extrinsic factors do also control HSC quiescence stability. HSC microenvironment is providing these signals, especially the osteoblastic niche that serves as a reservoir of LT-HSC in a dormant state. Hypoxic microenvironment such as HIF-1α presence helps the HSC quiescence maintenance as well as several factors produced and released in a paracrine manner mainly by osteoblasts[186,200] (Figure 11). Osteoblast-produced TGFβ is a quiescence inducer as well as other osteoblasts secreted proteins such as CXCL12 (= SDF-1) Angiopoietin1 (Ang-1), TPO(thrombopoietic) and osteopontin (Opn) that will respectively bind to HSC CXCR4,Tie-2 (angiopoietin receptor) ,MPL(thrombopoietin receptor) and CD44[186,199,201–203]. In addition, osteopontin (Opn), a multidomain phosphorylated protein secreted by osteoblasts do promote HSC quiescence[204]. It can bind to several integrins like α9β1 as well as CD44 expressed on HSC cell surface [204–206]. HSC quiescence is also fostered by its physical binding to the osteoblastic niche through N-Cadherin, integrin, CD164, mb-SCF and Jagged-1 interactions insuring HSC quiescence stability and maintenance through several intracellular induced signalling such as NOTCH and β-catenin WNT canonical signaling[186,207,208].

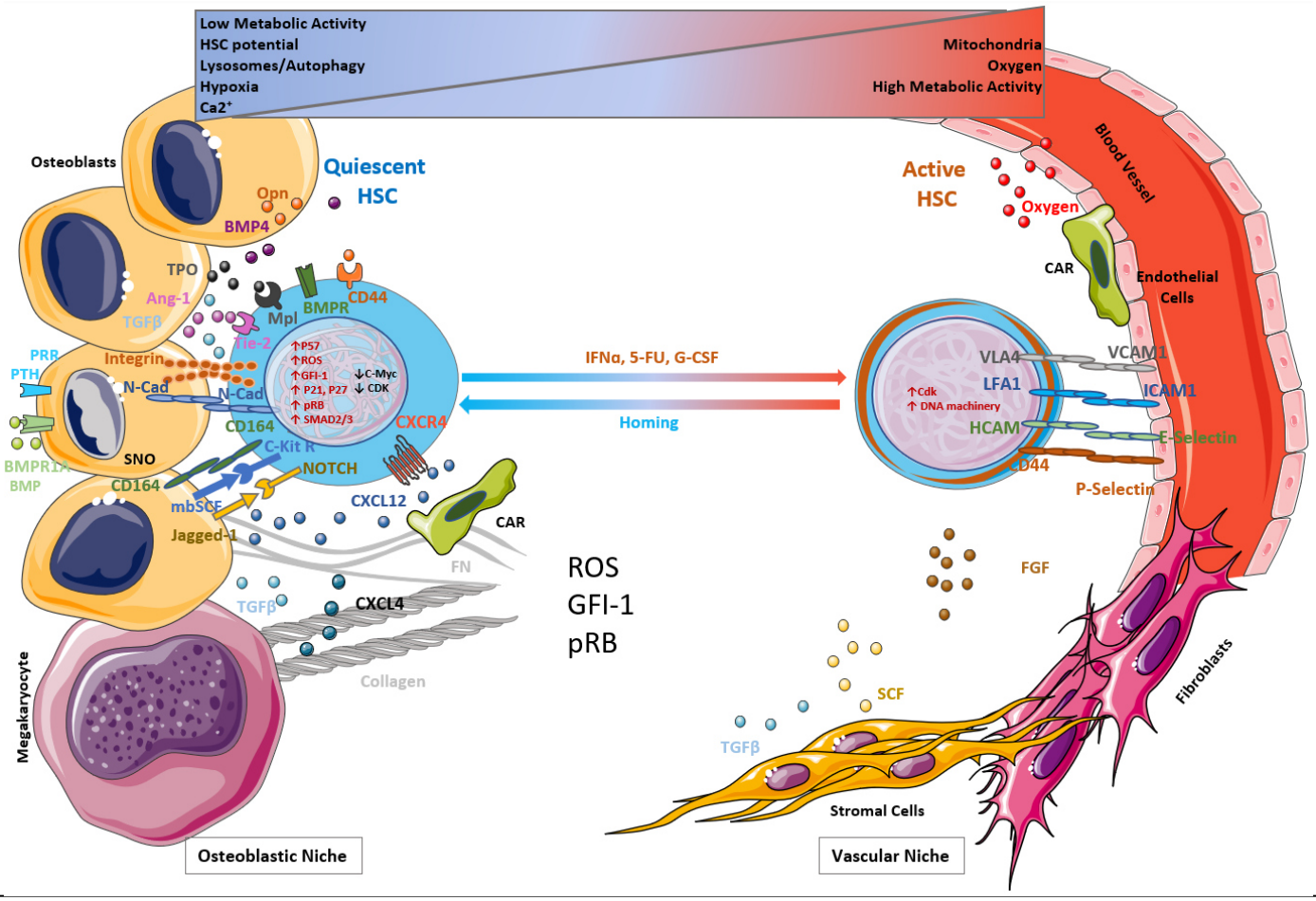


Figure 11: Balance between dormant and active hematopoietic stem cells in BM niche.

Within human BM, two different compartments do exist. The osteoblastic niche will mainly serve as a quiescent HSC reservoir where osteoblasts do maintain HSC quiescence through different cell to cell interactions such as Integrin, β-cadherin and membrane-SCF that will interact with HSC c-Kit receptor. Osteoblasts also promote and maintain HSC dormancy by releasing several soluble cytokines such as BMPs, TPO, Opn, Ang-1 and CXCL12 that will interact respectively with BMPRs, CD44, MPL, Tie-2 and CXCR4 present at quiescent HSC cell surface. Furthermore, megakaryocytes do also enhance HSC quiescence by releasing CXCL4 and TGFβ in the osteoblastic niche. Hypoxia will also play a key role in HSC quiescence maintenance. As well, quiescent HSC fitted with a high stem cell potential will have a low glycolytic metabolic and an increased autophagy and lysosome numbers. At the contrary, active HSC will have a high metabolic and mitochondrial activities. Several extrinsic factors such as IFNα, G-CSF, Oxygen or 5-FU do enhance HSC cycling. The vascular niche components do mainly produce cytokines that will promote HSC cycling such as SCF and FGF.

In addition, other immune cells such as megakaryocytes showed an involvement in the HSC quiescence process as it releases CXCL4 (=PF4) as well as TGF-β . These factors release will result in HSC proliferation inhibition as well as SMAD2/3 pathway activation in quiescent HSC fostering thus HSC quiescence in normal conditions. Nonetheless, during injury condition, the same megakaryocytes begin a

FGF1 release to induce an HSC differenciation as the system requires[202,209,210]. BMPs are also indirectly controlling HSC quiescence. Indeed, it plays on osteoblasts by triggering intracellular signals through BMPR1a as a knock down of this receptor induces an HSC proliferation increase[211]. It also acts directly on HSC by increasing NFATC1 transcription factor that will block CDK4 transcription fostering thus HSC dormancy[212].

Regarding the vascular niche that is mainly composed by stromal cells as well as endothelial cells, it negatively regulates this quiescence as it promotes HSC proliferation and differentiation with a high oxygen release by RBC present in the vessels or by releasing some soluble factors such as SCF or Fibroblast Growth Factor (FGF)[213] (Figure 11). Other factors may push dormant HSC to undergo a cycling such as IFNα, G-CSF or 5 Fluoro Uracil (5-FU)cytotoxic agent that kills cycling cells including HSCs[186]. We note that vascular niche can also secrete TGFβ contributing to the HSC quiescence in some cases[214].

1.1.2.2.2 Self-renewal and differenciation

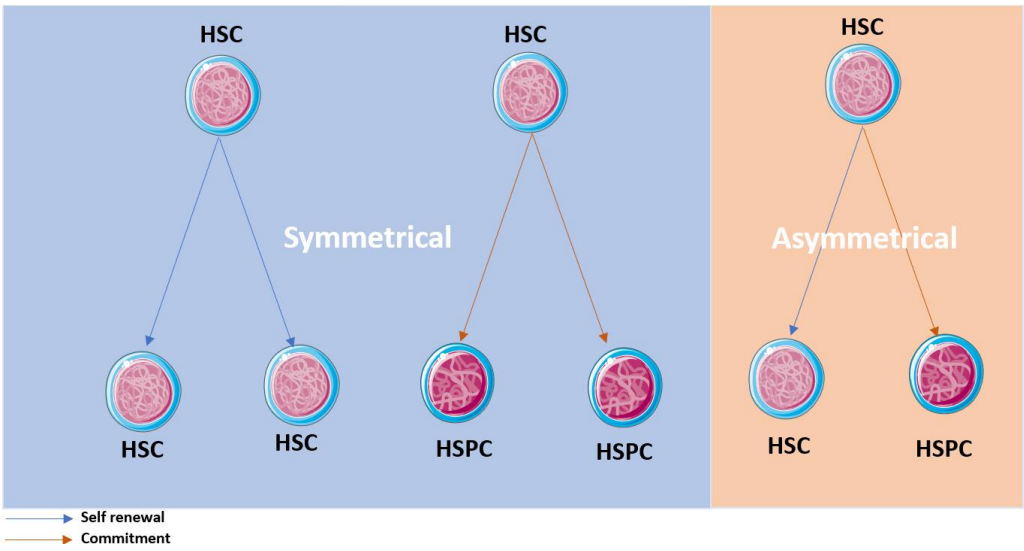


Figure 12: Symmetric and asymmetric HSC division. HSC fate is determined by its division mode.

If the HSC undergoes a symmetrical division, it can either give rise to two HSCs by a self-renewal process or two committed hematopoietic stem progenitor cells (HSPCs). When HSC undergoes an asymmetrical division, it will give rise to an HSC and a comitted HSPC. *(Adapted from Sica et al.,2020)*

HSCs are endowed with special features. They have the ability of self-renewal and differentiation through symmetrical and asymmetrical division[155]. Nevertheless, the HSCs are tightly regulated to respect the balance between the self-renewal and the differentiation

according to tissue homeostasis[215]. During the asymmetric division, one HSC will divide giving rise to two daughter cells, one HSC and one progenitor cell. During the symmetric division, one HSC will divide into two identical daughter cells either HSCs to preserve SC pool or progenitor cells to trigger a cell commitment [215,216](Figure 12). The asymmetric division process has been highly conserved across the evolution. This phenomenon generates from one HSC two daughter cells with different fates. This step is critically important for the purpose of generating differentiation during the development, given that one HSC can generate a whole blood tissue lineage. HSC division deregulation will lead to oncogenesis. The differentiation inhibition will trigger cancer progression and the differentiation increment will lead to the loss of the stemness pool[155]. A good combination between the two division modes will provide a balance between stemness and differentiation ensuring tissues homeostasis control. For the division triggering, both extrinsic and intrinsic cues are involved. The extrinsic signals play an important role in the differentiation control but the intrinsic cues play also a major role to control daughter cells' birth[217]. Several protein determinants have a leading role in specifying the daughter cell fate. Their skills make them a point of control in oncogenesis. The precursors of cell division are well established in invertebrates' models but remain largely obscure in mammalian organisms[217] . In the Drosophila model three different classes of proteins have been emphasized for their involvement in the asymmetric division. The polarity proteins as Par3, Par6 and aPKC; the regulators of spindle Pins, Dlg and Lis1; and the cell fate determinants like Numb or Musashi [217]. Therefore, the choice of the division mode will be done depending on the localisation of these proteins.

1.1.2.3 HSC Aging:

As the hematopoietic niche represents a complex and dynamic microenvironment monitoring HSC behaviour, it accumulates alterations along aging process and consequently alters HSC functions. As well, intrinsic factors independent from hematopoietic niche do contribute to HSC functions decline upon aging. Hematopoietic niche does not only represent a 3D architectural space where HSC exist, it plays important role in HSC fate regulation in both healthy and oncogenic conditions.

Upon aging, hematopoietic niche do gradually comprise changes that will affect HSC function.

1.1.2.3.1 Intrinsic HSC aging regulators:

Upon aging, HSC differenciation is flawed toward myeloid progeny at the expense of the lymphoid progeny accumulating an impaired regenerative ability[218,219]. Furthermore, it has been shown that aging pushes HSC as well as MPP early progenitor to maintain their quiescent state by increasing their self-renewal ability at the expense of their cell lymphoid and myeloid differenciation[220,221]. Also, HSC do loose its cell polarity because of aging and comprises several epigenetic alterations such as mitochondrial SIRT3 acetylation decrease [222,223]. In addition, aging will deregulate HSC autophagy ability[224]. It also increases intrinsic WNT5a dependant signalling pathway as well as Notch signalling leading to Hes1 transcriptional increase[221].

1.1.2.3.2 Extrinsic HSC aging regulators:

Besides intrinsic aging effects, the extrinsic HSC aging is regulated by its surrounding microenvironment. A decrease in the Insulin like growth Factor IGF1 has been described as a crucial HSC-Aging promoter factor acting on both HSC and microenvironment Mesenchymal Stromal Cells (MSCs) [225,226]. Another aging effect occurs at the vascular niche level where the endothelial cells number dramatically decrease and affects HSC behaviour during aging process[227,228]. Throughout aging, both arterioles and arteries do degenerate by minimising their length and impairing their orientation which will disorganize the niche making it not supportive anymore fore HSC preservation[228,229]. On top of that, nervous sympathic adrenergic system is also strongly affected upon aging process which consequently dysregulates HSC homing and egress from BM compartment[230].

1.1.2.4 HSC Therapeutic Use:

HSC represents one of the first described cells recognized as relevant for clinical research and application. Actually the first try for a human BM transplant went back to 1939 for an aplastic

anemia patient where the engraftment purpose was to reconstitute normal hematopoiesis within patients malfunctioning BM[231]. Then, in 1956 Barnes et al. showed an acute myeloid leukemia (AML) overcome thanks to a bone marrow transplant in a mouse model. Next, in 1958 the first Bone Marrow Transplant (BMT) between nonrelated human donor and receiver was done by Georges Mathé in Paul Brousse French hospital on Yugoslavian physicians that were accidentally irradiated by a nuclear reactor. The first HSC therapeutic use to cure leukemia was done by E. Donnall Thomas in Seattle. This doctor performed the first allogenic BM transplantation by an intravenous transfusion on six leukemic patients after a chemotherapy and a radiotherapy treatment. As the histocompatibility was not considered at that time, the transplanted patients died in the 3 months following the intervention[232].

The human leukocyte antigen (HLA) was firstly discovered by Jean Dausset in 1958 with firstly named it MAC based on the 3 first alphabets of his volunteers' names. Donnall Thomas, an American physician followed this therapeutical use, he performed around 100 transplantations but only 13 succeeded then, after 63 AML patients engraftment he won with his collaborators Nobel Prize in 1990 for his achievements[232]. In 1989 the World Bone Marrow Registry (BMDW) was funded and counts today more than 33 573 307 donors and 709 013 cord blood units[233].

The first HSC transplantation in CML case went back to 1979. This kind of transplantation is based on either an allogenic transplantation of HSC coming from a healthy donor to CML patient or an autologous transplantation that is based on CML patient's own HSC healthy stem cell collection before treatment, storage and transplantation to CML patient after he undergoes different cytoreductive regiment treatments such as chemotherapy and radiotherapy to destroy the unhealthy present stem cells whatever the received transplantation. The goal is to see the healthy HSC repopulating patient's defective hematopoietic system.

In this respect, a huge interest linked to these HSCs made it commonly used in scientific fields. However, their lab expansion remains very far from their real expansion within human BM which make their study pitfall because of the lack of real complex microenvironment mimicking their real niche.

1.1.3 Hematopoietic Niche:

Hematopoietic microenvironment has been considered as an anatomic and functional compartment supporting HSC maintenance and survival. In 1978, Schofield awarded it as the hematopoietic "Niche", a compartment located within the BM in adult human and responsible of HSC fate regulation[234]. This specialized BM area has been described as a set of two different « niches » with specific morphology, composition, and function. The "osteoblastic niche", also named "endosteal niche" and located at the inner surface of trabecular bone cavity and the "vascular niche" also called "endosteal niche" and located next to blood vessels (Figure 13). As described above, the osteoblastic niche that is mainly composed by osteoblasts contributes widely to HSC quiescence maintenance through different signals and serves as a long-term reservoir of quiescent HSCs meanwhile the vascular niche serves as a short-term HSC reservoir and will more regulate HSC mobilisation, proliferation, differenciation and migration through blood circulation. Both niches do maintain hematopoietic homeostasis (Figure 13).

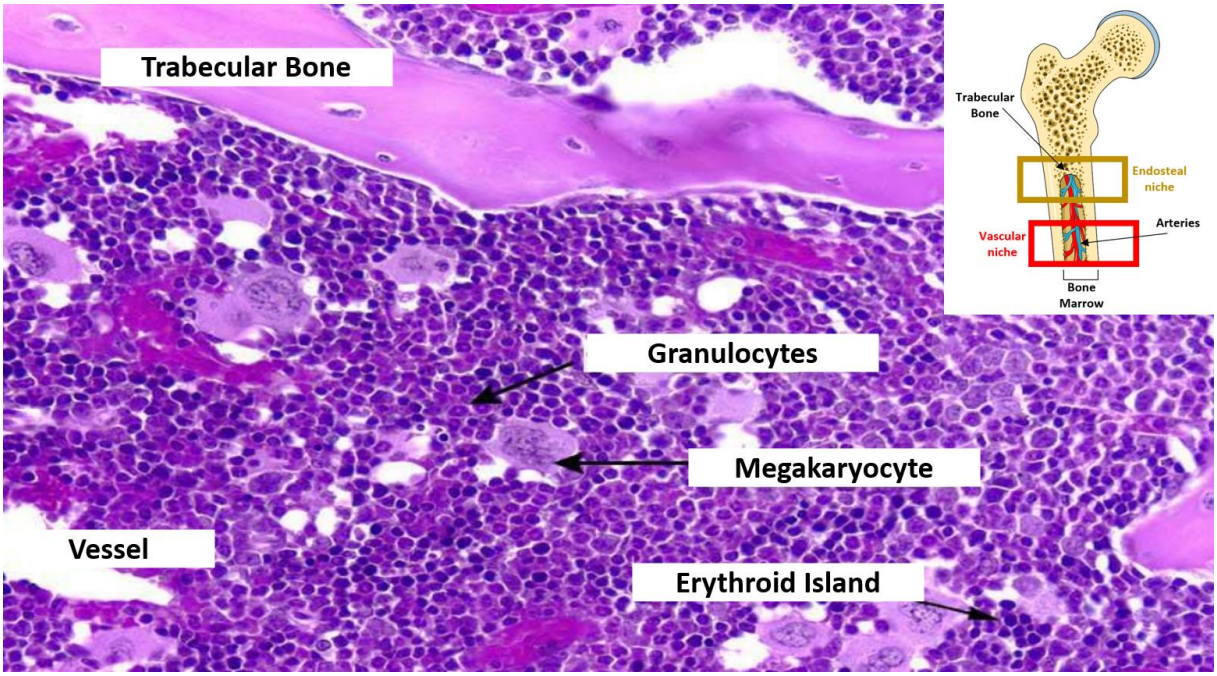


Figure 13 : Bone Marrow clot section.

A IHC on BM clot section shows a quantitative representation of different marrow components such as trabecular bone and vessels in addition to blood cells such as granulocytes, megakaryocyte, and erythroid islands .
(Taken from BM and spleen examination report by Jose M. Gonzalez-Berjon M.D. 08/05/2010)

1.1.3.1 Osteoblastic Niche:

The evidence of an osteoblastic niche existence was supported by several *in vivo* experiments. Indeed, a preferential post transplantation HSC homing close to the mice endosteum was observed, thus a HSC pool was maintained in this area[235,236]. Furthermore, HSC differenciation progress was also happening but homing elsewhere into a distinct location[236]. The endosteal niche is mainly comprised of osteoblasts that are monitoring HSC homeostasis through several signals[237] (Figure 11). To this end, these cells do produce several molecules such as CXCL12, BMP4, Ang1, Opn, CD164, mb-SCF and Jagged-1 (JAG1) and many others cited above that are critical for HSC quiescence, self-renewal, maintenance, homing and survival[203,204,238–243]. JAG1 is a surface protein able to bind four different NOTCH receptors that can be expressed on HSCs, following this binding interaction, a proteolytic notch receptor cleavage by a γ secretase complex will release a NICD intracellular domain that will translocate to the nucleus and activate specific NOTCH genes such as Hairy Enhancer of Split Homolog (HES) and Hes-related protein (HEY) genes[244]. Notch1 receptor is known to be express on LT-HSCs and it maintains HSCs undifferentiated state, on note, a loss of this receptor will cause a HSC impair *in vivo* and *in vitro*[208,245,246]. Furthermore, endosteal niche retains HSCs through adhesion molecules such as VL1-4 ($\alpha 4\beta 1$) expressed on immature $CD34^+$ HSCs that binds to VCAM-1 present on BM cells[242,247] (Figure 11).

Cell adhesion is also supported by Ang-1 that increases $Tie2^+$ HSCs adhesion to FN and collagen present in the niche[248,249] (Figure 11). Osteoblasts HSC adhesion function is not exclusive to this cell type, nonetheless, an osteoblasts sub type named spindle-shaped N-cadherin$^+$ CD45$^-$ osteoblastic cells (SNO) do localize within the endosteum and do express a high level of N-Cadherin that binds to HSCs and BMP signalling through BMPR1a present on these osteoblast do control niche size[211]. Furthermore, a high concentration of $Ca2^+$ in the endosteal niche orients HSC homing through its highly expressed calcium sensor [250].In addition a hypoxic microenvironment in this niche maintains HSC G0 state[251]. Osteoblasts can control endosteal niche size by monitoring HSC number within this niche. Other organs can also control the hematopoietic niche independently from the BM such as the thyroid released Parathyroid hormone (PTH) that increase *in vivo* Jagged-1 osteoblasts

expression will increase HSC recruited numbers[252]. As differentiating HSCs were migrating to another site distinct from endosteum, an in vivo evidence of a distinct vascular niche existence was highlighted[253].

1.1.3.2 Vascular Niche:

The first discovery of a distinct vascular niche within the bone marrow goes back to 2005[253]. This niche is located within axial bone part of the BM (Figure 13). This part of the BM is knows to support HSC differenciation, proliferation, active cycling and Short Term (ST) HSC maintenance[254] (Figure 11). In case of injury, HSC will migrate from the endosteal niche to the vascular niche to undergo cell proliferation and restore hematopoiesis[255]. In this part or the hematopoietic niche, oxygen concentration is increased as against endosteal compartment[256]. Furthermore, a study showed that a Lin^- HSC population is associated to blood vessels within the vascular niche suggesting that as osteoblasts in the endosteal niche, endothelial cells do create vascular niche for HSCs[257]. Going back deeper in the embryogenesis steps, HSCs were found close to endothelial cells whatever the development state of the concerned body. In addition, HSC seems to originate from a perivascular progenitor in embryonic development. Plus, HSC is present in the YS, AGM and vitelline arteries where it undergoes hematopoiesis in association with blood vessels[3]. An *in vitro* experiment showed that harvested endothelial cells with adult mice HSC lead to support and expansion of this HSC's pool[7,258,259]. To foster HSC localisation within vascular niche, erythrocytes do express adhesion molecules such as ICAM1 (CD54), VCAM1, P- and E-selectin (CD62P, CD62E) that bind respectively to LFA-1 (CD11a), VLA4(CD49d), CD44 and HCAM hyaluronan binding molecules expressed on HSCs [260–262] (Figure 11). Furthermore, these vascular niche cells do secrete FGF4 and SDF-1 (CXCL12) that is known to be deeply involved in HSC homing, mobilisation and engraftment [260,261]. Of note, endothelial cells role was widely investigated and it has been demonstrated that c-akt murine thymoma viral oncogene homolog 1 (Akt) activation is very important *in vivo* in addition to the up regulation of a numerous factors such as BMP4, Ang1 and FGF2 to increase HSC number and recovery after a myeloablation[263,264].

One crucial component of hematopoietic niche is reticular cells, specifically those producing

CXCL12 and called CXCL12 Abundant Reticular (CAR) cells. A study showed that HSCs were localized close to CAR cells within the BM, either in endosteal niche between CAR cells and osteoblasts or within the vascular niche alongside endothelial cells and CAR cells [265] (Figure 11).

Endosteal and vascular niche remain distinct within the bone marrow regarding their cell components and their specific localisation (Figure 13), both niches are critical in HSC maintenance, proliferation, differenciation and homeostasis. Under hypoxia LT-HSC remains in low cycling condition within the endosteal niche but when required it leaves this part of the niche to the vascular niche where it undergoes cell cycling to restore hematopoiesis[266]. We note that a HSC that moved from endosteal to vascular niche to undergo cycling is able to turn back to the endosteal niche where it re-enters G0 state again but decreases it HSC potential with the number of undergone cell cycles[187,251] (Figure 11).

1.2 Chronic Myeloid Leukemia

1.2.1 Leukemias

Leukemia comprises a group of malignant diseases that affect the two blood forming tissues that are BM and lymphatic system. The etymology of its name comes from Greek language "leukos" (λευκός) that means "white" and "haima" (αἷμα) that means "blood". Leukemias result in abnormal heterogeneous neoplastic proliferation of blood cells blocked at different differentiating stages[267]. Two types of abnormal events may lead to leukemia. The first one is the accumulation of several genes / non-coding RNA sequences mutations or epigenetic changes affecting HSC fate[268]. The second one is a partially differentiated cell features change leading a non-stem cell to acquire stem cell properties[269]. The progressive BM and blood tissue invasion leads to a clinical report and distinction of different leukemias[270] (Table 1 adapted from [270]). The major types of leukemia are four sub-groups. The Acute Lymphocytic Leukemia (ALL) is the most common leukemia affecting young children. Acute Myelogenous Leukemia (AML) is a common type of leukemia touching both young and adult persons. Chronic Lymphocytic Leukemia (CLL) is the most common leukemia affecting adults. Finally Chronic

Myeloid Leukemia (CML) mainly affects adults and subjects may have few or no symptoms during long time before the disease evolute into a more active stage (Table 1).

Other types of leukemia do exist such as Myelodysplasic Syndroms (MDS), Prolymphocytic leukemia (PLL), Large Granular Lymphocytic (LGL) etc[271] (Table 1).

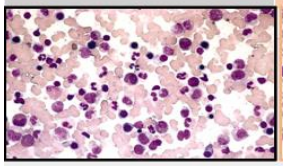
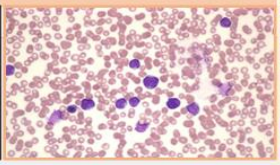
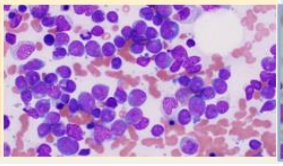
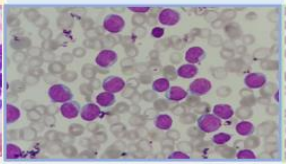
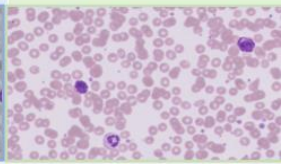
Present signs at diagnosis	ALL	AML	CLL	CML
Incidence age	Childhood, some adult cases	All ages	Middle age or advanced age	Adult age
WBC number	Increased in 50% of cases Normal or Low in 50% of cases Many lymphoblasts	Increased in 60% of cases Normal or Low in 40% of cases Many myeloblasts	Increased in 98% of cases Normal or Low in 2% of cases Presence of small lymphocytes	Increased in 100% of cases Many myeloid blasts
Anemia	Severe in more than 90% of cases	Severe in more than 90% of cases	Bit severe in 50% of cases	Bit severe in 80% of cases
Platelets %	Low in more than 80% of cases	Low in more than 90% of cases	Low in 20 to 30% of cases	High in 60% of cases
lymphadenopathy	Frequent	Occasional	Frequent	Rare
splenomegaly	In 60% of cases	In 50% of cases	Usual	Usual and voluminous
Other signs	Without prophylaxis CNS frequently involved	CNS rarely involved Presence of Auer rods in some myeloblasts	Sometimes presence of haemolytic anemia and hypogammaglobulinemia.	Low WBC alkaline phosphatase Philadelphia chromosome in more than 90% of CML patients
				

Table 1: Comparison between AML, ALL, CLL and CML.
Age incidence, white blood cells number and other symptoms do help to determine leukemia kind in leukemic patients.

(IHC BM staining are taking from ASH Image bank. The represented AML is PH^+ and CML BM come from a CML patients in the accelerated phase)

1.2.2 CML disease:

CML first reported clinical cases date back to 1842 where Alfred Donne described a leukemia case without deep disease detail[272]. Three years following this discovery, a case was reported in Scotland by John Hughes Bennett. In 1987, a similar case was reported in Germany by Virchow Carl Rudolph. Both cases presented splenomegaly, fever and high leukocytes blood number[273,274]. Virchow described “White Blood” as “Leukamie” and from here originates the

'leukemia' term. Later, a German pathologist called Ernest Franz Christian Neumann linked the leukemia origin to the BM compartment and postulated a common stem cell for all hematopoietic cells. After this scientific achievement, CML patients began to receive treatment administration such as low arsenic doses and spleen irradiation in the beginning of the 19th century[275,276] (Figure 22).

1.2.2.1 Epidemiology:

Chronic Myeloid Leukemia (CML) has been classified by the WHO as a myeloproliferative neoplasm (MNP) characterized by the expression of a unique fusion oncoprotein Bcr-Abl1 (p210) in the Hematopoietic Stem Cells (HSC) that could be revealed by different technics including karyotypes, molecular qPCR, protein quantification or gene position by FISH[277–279]. CML incidence counts for 15% of total leukemic diseases and was estimated to almost 900 new cases in France with a mild male preponderance. This disease is considered as rare as the world standardized incidence rate is 1 for men and 0,8 for women per 100 000 persons/year. In the United States, new CML cases for 2022 are estimated for 8 860 persons including 5 120 male & 3 740 female with an estimated death of 670 male and 550 female CML patients[280]. Even though this disease can appear at all ages but 58% of CML patients are 60 years old or more (ESMO).

The CML is a triphasic disease evolving from a controlled chronic phase (CP) to a more instable accelerated phase (AP) ending by a final blast crisis (BC). A patient bone marrow (BM) aspirate is required to perform a karyotype and a morphologic analysis to establish a diagnosis and evaluation of the disease phase [281,282].

1.2.2.2 Diagnosis:

BCR-ABL somatic fusion do characterize CML disease. In fact it has been demonstrated that the chromosomal translocation t(9;22) leading to BCR-ABL oncogene expression is responsible of CML disease initiation[283] (Figure 14).

The disease diagnosis is mandatory to detect the correct disease phase in order to adapt CML

patients' treatment (Figure 15). Most frequently the disease diagnosis is performed randomly during a routine checkup where the WBC are higher than normal. More than 90% of CML patients are diagnosed during the CML CP that remains asymptomatic or with mild symptoms such as weight loss, tiredness caused by a RBCs decrease or a belly swollen caused by extended spleen and detectable by clinician palpation and less than 15% of blasts in patients BM[284–286]. As this CP lasts from 5 to 6 years without treatment, the disease detection in CML patients at this stage remains variable and CML evolution post diagnosis changes according to the time CML patients spent in this phase before its detection[286]. Treatment objectives are to slow the disease evolution and to maintain it as long as possible in this controlled phase. CML diagnosis is further confirmed by accurate medical exams as reported in the Figure 15. Indeed, blood cell count and blood smear evaluation in addition to cytogenetic evaluation of chromosomal abnormalities and BCR-ABL1 transcript detection will help the clinician to further evaluate the disease stage (according to the NCCN protocol).

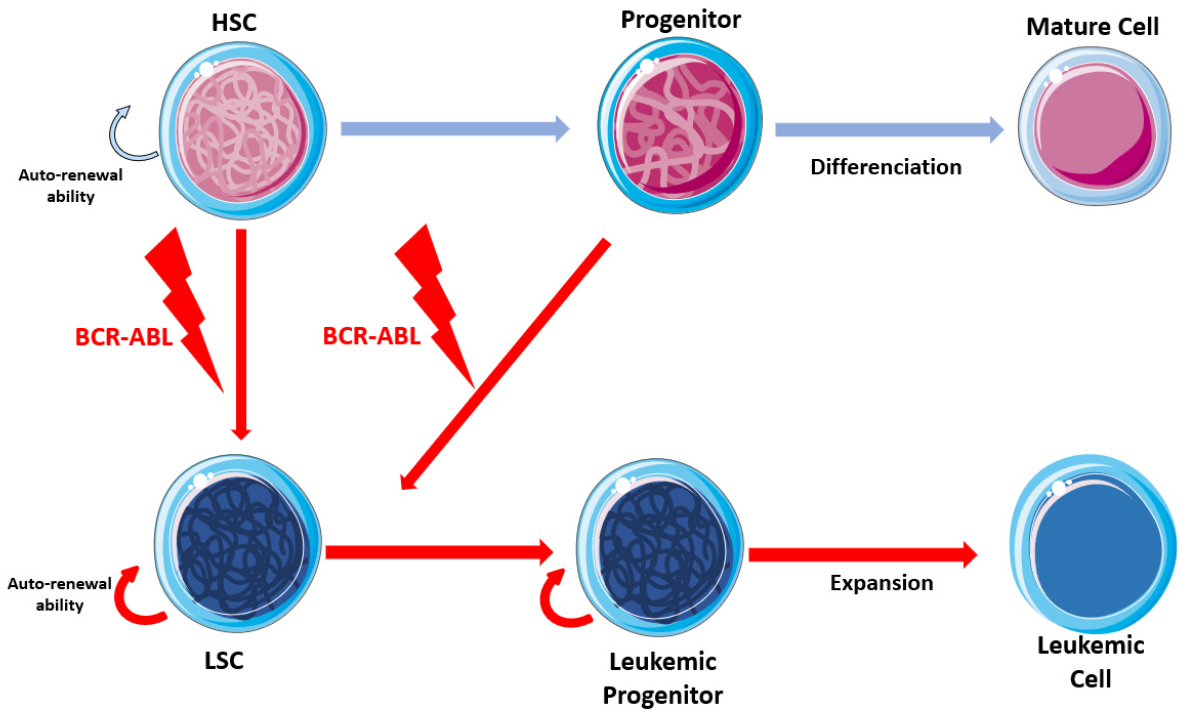


Figure 14 : Model of BCR-ABL leukemic transformation

In CML, Leukemogenesis is acquired through *BCR-ABL* oncogene expression. LSC BCR-ABL^{+} will have an increased auto-renewal abilities. The Leukemic progenitor is also fitted with auto-renewal ability and will give rise to undifferentiated leukemic cells.

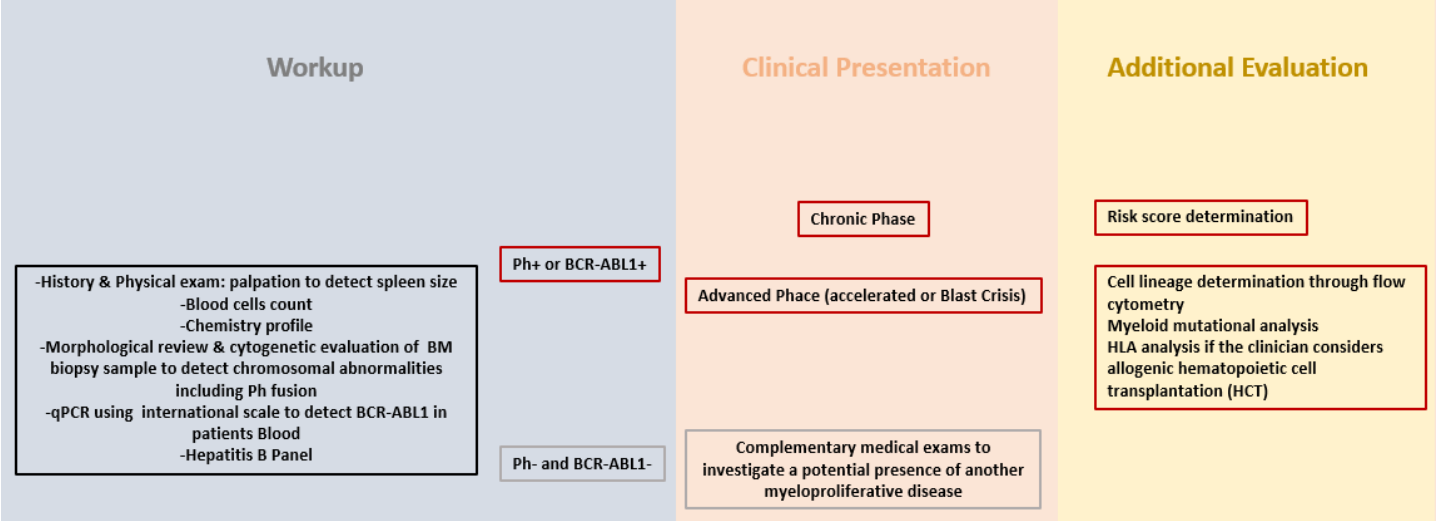


Figure 15 : CML evaluation guidelines
Patients BM initial evaluation as well as spleen palpation do provide a first morphologic disease statement . Then, a fluorescent in situ hybridization (FISH) can detect Phildelphia chromosome presence. A quantitative RT-PCR will detect the transcripts presence and form. This assessment will enable the disease stage determination by the clinician.
(*Adapted from National Comprehensive Cancer Network, version published the 27th of January, 2022*)

1.2.2.3 Prognosis

In the past, as any other cancer, CML was considered as a deadly disease. The treatment and diagnosis improved approaches, patient supportive care and awareness helped to significantly improve the CML prognosis. Before Tyrosine Kinase Inhibitors (TKIs) Era the patient's survival was mainly comprised between 3 to 5 years, nowadays, CML patients do have a median survival age of five years or more. These 5 years of patient's survival went from 31% in the 1990th to 70.4% in patients diagnosed in 2012[287,288] . More importantly, TKI discovery revolutionized the disease healthcare as far as a clinical trial revealed that CML disease related death was very rare in CML patients that achieved at least 2 years of CCyR after TKI uptake and their survival curve was not different from general population one. Indeed, 832 CML patients with a median Imatinib treatment of 5.8 years were included. Only 2.3% (19 cases) had to stop therapy because of the TKI toxic effects and only 0.16% (6 cases) died because of CML disease progression attesting through this successful trial that TKI drug prevents CML

disease progression for the majority of CML patients[289]. To Evaluate CML disease prognostic, different scores do exist to predict CML patients' survival based on their clinical diagnosis. such as the SOKAL score that is the frequently used one and derived from a study including European and American CML patients treated from 1960 to 1980 before the TKI era and takes in consideration patients age, spleen size, platelet count and myeloblast percentage in peripheral blood (PB) in the flowing equation "exp 0.0116 (age - 43) + 0 .0345 (spleen size [cm below costal margin] - 7.5 cm) + 0.188 [(platelet count/700)2 - 0.563] + 0.0887 (% blasts in blood - 2.1) "[290]. If the score is lower than 0.8 the patients risk is considered as low, if the score is between 0.8 and 1.2 the patients risk is ranked as intermediate and id the score is higher than 1.2, CML patients do present a high risk[290,291]. Regarding EUTOS (European Treatment and outcome study) score predicted the complete cytogenic response (CCR) and progression-free survival in 2060 CML patients treated by the first Tyrosine Kinase Inhibitor (TKI) treatment and categorized patients between high risk and low risk groups[292]. The Hasford score was also adopted to correlate and predict the molecular response to CML treatment[293]. Of Note, some criteria following the therapy such as short remission duration, high total dose of hydroxyurea or busulfan, poor suppression of Philadelphia chromosome of a longer time to achieve hematologic remission or other comorbidities may indicate a poor disease prognosis for CML patients[294]. As well, CML patients in a late stage (Blast Crisis) also do have poor prognosis as their survival without treatment is comprised between 3 to 6 months[294].

1.2.2.4 Disease evolution

The Chronic Myeloid Leukemia disease evolves from Chronic Phase (CP) to an Accelerated Phase (AP) ending by the final disease stage called Blast Crisis (BC). CP lasts between 5 to 6 years without treatment and around 90% of CML patients are diagnosed in this disease phase [284–286]. An untreated CML patient in CP is doomed to a quick evolution to AP and BC phases[295] (Figure 16).

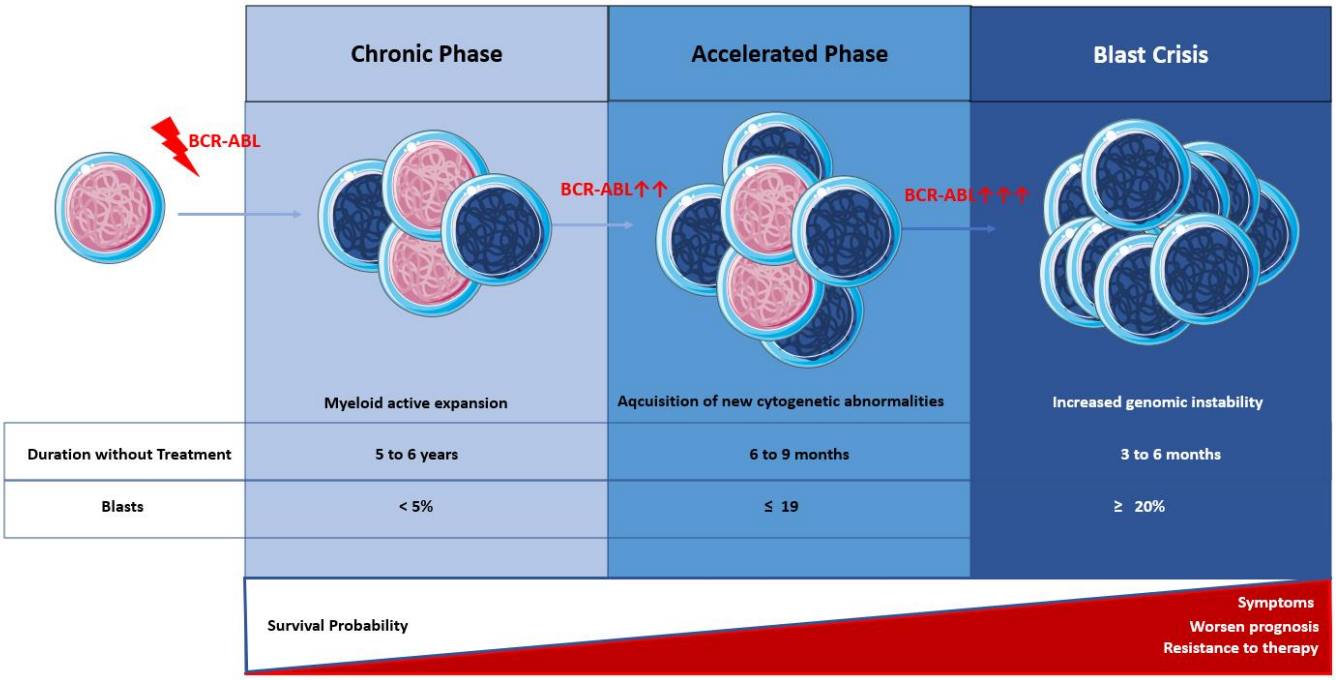


Figure 16: CML disease progression.

CML disease is characterised by *BCR-ABL* expression. Transformed immature cells will lead to a myeloid expansion during disease chronic phase (CP). The latter remains slightly symptomatic and is characterized by less than 5% of blasts in the blood circulation. This first disease stage may evolve to the accelerated phase (AP) where new cytogenetic abnormalities will appear. Plus, blasts number will increase up to 19% and some symptoms begins to appear. The AP can evolve into the final blast crisis BC where blasts number can exceed 20% of blood cells. BC is a rapid aggressive disease stage able to deteriorate rapidly patient condition if not managed and is characterised with more resistance frequency with heavier symptoms. *(Adapted from Losson et al., 2020)*

1.2.2.4.1 Chronic Phase (CP):

CML CP is more or less asymptomatic but patients can be diagnosed with an anemia including a hemoglobin values < 10g/dL[296,297]. This phase is characterized by less than 5% of BM blasts, a leukocytosis is also reported going from 12 to 1000 x 10^9/ blood litre with a median of 80 x 10^9/L (we note that a normal leukocyte blood count is comprised between 3.2 to 9.5 x 10^9/L) (Figure 16). Basophils do count for less than 20% and a common thrombocytosis. Other specific abnormalities can be reported such as an increased paratrabecular immature granulocyte with 5 to 10 cells thick while normal thick is comprised between 2 to 3 cells. Megakaryocytes can also display a small “dwarfs” with a hypo-lobated nuclei[296,298]. In this CML initial stage, a myeloid expansion begins gradually, and myeloid progenitors and mature cells

do accumulate within both BM and PB compartments. During CP, CML cells are heterogeneous comprising normal and leukemic cells leading to a more or less functional hematopoiesis with a modified small cell fraction that support CML disease maintenance and progression[298,299]. On one side, CML CP progression into an advanced phase (AP or BC) has been dramatically slowed since pre TKI Era. Indeed, nowadays around 1,5% of CP CML TKI treated patients do evolve into an advanced stage while 20% of CP CML patients used to evolve toward a more advanced stage before TKI ERA[300]. On the other side, it has been shown that a disease progression from CP to advanced stage involves several mutations without a unique signature leading to a hallmark of disease transformation[301,302].

1.2.2.4.2 Accelerated Phase (AP):

Disease Accelerated phase is characterised by a higher blast percentage in BM going up to 19%, 20% of more basophils, a persistent increasing leukocytosis counting more than 1000 x 10^9/L and new additional cytogenetic abnormalities and leukemic immature cells progression[296] (Figure 16). Unlike CP, the AP present valuable symptoms such as weight loss, enlarged spleen and general tiredness and has a median duration of 6 to 9 months without treatment [298].

1.2.2.4.3 Blast Crisis (BC):

Lastly, the ultimate disease phase and the more aggressive one in the BC[302]. This phase is characterized by more than 20% of BM blasts, extramedullary blast proliferation supplemented with large clusters of blasts in BC Patients biopsy[296]. This phase has a very tiny median duration without treatment comprised between 3 to 6 months[298,302](Figure 16). Furthermore, at this acute stage, the leukemic CML cells do proliferate and expanse quickly and imbalance normal hematopoietic cells presence, moreover these leukemic cells do accumulate a genomic instability due to several cytogenetic abnormalities. Finally, patients symptoms can be extended to extramedullary diseases caused by CML[298,302].

1.2.3 CML Leukemic Stem Cell

1.2.3.1 Special features

Cancer Stem Cells (CSCs) remain a key point to understand the persistence, patients relapse and resistance in cancers regarding their special feature of primary tumor initiated cells [303]. Several studies have demonstrated that CSCs are mainly responsible of the resistance to therapies in several cancers. To enhance tumorigenesis, they are also responsible of the metastatic increase potential in solid tumors through several inducer signals like WNT, Notch or Hedgehog[304] . Especially after conventional therapies, CSCs will turn dormant and after gaining several abilities to upregulate molecules and proteins such as TGFβ, Notch, drug efflux pumps, enhancing their telomerase activity and protection ability against ROS and DNA repair system, this very small subpopulation will be able to cause a Tumor relapse and re-generate the disease [305].

As normal HSCs, Leukemic Stem Cells (LSCs) can differentiate with limited proliferation and increased self-renew abilities to ensure a pool of tumor cells. To successfully eradicate tumor, this sub fraction of LSC should be targeted, otherwise, a constant reservoir of residual disease will lead to a CML relapse. This bulk of LSCs has been described for the first time in Acute Myeloid Leukemia (AML)[306]. In this study, the authors showed that a very small immature $CD34^{+}$ $CD38^{-}$ cell fraction counting around 0,1 – 1% is able to generate AML disease in Non-Obese Diabetic Severe Combined ImmunoDeficiency (NOD-SCID) mice. Of note LSCs population is variable and can represent up to 10% in some leukemias [277]. Furthermore, LSC study remained a big challenge during the past 30 years giving its crucial role in disease maintenance. The acknowledged fact that LSC do express same membrane markers as HSC made these cancer cells difficult to be specifically targeted. To this end, several studies were undertaken to identify a potential specific marker that could distinguish LSC from HSC. Several studies highlighted the relevance of more or less specific markers such as CD26, CD33, CD36,

CD44, CD47 or CD52[307–311]. Nonetheless, all these outcomes remain controversial and unconclusive but recent studies deciphering CD26 expression showed that this membrane protein may be used as a diagnosis and prognosis CML marker[312]. On account of microenvironment presence, LSC will be supported and protected by the surrounding cells and Extra Cellular Matric (ECM) components. Indeed, the same niche that preserves HSC maintenance and homeostasis do support and preserve LSCs which implies a niche modification to maintain cancer disease[313]. In spite of similarities between HSC and LSC, immature CML cells are present and measurable in peripheral blood while in normal conditions HSC do not get out of BM compartment without differentiating into a mature blood cell[314].

1.2.3.2 Philadelphia chromosome:

CML disease is a myeloproliferative disorder distinguished by the presence of Philadelphia chromosome (Ph) (Figure 17). This chromosome was described for the first time by Nowell in 1962[315]. It was only in 1973 where Rowley demonstrated that Philadelphia chromosome results from a chromosomal translocation that occurs between chromosome 9 and 22 [316]. Finally in 1986, A. De Klein et al. reported that c-ABL is translocated on the Ph chromosome and the BCR-ABL fusion gene was cloned and identified [317]. BCR-ABL oncoprotein effect has been investigated *in vitro* a well as *in vivo*. BCR-ABL induces apoptosis loss and growth factors self-sufficiency *in vitro*[318,319]. As well, a study where authors transplanted a BCR-ABL (p210) LSC into an irradiated mice model results in a myeloproliferative aggressive CML-like syndrome causing animals rapid death from 2 weeks following transplantation[320]. All these data supported the fact that BCR-ABL oncogene is responsible of CML disease initiation[283].

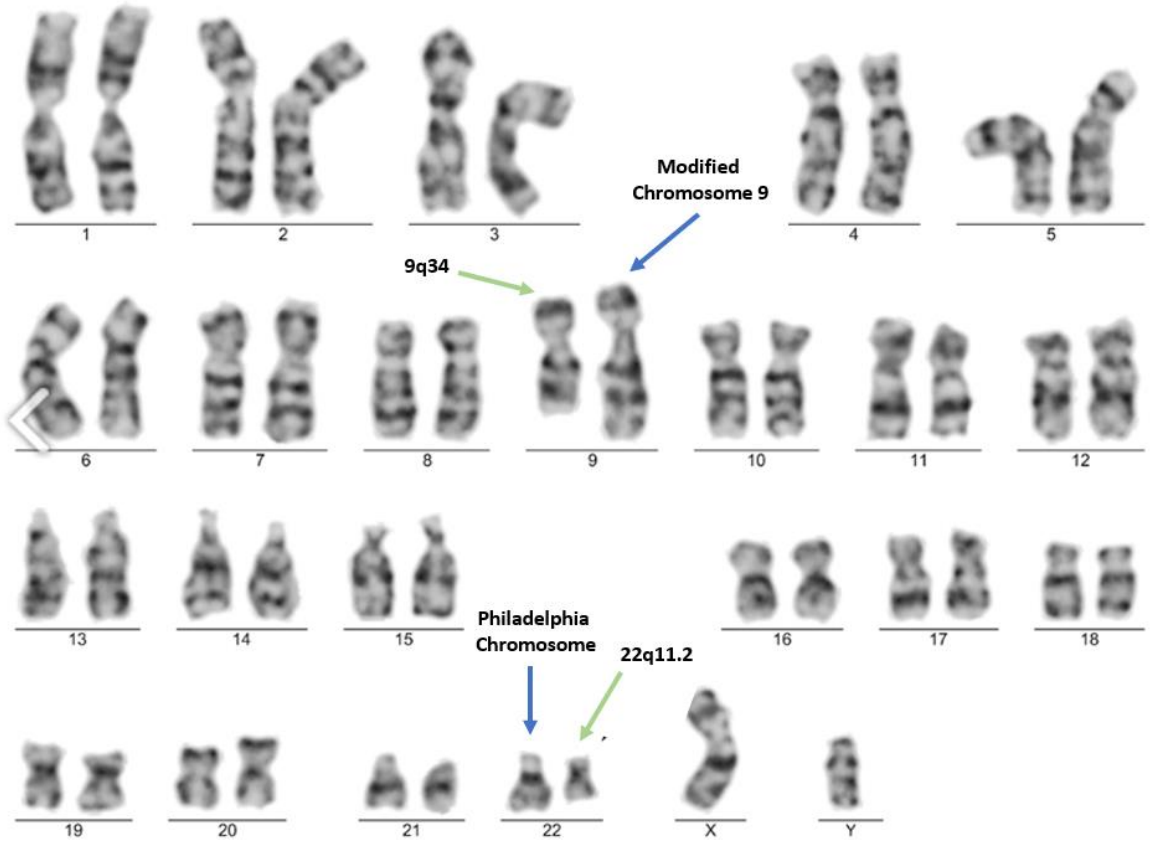


Figure 17: Karyotype coming from a male CML patient.

The chromosomal translocation between chromosome 9 and 22 results in a slightly longer modified Ch 9 and a longer Ch 22 that is the Philadelphia chromosome. *(Picture taken from atlas of hematological cytology)*

1.2.3.3 BCR-ABL Formation, Structure & Isoforms:

BCR-ABL fusion gene results from a chromosomal translocation between BCR gene that is normally located on the chromosome 22 and ABL gene that is located on the chromosome 9 t(9;22)[321] (Figure 18). This somatic mutation is generated from a break that happens in the chromosome 9 releasing ABL gene. The released ABL gene moves to the chromosome 22 to attach to BCR gene leading to a merged gene called BCR-ABL. The modified ckromosome22 that contains BCR-ABL is called Philadelphia chromosome taken its name from the city where it was discovered. Although BCR-ABL is commonly used to diagnose of follow CML disease

evolution, it is not exclusive for CML sub type and can also be expressed in some ALL and AML patients[322,323].

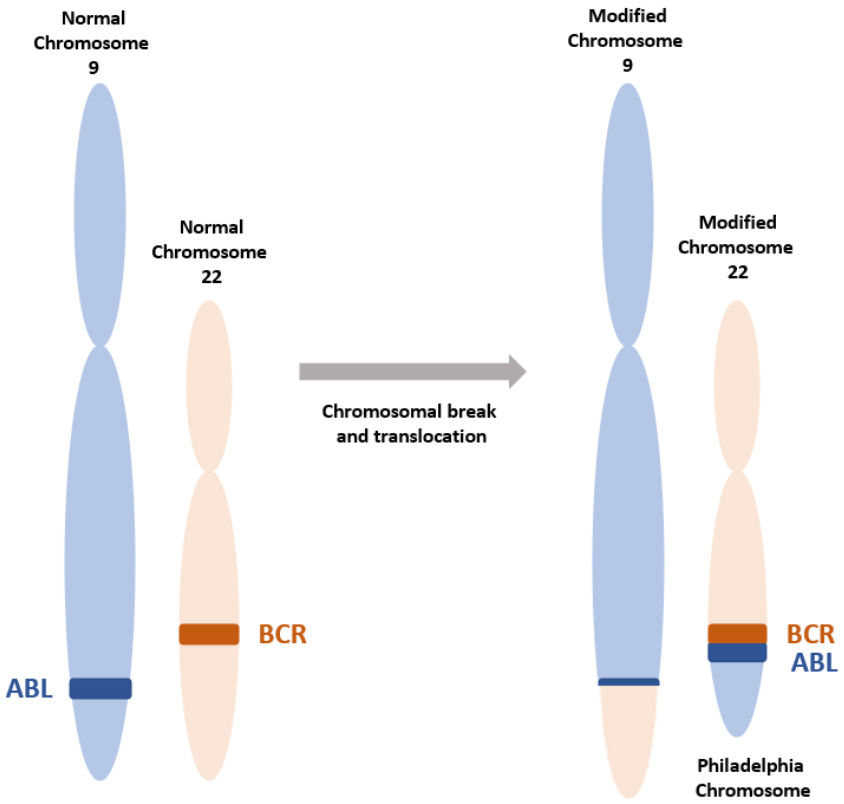


Figure 18: BCR-ABL translocation and Philadelphia chromosome formation.
After a chromosomal break, the ABL gene originating from the chromosome 9 will fuse downstream BCR gene located in the chromosome 22. The neo formed 22 chromosome is called Philadelphia chromosome. This translocation results in a slightly longer chromosome 9 due to the downstream BCR sequence that comes from the chromosome 22. Regarding the Philadelphia chromosome size, it is slightly shorter than the normal chromosome 22 because of the short chromosome 9 fused sequence.
(Adapted from Amin et Ahmed, 2021)

1.2.3.3.1 BCR-ABL structure:

Considering its key role in leukemic diseases, BCR-ABL remains a target for cancer research to understand and limit its oncogenicity. *BCR-ABL* oncogene is formed by a fusion of chromosome 22 *BCR* 22q11.23 and chromosome 9 proto-oncogene *c-Abl* 9q34.12 (also called *c-ABL1*) giving rise to the Ph chromosome[295,324,325] (Figure 18) . In normal cases, *c-ABL* encodes a non-receptor with an intimately regulated tyrosine kinase activity[326]. The oncogenic feature of *c-ABL* gene is enabled by the upstream *BCR* N-terminal coiled-coil oligomerization domain

(*BCR* $_{1\text{-}72}$ residues), SH3 *c-ABL* kinase regulatory domain will be inhibited by *BCR*, hence BCR-ABL1 constitutive activation leading to transformation[327–329].

Human BCR protein comprises 1271 aa and weights 160 kDa. This protein contains several domains such as an Oligo domain at its 1-72 N-terminal. A Serine Threonine kinase domain (S/TK), a domain homologous to the human Dbl and yeast Cdc24 proteins (Dbl homology domain) and finally a Rac GTPase activating domain (Rac GAP)[330]. Concerning c-ABL it is comprised of 1097 aa which corresponds to 150 kDa[325]. ABL protein contains 2 Src Homology Domains SH3 and SH2, a Tyrosine Kinase key domain TK, a Nuclear Translocalisation signal domain (NTS), a DNA Binding domain (DB) and an Actin Binding domain (AB) at its C-Terminal end[331]. The two key domains crucial for *BCR-ABL* transformation are *BCR* OLI (present at BCR N-terminal) and *ABL* TK (present at ABL N-terminal). *BCR* sequence can change depending on BCR-ABL form while *c-Abl* sequence remains the same whatever BCR-ABL form[321] (Figure 19). The different breakpoints in BCR gene occur either between S/TK and DH domains for ALL detected BCR-ABL^{+}, or between DH and Rac GAP domains for CML detected BCR-ABL^{+} leading to different fusion proteins forms weighting 190 kDa for ALL and 210 or 230 kDa for CML (9q34.12; 22q11.23) [321,331] (Figure 19).

1.2.3.3.2 BCR-ABL Isoforms:

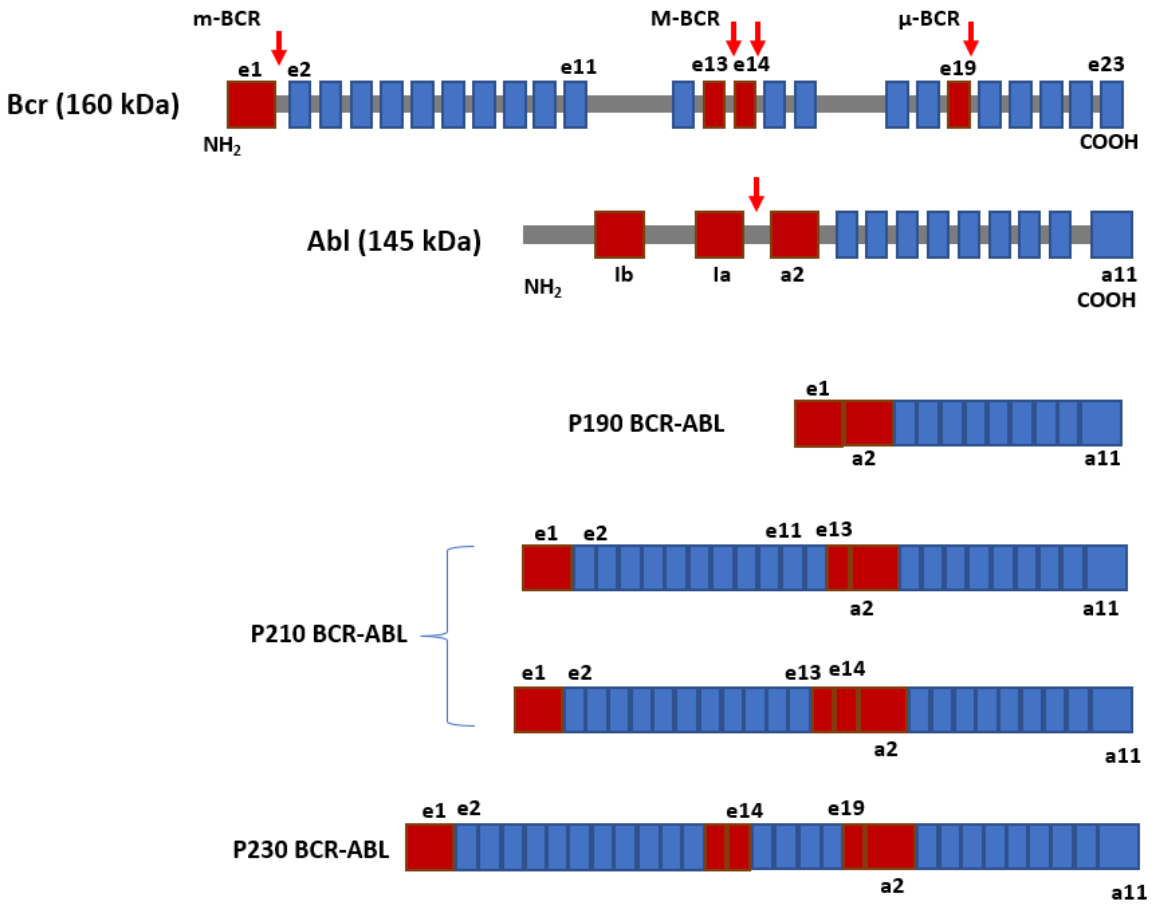


Figure 19: Structure of BCR-ABL.
According to where BCR will be cleaved (red arrows show cleavage sites), different BCR-ABL oncoproteins with different molecular weights will be generated.
(Adapted from Amin et Ahmed, 2021)

BCR gene sequence is divided into 3 main regions where the gene breakpoints happens, these regions are called Major *BCR* (*M-BCR*), minor *BCR* (*m-BCR*) or micro *BCR* (*µ-BCR*)[332] (Figure 19). More than 95% of CML patients do hold M-BCR breakpoints that lead to the transcripts b2a2 and b3a2 (where b2 & b3 corresponds to BCR exon13 and exon14 while a2 corresponds ABL exon2) also respectively called e13a2 and e14a3 corresponding to 210 kDa isoforms oncoproteins[333]. Another oncoprotein forms may be detected in CML patients such as p190 (e1a2) and p230 (e19a2) [321,334]. Nonetheless, other atypical BCR-ABL isoforms may be detected by multiplex PCR to target residual disease in CML patients as well as new BCR-ABL isoforms[334].

1.2.3.4 Molecular & functional mechanisms involving BCR-ABL:

To acquire a high activity potential, BCR-ABL monomer transduced from *BCR-ABL1* oncogene needs to undergo an oligomerization then autophosphorylation on two sites to be fully and highly activated (Figure 20). Indeed, an oligomerization will be done at the coiled coil (CC) BCR domain allowing a dimer oncoprotein that has an increased activity gradient compared to the monomer. A fist autophosphorylation is then achieved at the tyrosine Tyr1294 ABL regulatory domain[335]. This first autophosphorylation will induce a rapid phosphorylation of a second tyrosine kinase Tyr1127 that spaces SH3 inhibited regulatory domain resulting in a highly active kinase activity of the oncoprotein[335,336] (Figure 20). This constitutively activated oncoprotein will either recruit several adaptors or directly activate several downstream signaling pathways causing CML cell transformation.

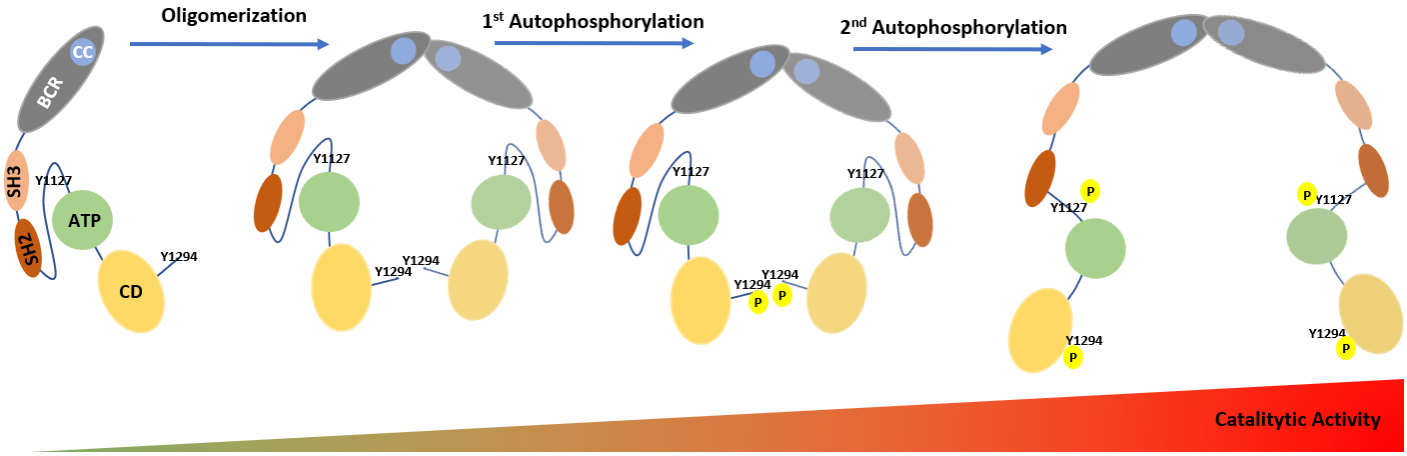


Figure 20: BCR-ABL activation.
In its monomeric form, BCR-ABL oncoprotein is unphosphorylated. Upon oligomerisation through the Coiled Coil (CC) BCR domain, BCR-ABL dimer will gain in activity gradient. A first autophosphorylation will occur at Tyrosine 1294 followed by a second one at the Tyr 1127. The second phosphorylation will disrupt the SH3 linker interaction enabling a full catalytic activity for the oncoprotein. *(Adapted from Amin et Ahmed, 2021)*

BCR-ABL1 oncoprotein can be involved in CML cells adhesion control as it can increase the leukemic cell adhesion by increasing β1 integrin clustering and cytoskeleton abnormal

remodeling[337] (Figure 21). As well BCR-ABL1 can induce the opposite effect as it underlies the Integrin mediated adhesion alterations in CML cells leading to the abnormal circulation of CML progenitors within the PB, which is never the case in normal homeostasis[338]. Furthermore, BCR-ABL1 controls CML cells proliferation, differenciation and cell survival through several pathways. Among these numerous pathways, BCR-ABL1 will increase *BCL-2* expression as well as PI3K/AKT, JAK/STATs, Ras/ERK and SRC pathways. Indeed, BCR-ABL1 does increase the expression of the anti-apoptotic BCL-2 through Ras activation[339]. In another way, the oncoprotein do activate PI3K/AKT GSK3β, Caspase9,FoxO3 and BCL-XL to block CML cell apoptosis increasing leukemic cells' survival[340]. Consequently, apoptosis intrinsic inhibition makes immature $CD34^+$ CML cells less sensitive to a wide panel of cytotoxic agents[341]. As well cell cycle progress is enhanced by an abnormal activation of STAT1 and STAT5 which results is a CML cell proliferation[342,343]. Furthermore, RAS/MEK/ERK signaling pathway will be abnormally activated to enhance mitogenic CML cell ability and increase cancer cell proliferation[344]. Besides, CML $BCR\text{-}ABL^+$ do set an autocrine production loop of TNFα, IL-3 and BMP4, furthermore, *in vitro*, BCR-ABL allows CML cells to grow independently from growth factors such as G-CSF and GM-CSF which fosters CML cells quiescence, survival, proliferation and resistance to TKI treatment[93,345–347]. In addition, another pathway involved in cell proliferation and differenciation will be highly increased in CML cells. BCR-ABL1 will activate and recruit several SRC protein family such as P53/56lyn, p59hck and SRC[348]. Numerous studies highlighted SRC proteins key role in BCR-ABL1 mediated transformation as well as Its signal transducing role through STAT5 & PI3K/AKT[349,350]. Several SRC protein kinases such as SRC, HCK and LYN are deeply involved in hematopoietic cells and can be recruited by BCR-ABL1 oncoprotein[348]. SRC kinases are also involved in CML disease resistance as they maintain BCR-ABL1 in an active structural conformation on which Tyrosine Kinase Inhibitors (TKIs) cannot bind[351]. Nonetheless, SRC involvement in BCR-ABL1 tumorigenesis remains unsteady as other studies shows that SRC is not necessary for *in vivo* BCR-ABL1 CML induction[352].

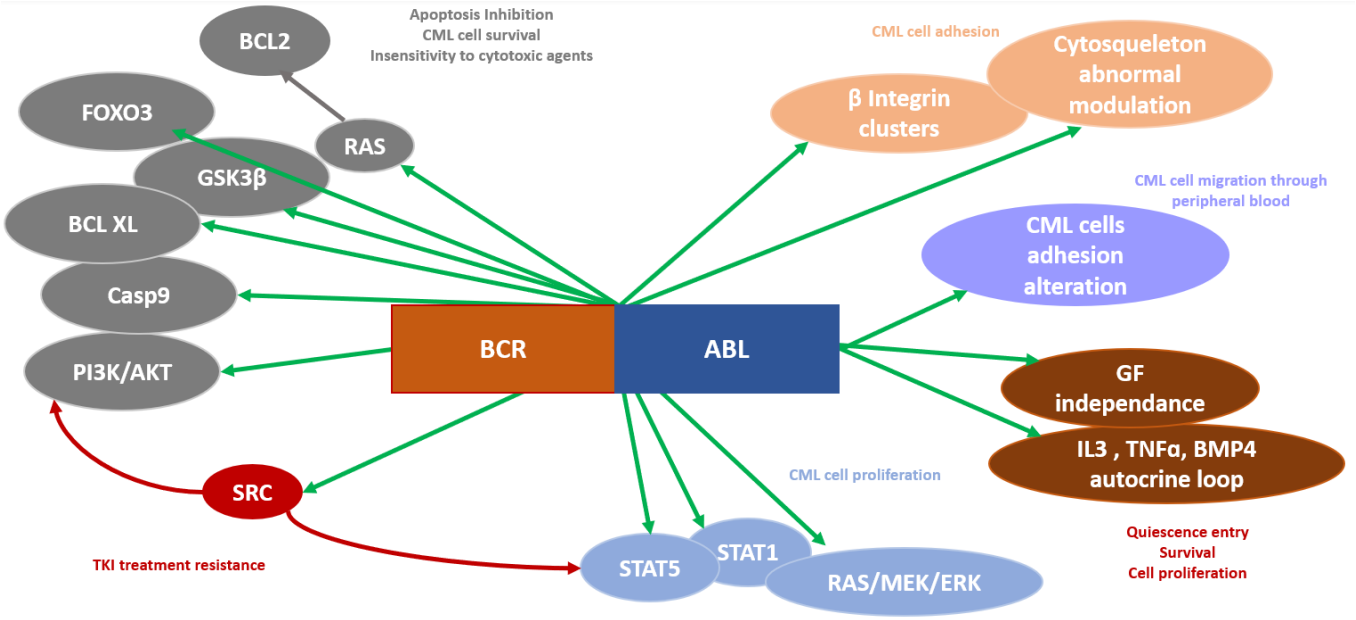


Figure 21: Main downstream pathways activated by BCR-ABL oncoprotein.
BCR-ABL activates a multitude of pathways deeply involved in leukemic cell survival, insensitivity, and resistance to treatments, CML cell quiescence, leukemic cell adhesion, proliferation and migration.
Green arrows show the directly activated pathways by BCR-ABL. Red arrows show undirect activation of PI3K/AKT and STAT5 by BCR-ABL through SRC proteins. Grey arrow shows and indirect activation of BCL2 by BCR-ABL through RAS superfamily.

1.2.3.5 CML treatment & resistance

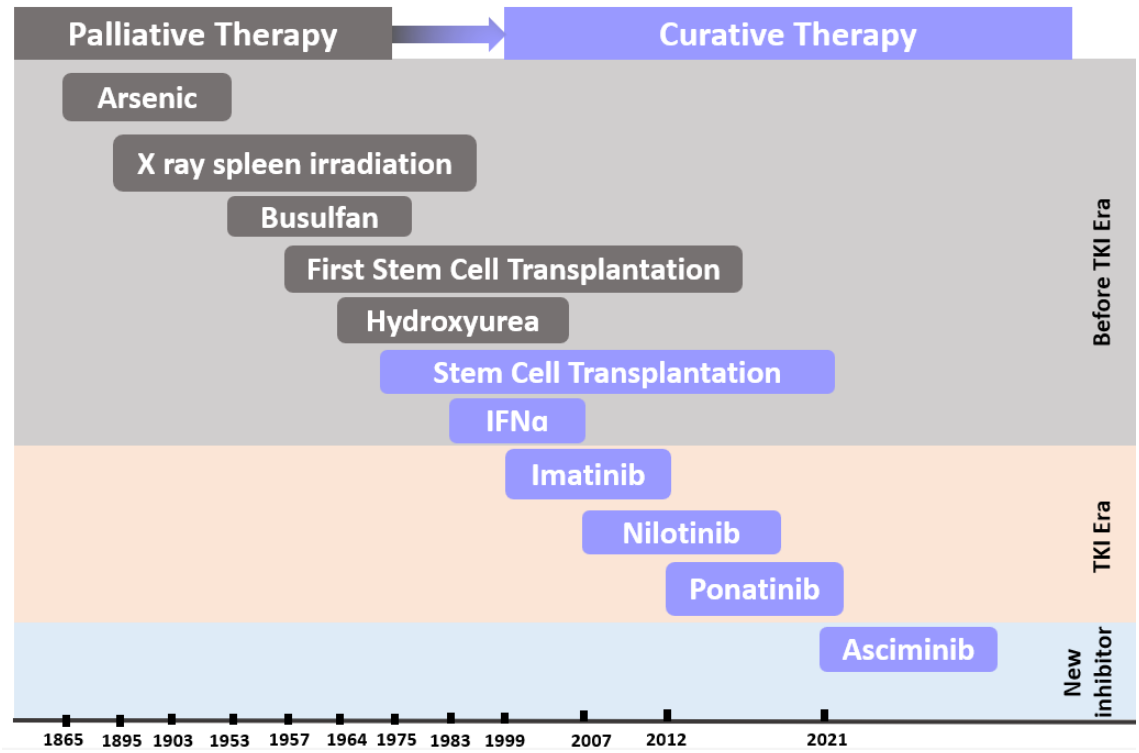


Figure 22: CML treatment evolution.
Over the years, CML treatment progressed from a palliative treatment to a curative and targeted treatment.

1.2.3.6 Therapy's evolution

1.2.3.6.1 Conventional cancer treatment (Chemotherapy & IFNs):

Before Tyrosine Kinase Inhibitors era, different unspecific drugs were used to treat CML patients. First CML treatment dates back to 1865 where the first Arsenic doses were administrated to CML patients[275] (Figure 22). Few years later, precisely in 1895 , X rays discovery by a German physician called W.C.Roentgen initiated its use as a palliative therapy for several diseased including CML. Indeed, in 1903 the first spleen irradiation was successfully performed[276]. In 1953 a nonspecific cell cycle alkylating chemotherapeutic agent named Busulfan (Myleran©) was introduced by a young clinical investigator David Galton to treat CML[353]. This chemotherapy succeeded to maintain CML patients in a partial hematologic response, nonetheless its toxicity lead to serious side effects such as BM aplasia or hormonal disorder that occurs in adrenal glands causing fever, weakness, pulmonary fibrosis or a high blood pressure[353]. Few years later, in 1964, Hydroxyurea (= Hydroxycarbamide) (Hydréa ©), a cell cycle and DNA synthesis inhibitor was proposed as a CML treatment[354]. This chemotherapy is less toxic than Busulfan but causes a reversible hematopoiesis inhibition, although it increases survival median of CML patients to 5 years whereas this median remains 3.75 years in CML Busulfan treated patients. Later on, a meta-analysis was performed to compare CML treatments and it proved that Hydroxyurea is more efficient than Busulfan to treat CML disease[355]. All in all, these treatments were breaking CML disease for a while but do not prevent its evolution into an aggressive stage. In all cases, CML patients treated by Busulfan or Hydroxyurea were doomed to evolve to the final Blast Crisis (BC)[356]. First Stem Cell Transplantations (SCT) dates back to 1957 when E.D Thomas et al. reported intravenous infusion of BM in leukemic patients already treated with chemo and radiotherapy[232]. Nonetheless, it is only in the early 1980 where SCT approach was pushed forward and developed to become the unique curative therapy at this time[357]. To be transplanted, HSCs were received from a donor (allogenic) or extracted from the concerned CML patient (autologous) and treated, then transplanted to the CML patient receiver after treating him

with chemo combined or not with radiotherapy to destroy his BM cells. The goal of healthy SCT within CML patients destroyed BM is to enable these normal HSCs to repopulate and replace CML patients BM[357–359]. In the 80th the use of Interferon α cytokine (IFN α) treatment began. The first CML patients arm treated with IFNα started in 1986 to be compared to a classical chemotherapy treatment [360]. This cytokine treatment turned out to be promising as it maintained cytogenetic remission in up to 20% of CML patients[361]. Considering its production by immune cells such as dendritic cells and leukocytes, IFNα is deeply involved in innate immune response[362,363]. Nevertheless its use as a therapy causes slight side effects in CML patients such as fever, headache, nausea and vomiting but with a long cytokine treatments patients suffered from unwieldy side effects such as autoimmune thrombocytopenia, heart dysfunction as well as several kidney diseases [364–366]. IFNα monotherapy is no longer recommended but its combination with different chemotherapies succeeded to increase CML patients cytogenetic remission[361].

1.2.3.6.2 Tyrosine Kinase Inhibitors:

Considering CML as a cancer disease caused by an acquired somatic abnormality, it helped to focus therapy research on BCR-ABL fusion oncoprotein with more or less specific drugs considering their unspecific action that reaches other tyrosine kinases independent from BCR-ABL (Table 2). Indeed in 1999 targeted therapies began with a molecule called Tyrphostin (AG490) that blocks JAK2 kinase activity resulting in an inhibition of immune T cells proliferation[367]. Afterwards, a small molecul initially called STI 571 was developed in Ciba-Geigy laboratories and then repurchased by Novartis group. STI 571 showed a dramatic major cytogenetic response induction in CML patients[368]. Besides, STI 571 success was a pioneer for cancer specific targeted treatment[369].

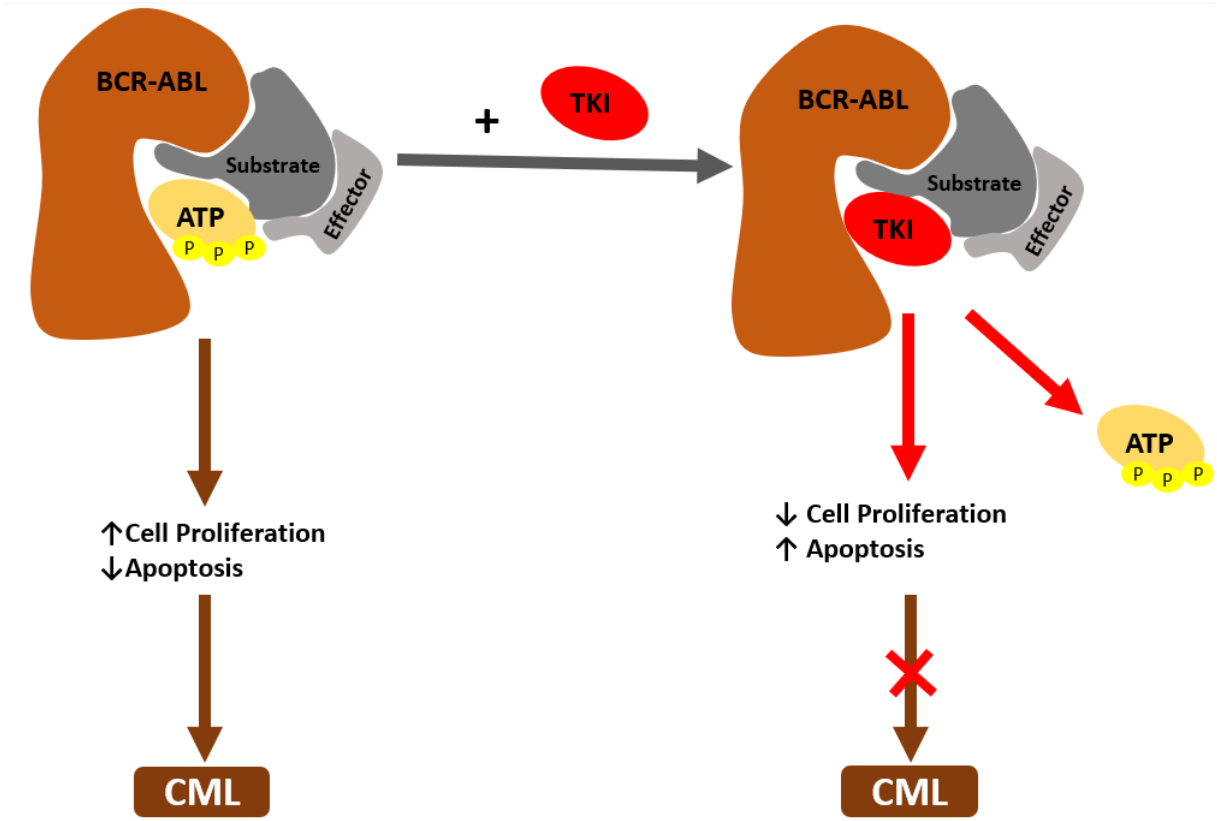


Figure 23: TKI mechanisms of action.
In absence of treatment, ATP will bind to BCR-ABL ATP pocket triggering downstream signalling that increases leukemic cell proliferation and decreases its apoptosis hence fostering CML leukemogenesis. Tyrosine Kinase Inhibitor will compete with ATP molecule to bind to the ATP pocket avoiding BCR-ABL activation. Thus, TKI treatment will decrease leukemic cell proliferation and increase its apoptosis avoiding CML disease progression. *(Adapted from Manouchehri et al., 2019)*

	Imatinib	Nilotinib	Ponatinib	Asciminib
FDA Approval	Mai 2003	October 2007	First approval in December 2012 2nd approval in 2014	29 of October 2021
Inhibited Kinases	BCR-ABL1 ABL1 ABL2 CSF-1R DDR1 & 2 SCF PDGFRA & B C-Kit NQO2	BCR-ABL1 ABL1 ABL2 CSF-1R DDR1 & 2 SCF PDGFRA & B C-Kit NQO2	BCR-ABL^{315I} BCR-ABL1 ABL1 ABL2 CSF-1R DDR1 & 2 SCF PDGFRA & B C-Kit VEGFR1 & 2 & 3 Ephrin receptors NQO2EPH receptors SRC superfmilly RET, TIE2,FL3	BCR-ABL1 BCR-ABL^{315I} (not yet available)
CML Treatment Indication	First line therapy	First of second line therapy	First line for patients holding BCR-ABL^{315I} Second line therapy if absence of response for prior TKI	First line for patients holding BCR-ABL^{315I} Second line therapy for CP-CML patients that failed at least to 2 TKIs treatments

Table 2: CML treatment comparison.

CML TKIs are not exclusively specific to BCR-ABL. TKIs can also target a wide panel or tyrosine kinases as shown in the table. Of note, Asciminib other targets is not readily well assessed.
(Greuber et al.,2013; Manley et al., 2020; Yeung et al., 2022)

1.2.3.6.2.1 First generation

Since the 1999, the Tyrosine Kinase Inhibitors (TKIs) treatment era began for CML treatment (Figure 22). Indeed, TKI drugs targets the oncoprotein by competing with ATP in two different modes, Type I or Type II binding. For Type I binding, TKI bind to the active conformation of BCR-ABL1 while the type II binding consist in a TKI binding to the inactive form of BCR-ABL1[370,371] (Figure 23).

Nowadays, the first line treatment for this disease remains TKI such as Imatinib (= STI 571; Gleevec©) (Figure 24), that allowed to increase CML patients overall survival by 40% compared to the Interferon treatment and by 60% compared to the chemotherapy treatment

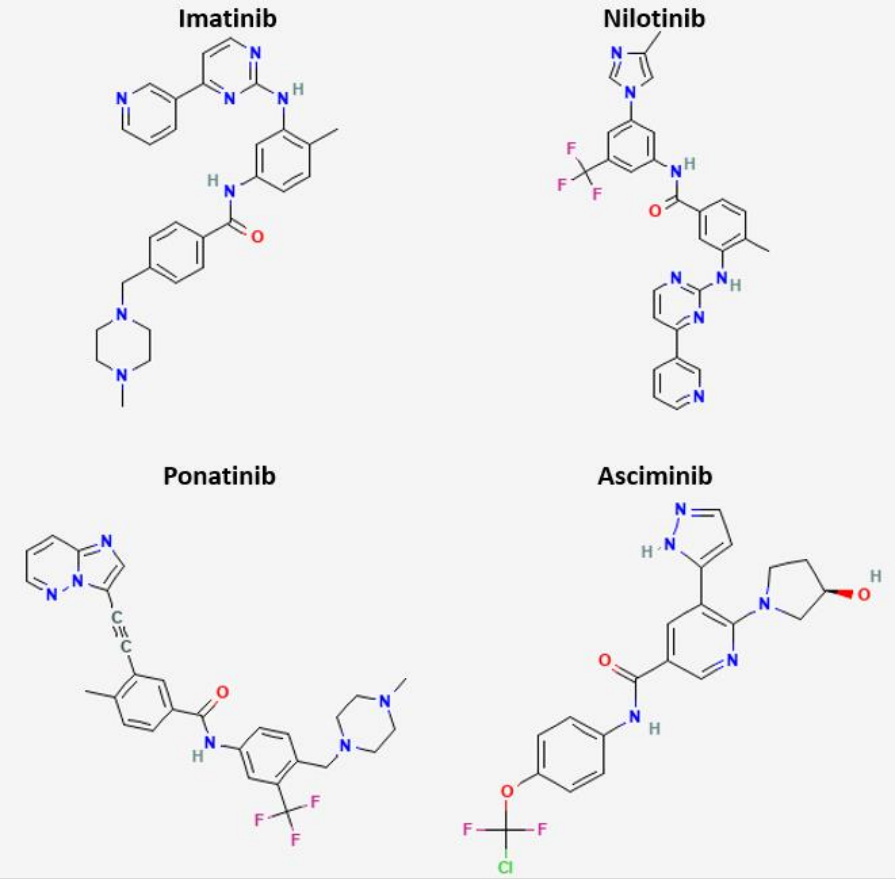


Figure 24: TKIs and Asciminib 2D structures.
Pubchem CID: 5291 (Imatinib), 644241 (Nilotinib), 24826799(Ponatinib), 72165228(Asciminib) *(Pubchem, NCBI)*

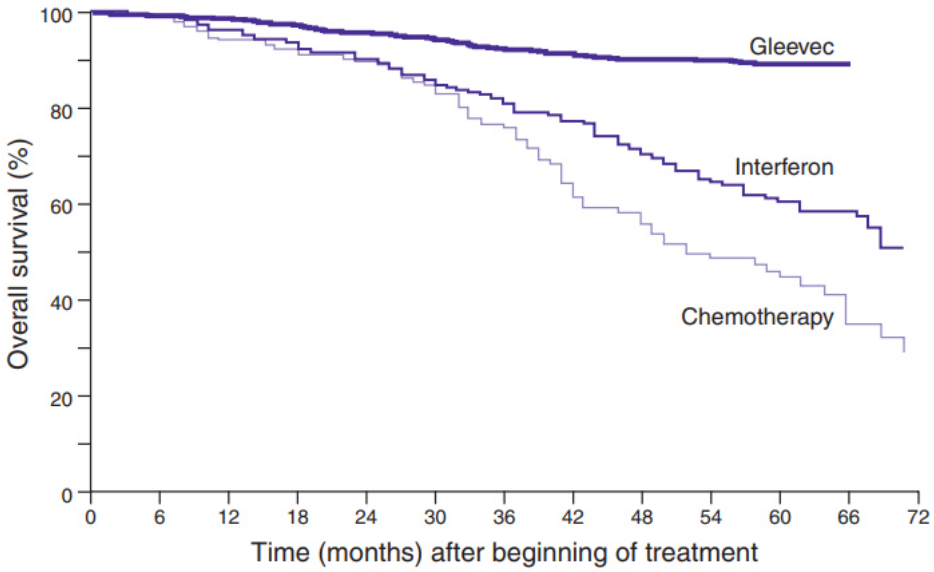


Figure 25: Imatinib (Gleevec©) treatment efficiency for CML patients.
Imatinib treatment dramatically improve CML patient overall survival while interferon treatment shows a minimal benefit on CML patients survival curve. Chemotherapy conventional treatment shows the weakest effect on CML patients' survival. *(Druker et al., 2009)*

(effects observed after 60 months of treatment) [372] (Figure 25). Furthermore, a randomized study (IRIS) compared imatinib effects with combinatory IFNα/cytarabine treatment. Imatinib treatment was noteworthy insofar its dramatic cytogenetic and molecular induced responses in CML patients. Indeed, 85.2% of imatinib treated patients achieved Major Cytogenetic Response while only 22.1% CML patients from the combinatory treatment group achieved it[373]. This revolutionary treatment is a 2 phenylamino-pyrimidine derivative that acts in a specific way on the Tyrosine Kinase (TK) BCR-ABL activity[374] (Figure 23). Its first *in vitro* effects resulted in ABL1 kinase activity inhibition in CML like cells[375]. As well, it decreased CFC colonies forming of seeded CML derived MNCs (mononuclear cells) cells by 92-98%[375]. Imatinib molecule binds to the ATP pocket of the onco-kinase, avoiding its phosphorylation and stopping BCR-ABL1 downstream induced signal decreasing CML cell proliferation and enhancing its apoptosis [376]. Interaction between BCR-ABL1 oncoprotein and Imatinib is possible even when the oncoprotein is in an inactive unphosphorylated form as Imatinib proceeds to a Type II binding to BCR-ABL[377].

This involved a stabilization of the oncoprotein in its inactive form reducing oncogenic signal transmission[378]. Plus, Imatinib is not exclusively targeting BCR-ABL oncoprotein but does also inhibit c-KIT, PDGFR, NQO2 and DDR1[379] (Table 2).

Thus, Imatinib/BCR-ABL1 oncoprotein targeting revolutionized the CML treatment, Nevertheless, several resistance mechanisms shape through the disease evolution causing a restriction for this drug efficiency. Nonetheless, Imatinib side effects are nowadays well documented and turn out to be rare. Indeed, more than 10% of CML treated patients can show short term toxicities such as nausea, muscle cramps, headache, fatigue or muscle cramps whereas less than 1% of CML Imatinib treated patients will suffer from long term toxicities such as renal failure, cardiac toxicities or cerebral edema[380]. Although its satisfactory results, several CML patients do unfortunately develop drug resistance and response loss to Imatinib, the reason why other 2nd TKI generation more potent than Imatinib was developed[381,382].

1.2.3.6.2.2 Second generation

Second generation of TKIs do comprise several molecules such as Nilotinib, Dasatinib or Bosutinib. Its development emerged to face CML resistance to Imatinib.
In 2007, Nilotinib, also called AMN107 molecule (Tasigna ©) was approved from US FDA (United States Food and Drug Administration) as an Imatinib drug analogue with a higher affinity for BCR-ABL1 for Imatinib resistant or intolerant CM patients (Figures 22, 24, table2). Nilotinib is an amino-pyrimidine developed from Imatinib to comprise alternative N-methylpiperazine groups to bind ABL[383]. Its development goal is not restricted to CML but also to ALL Ph^{+} wases that do respond transiently to Imatinib therapy[384]. Its potency is more than 20 folds higher than Imatinib to inhibit BCR-ABL ATP pocket as well as it is efficient to target a wide panel of imatinib resistant mutations except T315I[383,385]. This drug proved its efficiency *in vivo* as it significantly increased survival of mice injected with Imatinib resistant BCR-ABL^{+} CML cells[384]. A phase 1 study included CML patients resistant to Imatinib as well as Ph+ ALL patients, an increase up to 35% of CCyR was observed in CML resistant patients[385]. In addition, Phase 2 study revealed that Nilotinib administration to CML Imatinib intolerant /resistant patients increased their CCyR to 45% and their overall survival upon 4 years to 78%[386] . Phase

3 of a clinical trial (NCT00471497) from Novartis group compared Nilotinib with Imatinib and revealed that Nilotinib treated CML patients achieve 83.3% of CCyR (Complete Cytogenetique Response) against 66.7% for Imatinib treated group[387]. Of Note, Nilotinib acts through a Type II inhibition to target BCR-ABL, as well, its inhibitory effect is not exclusive for BCR-ABL1 ATP pocket. Indeed, its large target spectrum includes PDGFR, c-KIT, NQO2, DDR1, EphB4 epinephrin receptor and CSF-1 Receptor[379] (Table 2). Furthermore, the use of Nilotinib in CML patients may cause several diseases such as diabetes and heart diseases like coronary artery disease, peripheral arterial occlusive disease or cerebrovascular disease[388].

1.2.3.6.2.3 Third generation

Many molecules belonging to a third TKI generation were designed for a shared purpose of targeting T315I mutation. Among these molecules, Ponatinib also known as AP24534 molecule (Iclusig©) showed a clinical activity (Figures 22, 24, table2). Indeed, Ponatinib is the first TKI effective against T315I BCR-ABL1 mutation that counts for 20% of total clinical Imatinib CML resistance[389,390]. As Imatinib and Nilotinib, Ponatinib targets BCR-ABL inactive form. Its therapeutic use is not limited to CML treatment but it is extended to a large panel of cancers considering its action against PDGFRα, c-SRC ,VEGFR2, c-KIT, and FGFR1 in haematological cells[391]. As well against c-KIT and FGFR2 in Gastrointestinal cancer[392,393] in addition to several other targets such as RET in thyroid cancer, and FGFR3 in bladder cancer[393,394]. In December 2012, after a successful phase 1 and phase 2 studies, the FDA approved Ponatinib use in CML and BCR-ABL^{+} ALL patients as a second line treatment[395]. Nonetheless, regarding serious vascular adverse effects, US FDA decided to withdraw Ponatinib from the medical market until 2014 where a CML patient follow up study outlined satisfactory results. Since then, Ponatinib was placed back into the medical market[396,397]. Notice that because of its severe side effects such as blood clots that do block blood circulation within blood vessels, Ponatinib clinical use is still outlined[398].

Thanks to TKI discovery, CML changed from a life-threatening to a manageable disease. The effect of TKI binding do decrease CML tumorigenesis burden and improves CML patients' survival as the majority of TKI treated CP CML patients do have a normal life expectancy[399] . Nonetheless, even with the therapeutic evolution and efficacy supplied by TKIs, some CML

patients intolerance, poor initial response or loss of response[400,401] to TKI drugs besides numerous mutations in BCR-ABL1 (BCR-ABL1^{mut}) ATP site do drive CML tumorigenesis by outrunning the treatment effect[402,403]. For instance, the T315I mutation is a substitution of The Threonine at 315 position by an Isoleucine. It is found in around 20% of CML resistant or relapsed patients[397]. The unique TKI able to target this mutation that involves a gatekeeper residue is the Ponatinib (Iclusig ©)[404]. Nonetheless, this drug's prescribed dosage remains low in CML patients regarding its life-threatening side effects[405] . Indeed, several Ponatinib CML treated patients do develop serious cardiovascular diseases because of its serious side effects[398,406].

1.2.3.6.3 New Inhibitors

In this research perspective toward new drug that specifically targets ABL1 with lower side effects, a new drug was discovered.

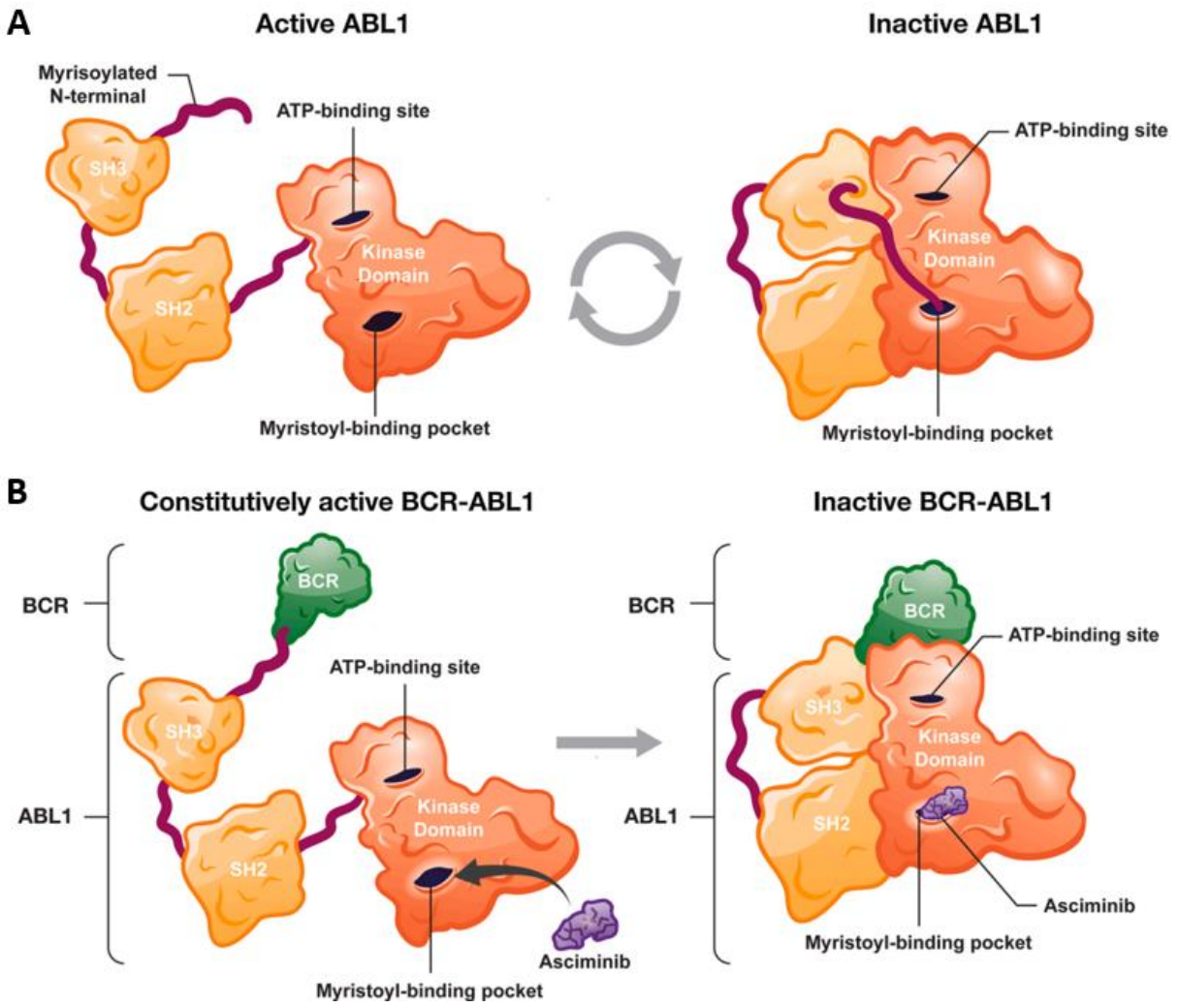


Figure 26: Asciminib mechanism of action.
(A) Normal ABL is active when myrisoylated domain is out of the myristoyl binding pocket. When myrisoylated domain binds to the myristoyl binding pocket the kinase conformation changes and became inactive.
(B) In CML, BCR occupies ABL myrisoylated hence hindering ABL conformational change to become inactive. Asciminib use will recover this hindering by binding to the myristoyl available site to switch BCR-ABL from an active to an inactive conformation. *(Réa, Hughes, 2022)*

In 2003, a scientist group reported that a myristoyl group is deeply involved in ABL1 & ABL2 kinases autoregulation[407–409] . It has been demonstrated that an active ABL1 has a free Myrisoylated N-Terminal at its SH3 domain and this Myrisoylated N-terminal will go into the Kinase Domain (KD) (Figure 26).

Myristoyl binding pocket leads to an SH2 and SH3 domains docking against the KD to leave the ABL1 in an inactive conformation[410](Figure 26). However, when the BCR combines with ABL1, the latter's myrisoylated domain is not available anymore and the binding of BCR causes the loss of ABL1 natural autoinhibition leading to a constitutively activated oncoprotein kinase[407,410]. Nevertheless, even if BCR binding hinders ABL1 autoinhibition, the myristoyl binding pocket remains available and the concept of using this second BCR-ABL1 target began with numerous molecules for a potential drug discovery.

Among these potential inhibitors, GNF-2 succeeded to inhibit BCR-ABL1 allosterically and showed an anti-proliferative effect on BCR-ABL1^{+} cells[411]. Later, its GNF-5 analogue was developed with a better pharmacokinetics features and did show an *in vivo* inhibition efficacy but none of these inhibitors achieved the clinical developpement[412].

In 2017, a potential myristoyl pocket inhibitor called ABL001 (lately called Asciminib or Scemblix©) was tested and showed a potent inhibition of BCR-ABL1 mediated leukemic cell proliferation[413](Figure 24, table 2). This molecule mimics the myrisoylated ABL1 N-terminus and lodges within ABL1 Myristoyl binding pocket restoring thus BCR-ABL1 catalytically negative conformation[410]. Its promising effects lead it to a pivotal phase I and phase III trials where it proved a significant efficacy along with attractive CP CML patients safety[414,415]. In October 2021, Asciminib monotherapy was approved by FDA to treat CP CML Ph^{+} patients that received at least 2 TKIs as well as CP CML Ph^{+} BCR-ABL1^{T315I} [416](Figure 22, table 2)) .This first in class STAMP (Specifically Targeting the ABL Myristoyl Pocket) inhibitor may present an efficient and relatively safe option to treat CML patients that do not tolerate or resist to

several TKIs[414]. Furthermore, its targeting to the myristoyl pocket makes it more selective then TKIs as it binds exclusively to a particular kinases[417] . Plus, its non-competitivity with TKIs to bind the ATP pocket allows a prospect for a future combinatory use of these two kind of drugs to target and limit tumorigenesis in CML. Asciminib side effects remain mild such as nausea, diarrhoea, tiredness, headache, dizziness or stomach. Nonetheless, its clinical studies showed that for a short CML patient follow up less than 10% of included CML patients had to discontinue treatment because of its side effects[418]. Of note, regarding its recent beginning use as a CML drug, significant data concerning its side effect linked to a long-term administration are not yet available.

Nevertheless, at least 3 patients in the ASCEMBL trial developed a mutation in the myristoyl site causing drug resistance [419]. Indeed, resistance to this myristoyl site inhibitor will certainly emerge over CML patients' treatment time due to mutations occurring in this site, the reason why a potential combination between a TKI and Asciminib may supress resistance emergence.

1.2.3.6.4 Transplantation

As discussed above, the first HSC allogenic transplantation use as a therapy for CML patient dates back to 1979 (Figure 22). This kind of transplantation can either be an allogenic transplantation using HSC coming from a healthy compatible donor or an autologous transplantation that is based on CML patient's own healthy stem cell collection before treatment, storage and transplantation to CML patient after undergoing several treatments to destroy the abnormal present stem cells. The goal is to see the healthy HSC repopulating patient's defective hematopoietic system. SCT was the first adopted CML curative therapy, nonetheless, this technic is related to a high mortality cause that can reach 78% of transplanted patients[420]. This therapy lethality is mainly related to opportunistic infections because of the induced pre-destruction of the patients immunity system before transplantation as well as Graft Versus Host Disease (GVHD) that occur in transplanted patient[420]. However, this data was generated in CML transplanted patients receiving intensive chemo and/or radiotherapy before Imatinib era. Recent outcomes are focused on CML patients that previously received TKI but do not respond or tolerate this therapy[421]. Indeed, after frontline therapy failure, CML patients do have more therapeutic options such as a TKI

dose increase or a try for a second or third TKI generation. The choose of these options mainly rely on the CML patients' comorbidities, age, disease stage, and BCR-ABL1 eventual mutations.

Of note, allogenic SCT is no longer proposed in the first line treatment but it is still adopted for CML patients in CP or advanced phase that failed to at least 2 TKIs[421] . Numerous studies showed that some transplanted CML patients do respond well to this curative therapy as a case report showed a late CML patient relapse around 25 years post allogenic SCT[422].

1.2.3.7 Patients follow up & MMR determination

TKI therapy's use goal is to increase CML patients' overall survival minimizing CML disease related symptoms and complications by improving overall life quality of these patients. To follow up CML patients' treatment responses, a universal and standardized method is adopted. In addition to blood cells count and spleen size checking, a sensitive measurement of Ph^+ chromosome and *BCR-ABL1* transcript level using real time quantitative polymerase chain reaction (RT qPCR) world widely standardized is necessary to access patients initial response. This quantitative RT qPCR will also predict patients disease evolution as well as their

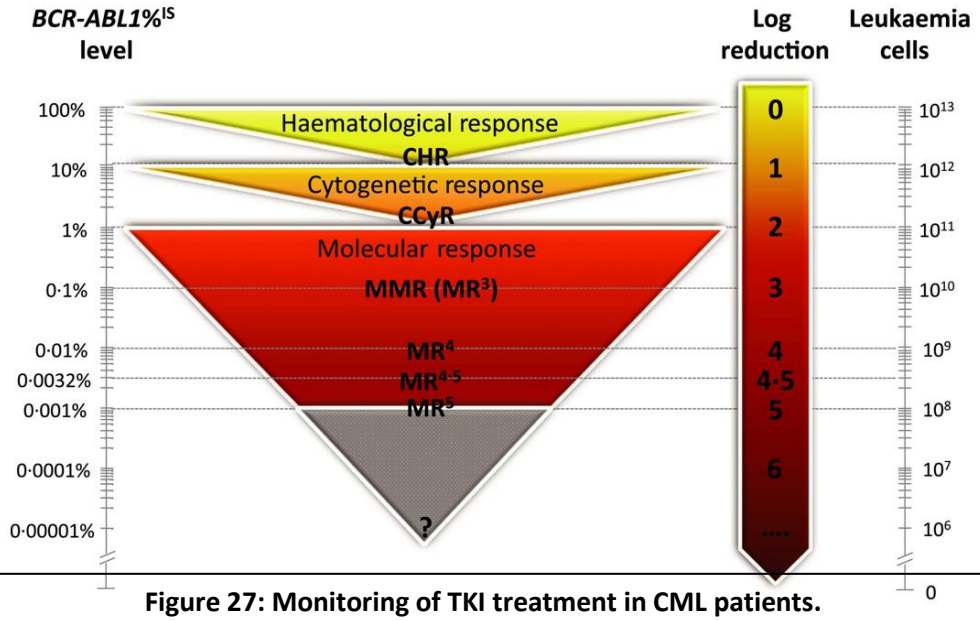


Figure 27: Monitoring of TKI treatment in CML patients.
Key levels of molecular response are reported on an International Scale (IS) for treatment monitoring. Hematological response (HR) is the first objective that CML patients are expecting to reach. At this level blood cells' count reaches the normal values. A deeper response to achieve is the cytogenetic response (CCyR) and is related to the percentage decrease of Ph^+ cells. Lastly, the molecular response (MMR) reflets *BCR-ABL* transcript levels. MMR is defined in terms of *BCR-ABL* transcript log reduction from a standardized baseline and several MMR levels do exist depending on how deep the response is. (Soverini et al., 2016)

level of achieved remission to plan a future potential treatment arrest [423] (Figure 27).

1.2.3.7.1 Hematological response

Hematologic response monitoring of treated CML patients remains mandatory to report accurately treatment efficiency and predict an emerging treatment resistance. This hematological response is accessed around 3 months after the beginning the TKI treatment uptake. The Complete Hematological Response (CHR) is achieved when leukocytes number is under 10 x 10^9/L with a complete absence of immature cells present in peripheral blood, plus the spleen size should be back to normal. When a condition of the cited above is missed we can say that the patient achieved a Partial Hematological Response[423,424] .

1.2.3.7.2 Cytogenetic response:

The cytogenetic response is deeper than the hematological one. This response is done after 3 and 6 months following the treatment beginning. It can also be checked later than 6 months following treatment beginning until it's achieved by the patient. To be determined, a karyotype using either Fluorescent *In Situ* Hybridization (FISH technic) or Chromosome Banding Analysis (CBA) of BM resident cells is performed. When no Ph+ cells are detected in the BM, the cytogenetic response is considered as complete (CCyR). Nonetheless, when more than 95% of BM cells are Ph+ there is no cytogenetic response. When Ph+ BM cells are between 35 to 95% the cytogenetic response is considered as minor and when Ph+ BM cells count for less than 35% the cytogenetic response is considered as partial (PCyR)[425].

1.2.3.7.3 Molecular response:

This response relies on *BCR-ABL1* transcript expression in BM and PB blood cells. The oncogene transcript level measurement by RT qPCR in nowadays the principle standardized international method to monitor disease evolution. Several degrees of molecular response can be assessed. An early molecular response can be observed after 3 to 6 months following

the treatment and corresponds to 1 to 2 log reduction or *BCR-ABL1* transcript compared to the standardized baseline (MR^1 & MR^2). The Major Molecular Response MMR is assessed 12 months after treatment beginning and is achieved when *BCR-ABL1* detected transcript is very low and ≥ 3 log reduction compared to the standardized baseline (MR^3) [426]. A complete molecular response CMR (also called Deep Molecular Response DMR) is achieved when the transcript levels are ≥ 4.5 log reduction compared to the standardized baseline ($MR^{4.5}$)[425,427,428].

1.2.3.7.4 Treatment Free Remission (TFR):

The Standard of care in CML disease is treatment-free remission (TFR). Considering TKI treatments wide side effects, its cost and the psychological burden of a daily pills administration, a TFR should be considered in CML patients that achieved a deep molecular response (DMR). The latter is fixed by the international scale (IS) as being equal or less than 0.001% of BCR-ABL1 transcript. It has been shown that patients treated with a 2nd TKI generation have a high DMR incidence compared to ones treated with Imatinib[429]. The concept of CML treatment arrest began with a French pilot study called STIM1 (STop IMatinib) protocol where, after 6 months of treatment arrest for patients with at least 2 years of negative results for BCR-ABL1 detection, 61% of them relapsed, but after 6 and a half year, 38% of patients maintained a molecular remission[430] . Another similar study called EURO-Ski included the participation of 11 European countries and 61 sites. Its main goal was to define optimal conditions for CML treatment discontinuation[431]. Inclusion criteria were patients in CP, with a MR^4 for one year or more and at least 3 years of TKI uptake without any treatment failure. Following the treatment arrest, some patients showed a molecular recurrence and had to undergo a TKI re uptake. The latter was based on the patients' tolerance, comorbidities, risk of toxicity and delay toward a new TFR attempt. The probability to reach a second TFR after a 1st or 2nd TKI administration was about 50%. Unfortunately, some patients evolved to a blast crisis. Around 25% of included patients developed muscle & joint pain following TKI treatment discontinuation and these misguided effects required temporary anti-inflammatory treatments administration. As well, most of patients re-entering a TFR suffered from

numerous unfortunate affects such as depression, anxiety and fear, this is why a psychological monitoring should be included from the beginning of these studies for patients relieve.

TFR achievement is the actual perspective seeing TKIs as a curative treatment and is currently considered after 5 years of remission. Nonetheless, a very significant proportion of patients relapse after a TFR achievement and displayed again BCR-ABL1 positive cells in BM in contrast to the peripheral blood, indicating the long-term persisting CD34+ CML cells [432] . These observations open the road for new opportunities to eradicate these rare and persisting CML SCs during the remission phase. It will certainly give a new twist to the CML cure achievement but request a deep understanding of cellular and molecular mechanisms that sustain this LSCs long-term *in situ* quiescence.

1.2.3.8 CML Persistence and LSC quiescence:

Although TKIs dramatically improved CML patients care and enabled the achievement of remission, these patients still do carry minimal residual disease as long as TKIs fail to eradicate quiescent LSCs. An undetectable reservoir of these LSCs is still present in CML patients at remission. The first study highlighting presence of reversible quiescent leukemic cells in CML patients dates back to 1999[376]. Later, it has been demonstrated that these quiescent LSC do not respond to TKI treatment and are not eradicated by CML treatment[433]. In 2010, The STIM protocol was performed to see if patients at remission for several years can stop imatinib treatment uptake. Included CML patients had no detection of the *BCR-ABL1* transcript during at least 2 years. The outcome showed that around 61%(42 among the 69 followed patients) of CML patients in remission relapse in the 6 months following the treatment cessation [430]. The fact that when the drug pressure is removed, sensitive CML LSCs are emerging to re-initiate the disease progression. Therefore, despite the good response to imatinib, a LSC fraction survives through the niche interaction during the treatment and is responsible of the persistence development and patients' relapse after treatment cessation [430 434].

LSC population is heterogeneous. Some LSC do derive from a BCR-ABL1 transformed HSC while other LSC present in CML patients at advanced disease stage do originate from progenitors but acquired SC features and other putative LSCs lack leukemogenesis potential[435,436]. Therefore, LSC ability to act as Leukemia Initiating Cells (LIC) is heterogeneous within BM compartment and relies on intrinsic and extrinsic controlling factors. Disease re-progression after remission and therapeutic failures do result from LSC quiescence within the BM. In reality quiescent $CD34^{+}$ $CD38^{-}$ LSCs represent less than 1% of CD34+ cells[433] . Nonetheless this tiny population threatens patients' healthcare and serves as a residual disease reservoir able to induce leukemogenesis any time.

It has been demonstrated that *BCR-ABL1* downregulates CXCR4 expression in CML cells[437]. In addition, CML cells are also able to reduce CXCL12 production by its surrounding cells within the BM and consequently alters its adhesion to the niche leading to an extravagation and presence of leukemic cells in PB[438]. Other LSCs do sustain their quiescence through intrinsic or extrinsic factors avoiding TKI CML cells eradication. Intrinsic activation of *BCR-ABL1* do activate an endogenous inhibitor SET that negatively regulates Protein Phosphatase 2A (PP2A) resulting in CML LSC quiescence maintenance[439]. Also, the Promyelocytic leukemia gene (PML) that is involved in a chromosomal translocation t(15;17) responsible of Acute Promyelocytic Leukemia (APL) development is also involved in CML. This gene do encode a protein that regulates cell proliferation, senescence and apoptosis. In CML, an increase of PML seems to be critical for LSC quiescence maintenance[440]. Furthermore, extrinsic signals have been reported as critical for CML LSC persistence within the BM. Niche endothelial cells do supply CML LSCs with miR-126 enhancing their self-renewal and quiescence maintenance[441]. In addition, BMP4 has been described as an actor in this process as it is produced by MSCs and do enhance LSC quiescence through BMPR1B that can also be overexpressed on CML LSC cell surfacen[93,434,442]. Thus, a recent study showed that CML LSC co-activate BMP4/BMPR1B/SMAD1,5,8 and JAK2/STAT3 to maintain its quiescence by adhering to stromal MSCs[434] (Figure 29). Integrins such as VLA-4 and VCAM-1 have been described to increase LSC adhesion to the niche[443]. BM endothelial cells E-Selectin and BCR-ABL1 dependent CD44 overexpression do also play a role in LSC dormancy as well as its resistance to TKIs[444]. NOTCH LSC overexpression and Osteoblasts JAGGED-1 do also play a critical role in LSC quiescence support as osteoblasts ablation causes quiescent LSC sub-fraction loss [445]. Furthermore, miR-

300 released and fostered by MSCs exosomes and hypoxic microenvironment do also play a key role in LSC quiescence induction and maintenance[446]. Recent studies show that TKI treatment enhances LSC quiescence. As matter of fact, miR-126 is downregulated by BCR-ABL1 that inhibits RAN-exportin-5-RCC1 complex that maturates miRNAs. BCR-ABL1 targeting by TKIs will avoid this inhibition resulting in a great supply of miR-126 to LSCs increasing this way their quiescence and persistence[446]. As well, Imatinib immature persistent cells treatment showed an up-regulation of these cells quiescence by decreasing their KI67 expression *in vitro*[434].

1.2.3.9 Clinical Resistance:

Several resistance mechanisms to TKI therapy in CML remain unclear, some of them have been described in the literature. Nonetheless, at the molecular level, the mechanisms must be deciphered in order to better manage the CML disease and its treatment. Resistance mechanisms are subdivided in two categories, "primary resistance"[447] which is dependent to the BCR-ABL and "secondary resistance"[447] which is independent to the oncoprotein [448].

Concerning BCR-ABL independent mechanisms, it includes upregulation of drug efflux pomp that will drive out the imatinib preventing its action on the tyrosine kinase. Other mechanisms like secretion of molecules binding to the drug or activation of alternative pathways are included in this resistance mechanism.[449]

1.2.3.9.1 Oncogene-dependent resistance mechanisms: primary resistance:

BCR-ABL dependent mechanisms include several mutations in the ABL kinase ATP pocket, avoiding the fixation of the Imatinib. It also includes the amplification of BCR-ABL1 oncogene that will increase the level of BCR-ABL1 mRNA level leading to translation of the oncoprotein (Figure 28).

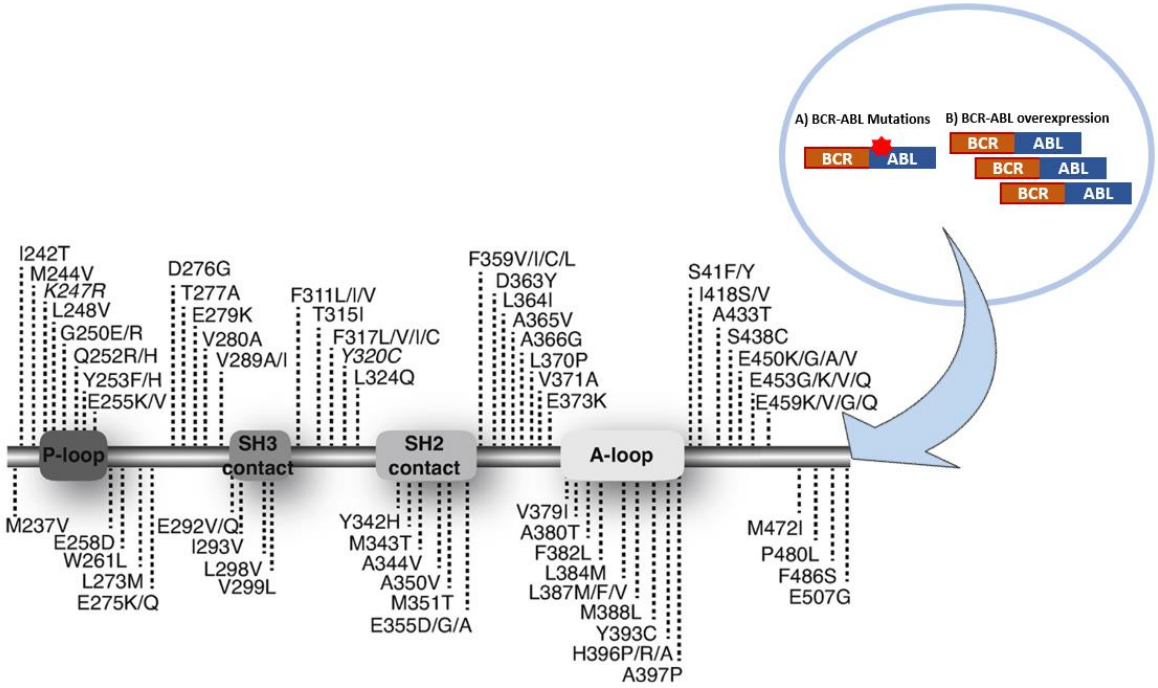


Figure 28: BCR-ABL dependant resistant mechanisms.
(A) Numerous mutations can occur in BCR-ABL oncoprotein leading to a TKI treatment resistance.
(B) BCR-ABL amplification can also cause patients resistance in CML patients.
(Adated from Soverini et al., 2014)

1.2.3.9.1.1 BCR-ABL mutations:

When a resistance is linked to a BCR-ABL1 mutation, it can be called "On target". This kind of resistance is linked to either *BCR-ABL1* oncogene or BCR-ABL1 oncoprotein modifications. The first BCR-ABL1 CML dependant resistance mechanism was reported in 2001 when CML patients at an advanced disease stage who presented an initial treatment response to imatinib relapsed [450]. BCR-ABL1 dependant resistance can either be quantitative or qualitative depending on BCR-ABL1 overexpression or mutations occurring in the kinase domain of the oncoprotein [451](Figure 28). The most described BCR-ABL1 mutation and the first discovered one in resistant CML patients remains T315I mutation. It results in a substitution of the Threonine at 315 BCR-ABL1 position by an Isoleucine[447,450,452]. As Threonine at 315 stabilizes Imatinib binding to the BCR-ABL1 ATP pocket through hydrogen bond interactions, the substitution of this aa with an Isoleucine will render the ATP pocket hydrophobic hindering this way imatinib fixation to the oncoprotein[450].

Aside from T315I BCR-ABL1 dependant mutation, a broad range of different mutations affecting BCR-ABL1 oncoprotein was reported whether in the N-terminal P-LOOP such as M244V, in the SH3 domain like I293V mutation, in the SH2 domain M343T or at the C-Terminal A -LOOP like L384M mutation domains[453](Figure 28). All these mutations do disturb conformational binding of TKIs to BCR-ABL1 ATP pocket leading to different degrees of resistance to TKIs treatment.

Recent study reported that some mutations do disturb TKI binding kinetics and not its binding to the oncoprotein[454]. Indeed, the authors reported different ABL^{mut} mutations that still bind Imatinib like the wilt type ABL or even with a higher affinity, nonetheless the drug dissociation from the ABL^{mut} was quick in such a way, TKI do not have enough time to induce an efficient oncoprotein inhibition[454].

1.2.3.9.1.2 BCR-ABL Amplifications:

Another treatment resistance is caused by *BCR-ABL1* amplification (Figure 28). Oncogene amplification is widely frequent in cancer diseases and leads to anticancer treatments drug resistance. This kind of resistance is mostly observed in advanced CML stages[455]. *BCR-ABL1* transcript overexpression leading to a high BCR-ABL1 oncoprotein tradition can occur as well as the acquisition of a second Philadelphia chromosome holding several copies of *BCR-ABL1* appears in late disease stages[456–458]. Philadelphia chromosome duplication was identified in 3/11 of BC CML patients that were resistant to Imatinib therapy[450]. Another clinical trial showed that among 66 resistant CML patients, only 2 presented BCR-ABL1 overexpression suggesting that BCR-ABL1 overexpression may be rarely responsible of CML TKI resistance[459]. It appears that BCR-ABL1 overexpression helps CML patients to rapidly reach 1st or 2nd TKI generation resistance because of an early appearance of resistant TKI mutant clones[460,461]. This case of resistance translates into a high level of available BCR-ABL1 active oncoprotein that exceeds TKIs inhibiting ability.

1.2.3.9.2 Oncogene independent resistance mechanisms: secondary resistance:

1.2.3.9.2.1 Drug Influx/Efflux unbalance:

TKI influx/efflux balance is necessary as these small molecules act in an intracellular manner. ATP-binding cassette (ABC) and the solute carrier (SLC) membrane transporters are recognised as the most relevant transporters controlling TKIs absorption, distribution, excretion and toxicity[462]. They are ubiquitously present in several human tissues recognizing and transporting a large panel of small molecules enabling drug intracellular interactions. Their presence is essential to evaluate mechanisms of Multi Drug Resistance MDR[463] .

TKI transport into CML cell is performed in an inactive and active way through ABC transporters[463,464]. Based on its permeability TKI can be uptake by the cell following its extracellular concentration and its permeability, of note this transport is slow and low number of TKI molecules are transported compared to the active drug transport[465]. SLC22A1 Organic Cation Transporter (OCT1) active transporter is encoded by *SLC22* gene and mediates TKI transport from the extracellular compartment to the CML cell cytoplasm[465,466]. OCT1 protein expression has been correlated to BCR-ABL1 activity in primary CML cells[467]. Furthermore, Prazosin, an α1 blocker used to regulate high blood pressure can interact with OCT1 by inhibiting it decreasing Imatinib cell uptake which suggest that some drugs that cure diseases other than CML can interfere with Imatinib uptake if CML patient do take it in parallel with TKI, suggesting also that other actors may be involved in TKI cell uptake[467] . It has been demonstrated that genetic variants of human OCT1 affect intracellular Imatinib drug import[466]. Consequently a variant with a decreased activity compared to $hOCT1^{WT}$ will cause a decrease of MMR achieving by 34% and decrease of 26% of event free survival in CML patients after 5 years of treatment[468].

TKI Efflux is ensured by active transporters from ATP-binding cassette proteins (ABC) family. ABCB1 (also called P-glycoprotein or Pgp pump) is involved in Imatinib expulsion out of the CML cell and this membrane protein is encoded by Multi Drug Resistance 1 *MDR-1* gene. ABCB1 pump enables TKI drug expulsion from the cell[463]. This transporter is overexpressed in

BC CML patients cells and is at least partially responsible of Imatinib resistance as it carries Imatinib out of CML cell before the drug has time to target specifically BCR-ABL1 oncoprotein[469,470].

1.2.3.9.2.2 Secondary signalling pathways activation:

TKI resistance can be independent from BCR-ABL1 passing by numerous signalling pathways overactivation or de novo activation[451]. Key cellular pathways such as RAS/MEK/ERK, PI3K/AKT and JAK2/STAT3 & STAT5 can be overactivated in CML. Their downstream activation can influence CML cell sensitivity to TKI treatment increasing CML treatment resistance[344,471,472]. Indeed, in CML primary cells, Jak2 overactivation enhances BCR-ABL1 oncogenicity by activation PI3K/AKT signalling that fosters *c-MYC* expression which contributes to TKI drug multiresistance[473,474]. In addition, SRC kinases family are deeply involved in BCR-ABL1 downstream signalling[352]. It has been demonstrated that LYN member is highly activated in Imatinib resistant CML patients that do not have any BCR-ABL1 domain kinase mutations[475].

1.2.3.10 Microenvironmental deregulations

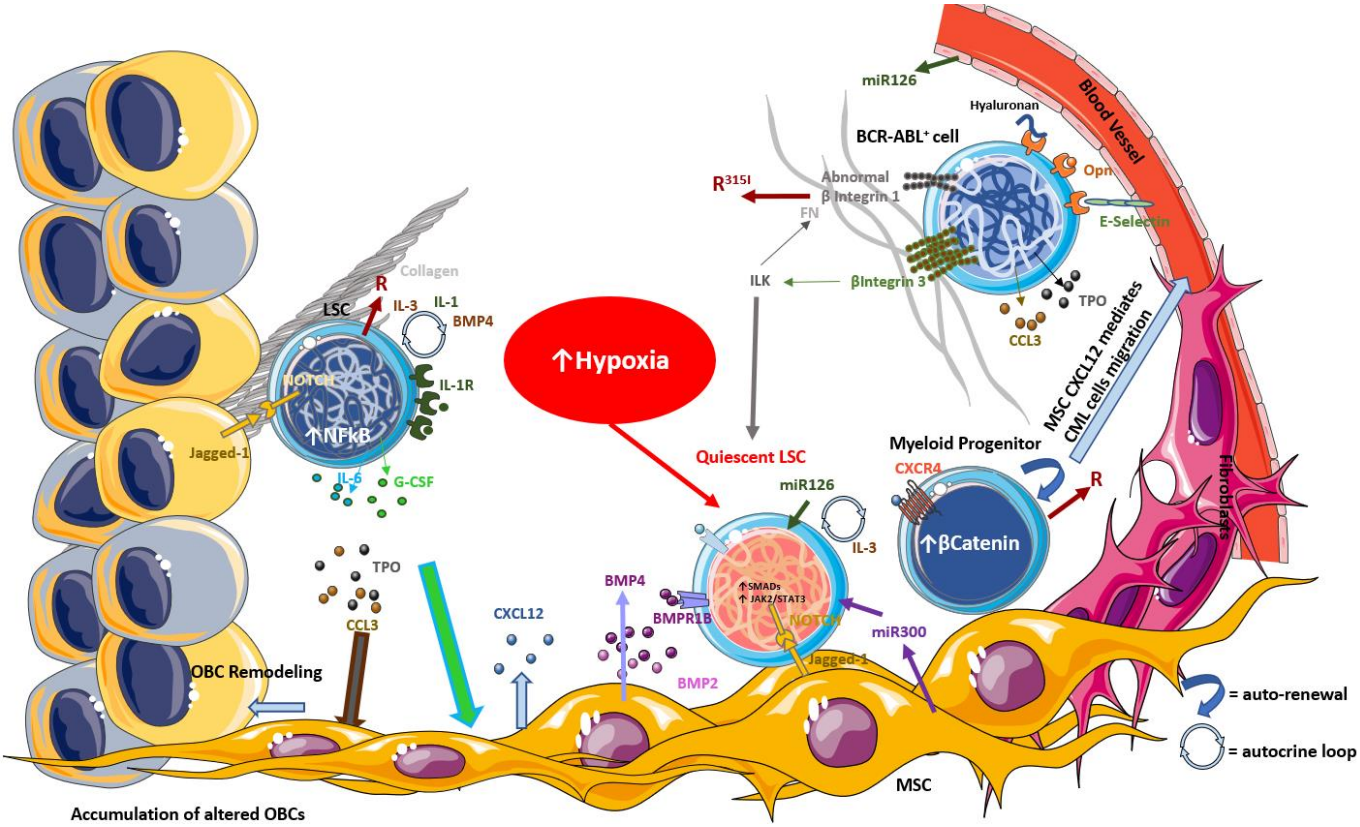


Figure 29: Overview of the modifications within CML BMM.
LSCs will acquire different features such as cytokines auto-production. CML LSCs will modulate the bone marrow microenvironment resulting in their increase support and maintenance. Through G-CSF and IL-6 release, LSC will enable its transmigration by stimulating CXCL12 MSCs production. As well, LSC increases in quiescence and maintenance in the niche by fostering fibronectin (FN) /collagen production and enhancing its cell-to-cell adhesion. Thus, LSC TPO and CCL3 release will push MSCs to remodulate osteoblasts (OBCs) leading to an abnormal accumulation of altered OBCs within the leukemic niche.

Regarding its pivotal role for HSC hematopoiesis homeostasis, BM microenvironment (BMM) is also essential for LSC support, growth, and maintenance. It has been widely accepted that with leukemogenesis, LSC balance and control hematopoietic cell's fate within the BM by modifying its surrounding niche. In CML, BM microenvironment benefits Leukemic cells in providing a mechanical protecting scaffold as well as a source of cytokines and growth factors that will help CML LSC and progenitor cells to be maintained and protected within the BMM.

1.2.3.10.1 Extracellular matrix modification

Through CML disease evolution, leukemic cells will be able to modify their BMM directly by producing and expressing adhesion molecules and cytokines or by stimulating their surrounding cells pushing them to behave differently than homeostasis case.

BCR-ABL1^{+} CML cells do express dysfunctional β1 integrin at its cell surface and the latter will interact with ECM FN altering hence CML cells adhesion within the BMM (Figure 29). Indeed, *in vitro* experiments showed significant interaction between BMM FN and CML cells β1 integrin resulting in a CML cell proliferation decrease[476]. Surprisingly, IFNα is able to restore normal FN/β1 integrin mediated interaction leading to a normal leukemic CML cell adhesion[338]. Thus, CD44 that is overexpressed at CML LSC cell surface will bind hyalyronan, osteopontin as well as E-Selectin resulting in a LSC maintenance and self-renewal within the BMM[444]. In addition to IFNα, PTH hormone treatment will decrease LSC number through TGF β1 release by ECM[477]. In addition, BCR-ABL^{T315I} CML cells overexpress β3 integrin at their cell surface resulting in an increase of Integrin-Linked Kinase (ILK) expression that will enhance FN deposition. ILK has been reported as playing an important role in TKI mediated resistance in T315I mutation cases as well as in quiescent LSC[478,479]. Following osteoblasts overproduction by TPO and MIP-1α(=CCL3) stimulation, Collagen 1 will also be overproduced and deposed in BMM. Furthermore, BMM modifications will lead to an increased trabecular bone formation and modification of osteoblastic cells leading to fibrosis[480] (Figure 29).

1.2.3.10.2 Cytokines production and some important deregulated pathways

Within leukemic niche, chemokines as well as cytokines production by LSCs or surrounding cells create a wide communication network controlling LSC and HSC fate. It has been demonstrated that CML LSC and BCR-ABL^{+} cells do secrete IL-1a, IL-1b, IL-3, IL-6, G-CSF and TNFα[438,481]. CML LSCs do inhibit miR-146a resulting in an overexpression of IL-1[482] . In parallel, CML LSCs do also increase IL-1R at their cell surface enhancing their sensitivity to this

cytokine[482] . This IL-1 autocrine loop will activate NF-kB intracellular pathway resulting in overexpression of several inflammatory cytokines within the BMM[482] . Furthermore CML LSCs will also secrete TNFα that will stimulate intracellular NF-kB leading to IL-3 autocrine production loop that controls LSC quiescence within BMM[345,346,481]. Thus, CML cells so also enhance their differenciation through G-CSF and IL-6 auto production to activate STAT5 intracellular pathway[345,346,483]. CML cells production of G-CSF in addition to TNFα can affect MSCs by decreasing their CXCL12 resulting in CML cells expansion and mobilization out of the BMM[265,438,484] (Figure 29) .

A widely deregulated pathway in leukemogenesis is the WNT pathway that alters both the mesenchymal niche and directly the LSC[485]. Indeed, CML is the first leukemic malignancy in which deregulated WNT pathway has been reported. The oncogene BCR-ABL indirectly enhances the beta-catenin activity by sequestering BCR which is a normal beta-catenin inhibitor and also by over activating the oncogene C-myc[486]. In CML, aberrant WNT signaling leads to a beta-catenin nuclear accumulation, followed by a deregulation of the WNT target genes[487]. A study performed on 33 CML patients samples and a CML blast crisis cell line (K562) showed that the beta-catenin gene is not mutated in its N terminal part so the beta-catenin mutation in CML is absent or very rare and other unclear mechanisms are over-activating this pathway to maintain and increase the CML pathogenesis[488]. Another experimental study showed that the deletion of β catenin in a BCR-ABL model decrease the LSC self-renewal and leukemia's development[489]. In addition, the deletion of β catenin in an established Leukemia synergize with TKI to efficiently eradicate CML LSC which confirms WNT involvement in CML tumorigenesis[490]. Finally, the WNT pathway is over activated in all CML stages but the accelerated phase, in which the gain of the self-renewal capacity by the granulocyte macrophage progenitors seems to be caused by the beta-catenin nuclear accumulation[455]. To summarize, in CML, the aberrant WNT signaling confers to the progenitors a self-renew feature without differentiation comparable to stem cell self-renewal ability that could preserve them from efficient killing by TKIs[455,491].

Since LSCs are constantly in interaction with their microenvironment, a study harvested primary CML cells with a conditioned media that have been in indirect contact with BM stroma. These data showed that soluble factors from the BM microenvironment helped CML

cells to escape their treatment by decreasing TKI-induced cell death[492]. A paracrine β catenin signaling appears to be involved in this CML cell's protection mediated by niche's stroma.

Another study based on co-culture of CML $CD34^+$ cells with MSCs showed that TKI treatment of CML LSCs and Progenitors will increase their apoptosis but if co-cultured with MSCs, their cell death will decrease significantly and the N-Cadherin levels will decrease at adherent CML cells' surface[493]. Consequently, N-Cadherin contributes to MSC mediated protection of CML LSC & progenitors from TKI and increases their treatment resistance. Furthermore, a TKI treatment of CML 34^+ decreases the level of beta-catenin in CML cells meanwhile a co-culture with MSCs will highly increase the beta-catenin expression in CML 34^+ whatever treated or not with TKI [493] (Figure 30).

2 Aim of the Study :

Scientific contest

La Leucémie Myéloïde Chronique (LMC) représente environ 15% des leucémies, c'est une pathologie touchant les cellules souches hématopoïétiques (CSH), cellules générant l'ensemble des cellules hématopoïétiques et

Lymphocytaires. L'origine de cette pathologie est une translocation chromosomique t(9 ;22) causant l'expression de l'oncoproteine BCR-ABL. Cette kinase sera constitutivement active et induira la phosphorylation d'une multitude de voies de signalisation causant ainsi différentes dérégulations touchant de nombreuses fonctions cellulaires ainsi qu'une expansion de cellules de type myéloïde. L'oncogène BCR-ABL rend la LMC spécifique, il a permis le développement de la première thérapie ciblée, les Inhibiteurs de Tyrosine Kinase (ITK) ciblant spécifiquement la poche ATP de la kinase BCR-ABL. Ce traitement de première ligne a révolutionné la prise en charge de la maladie mais demeure cependant non curatif. En effet, après l'arrêt du traitement aux ITKs, une rechute d'environ 60% est constatée chez les patients en rémission (Mahon et al Lancet Oncol. 2010).

Différents mécanismes de résistance sont responsables de cette rechute, certains sont connus comme la

mutation de la poche kinase de l'oncoproteine BCR-ABL ou encore la surexpression de l'oncogène ce qui

empêche la thérapie ciblée d'agir efficacement.

D'autres mécanismes de résistances demeurent mal identifiés comme l'implication de certaines Cellules Souches Leucémiques (CSL) qui semblent résister aux ITKs en se nichant dans la Moelle osseuse

(MO) des patients.

Lors de cette troisième année de thèse, j'ai pu travailler sur deux grands axes, tous inclus dans mon

projet de thèse. Le premier est basé sur l'évaluation de plusieurs aspects dont la quiescence, la production de BMP4 et l'expression de BMPR1B dans un modèle 3D mimant la niche médullaire. Le deuxième axe est l'étude de la quiescence des CSL à travers un modèle FUCCI BCR-ABL+ généré dans le

laboratoire et permettant de suivre de façon dynamique cette quiescence. Enfin, J'ai pu étudier

l’évolution de la quiescence des CSL FUCCI BCR-ABL+ sous ITK au sein de notre modèle 3D.

ce qu’on a trouvé et publié et ce qui est en cours

3 Materials & Methods :

3.1 Molecular Assay:

3.1.1 Proteins assay:

3.1.1.1 ELISA:

Bone marrow plasma is retrieved from patients or healthy donors BM after a high-speed centrifugation as shown below.

BM Plasma, retrieved supernatants from BA (sensitive CML LSC) BAP(Persistent CML LSC) after 3 days of culture or retrieved supernatant after a long time culture from the 3D system. Samples were precleared of cellular debris by rapid centrifugation and processed for BMP4 ELISA quantification (R&D Systems) kits following the manufacturer's instructions. Measures were performed in duplicate.

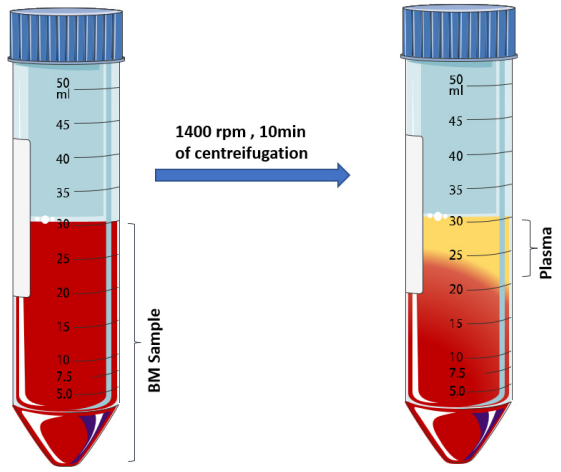


Figure 30: Bone marrow sample processing.

3.1.1.2 Western Blot:

Western blot was performed using TF1 FUCCI BA cells treated different drugs for 24 hours. Proteins were then extracted using RIPA buffer and 30 mg of proteins were loaded.

Membranes were incubated with monoclonal-antibodies against P-ERK (#4370), ERK (#4695), P- Caspase 3 (#9662). P-ABL (#2865), P-CRKL (#3181), and GAPDH (#8884) (Cell Signalling Technology).

3.1.1.3 Cytometry Analysis:

Used antibodies are : CD123 BV711 (BD, 751833), CD45 APC (BD, 550855), CD34 APC (BD, 345804),CD38 PeCy7 (BD, 335825) KI67 AF647 (BD, 561126) and DAPI staining (Chemometec, 910-3012) diluted 1/2000 in PBS 1X.

Staining lasted 30 minutes at 4° in the dark. Conjugated isotype-matched controls were used to assess nonspecific fluorescence. Flow cytometry analysis was performed using a FACS Fortessa flow cytometer (BD) gated on viable cells (using DAPI negative gating). For intracellular staining of KI67 a fix-perm protocole was used.

TF1 FUCCI BA Cells were fixated with PFA4% for 10 minutes at 37°C then put on ice for 1 minute. Resuspension of cells has been done in PBS 2% FBS before 1 centrifugation at 1350 g for 5 minutes. Membrane staining has been performed with CD45 APC for 30 minutes at 4°C. After 1 wash of PBS1X 2% FBS, cells were permeabilized with cold ethanol 80% during 20 minutes on ice. Ki-67 staining is done during 30 minutes on ice, cells were then washed twice with PBS1X 2% FBS before analysis on FACS Fortessa flow cytometer.

Furthermore, cytometry analysis was used to assess the G0 percentage by using mCherry high mVenus negative gating to delimit G0 fraction without fixing and permeabilising the sample thanks to the FUCCI vector.

3.1.2 Transcripts Analysis:

3.1.2.1 RNA Esxtraction and analysis:

Dry pellets from TF1 FUCCI BA sorted samples in Biorad S3E (Using FUCCI to gate the different cell cycles) were stored (80°) and lysed in TRI-REAGENT (Sigma-Aldrich).

Total RNA was purified using mini-RNA extraction kit (Qiagen). For RT-qPCR, cDNA was produced using Superscript II (Invitrogen) and quantified using Sybr-green (Quantifast, Qiagen) and Real-Time PCR system (Roche, LC480). TATA-binding protein (*TBP*) and hypoxanthine-guanine phosphoribosyl transferase (*HPRT*) genes were used for normalization.

The primer sequences were the following ones:

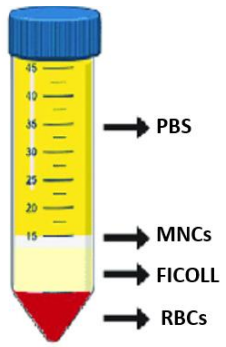
HPRT (F:TGACCTTGATTTATTTTGCATACC; R:CGAGCAAGACGTTCAGTCCT),
TBP (F:CAGCGAACCACGGCACTGATT; R:TTTTCTTGCTGCCAGTCTGGAC)
P21: (F:CGATGGAACTTCGACTTTGTCA; R:GCACAAGGGTACAAGACAGTG)
CD123: (F: TATGCCGGCAGTGAACAATA; R: AAGGCTTCCCACTGTTCTCA)

3.2 Functional Assay:

The content of treated cells in distinct hematopoietic progenitor categories was determined using the colony forming cell (CFC) assay. The assay is based on the ability of hematopoietic progenitors to proliferate and differentiate into colonies in a semi-solid media in response to cytokine stimulation. Unsorted TF1 FUCCI BA bulk as well as previously sorted cells in the Biorad S3E gating on different cell cycle phases (G0, G1, S and G2M) were plated in methylcellulose medium (Stem Cell Technologies) . The latter contains erythropoietin (EPO), SCF, IL-6, GM-CSF, G-CSF and IL-3 (Stem Cell Technologies) . Cells were plated in a 35-mm small Petri dishes disposed in a normal petri dish where a small 35 mm dish was filled with sterile water and disposed open inside of it to avoid methylcellulose dry. After approximately 13 days of incubation at 37°C, the colonies formed were enumerated.

3.3 Cell Culturing:

3.3.1 Primary Cells:



Normal bone marrow samples were obtained either from healthy BM donors for allogeneic transplant.

Leukemic BM comes from CML patients diagnosed to be in CP prior to or post TKI treatment (from patients achieving CCyR). All donors provided written informed consent in accordance with the Declaration of Helsinki. Studies were approved by local ethics committee bylaws. Definitions of response respected the last European LeukemiaNet Recommendations.

Figure 31: Sample fractions post Ficoll.

Mononuclear cells (MNCs) were firstly separated using a Ficoll gradient after a 40 minutes high speed centrifugation (shown below). Then, MNCs were subjected to CD34 immunomagnetic separation using the EasySep™ CD34 Positive Selection Kit from StemCell Technologies. Isolated CD34+ cells were then used for our experiments.

3.3.2 Cell lines:

TF1 cell lines were obtained and authenticated as described.(Arizkane et al. Hematologica 2021). TF1-BA, TF1-BAP cells were cultured in RPMI1640 containing 10% fetal calf serum (FCS). HS27A cells were obtained from ATCC and displayed properties of immature bone marrow stromal cells (BM-SC) . Cells were cultured in RPMI1640 containing 10% FCS. HMEC-1 (Cell Culture Development; Ref#CDC.HMEC-1) human microvascular endothelial cell line 40 was grown in MCDB 131 medium (Gibco), supplemented with 10% FBS, 1% Penicillin-Streptomycin, 1% Glutamax 100X (Gibco), 1 mg/L hydrocortisone (Sigma) and 10 µg/L Epidermal Growth Factor (Sigma).

BCR-ABL+ FUCCI cells were obtained from original TF1 ATCC cells (CRL-2003™) transfected with a FUCCI Vector (*described in Tomura et al. Plosone 2013*). Once cells acquired dependency to *BCR-ABL* oncogene, no GM6CSF was added to the culturing media.

à completer avec le design et vecteurs du fucci + HS271 shBMP4 et OEBMP4

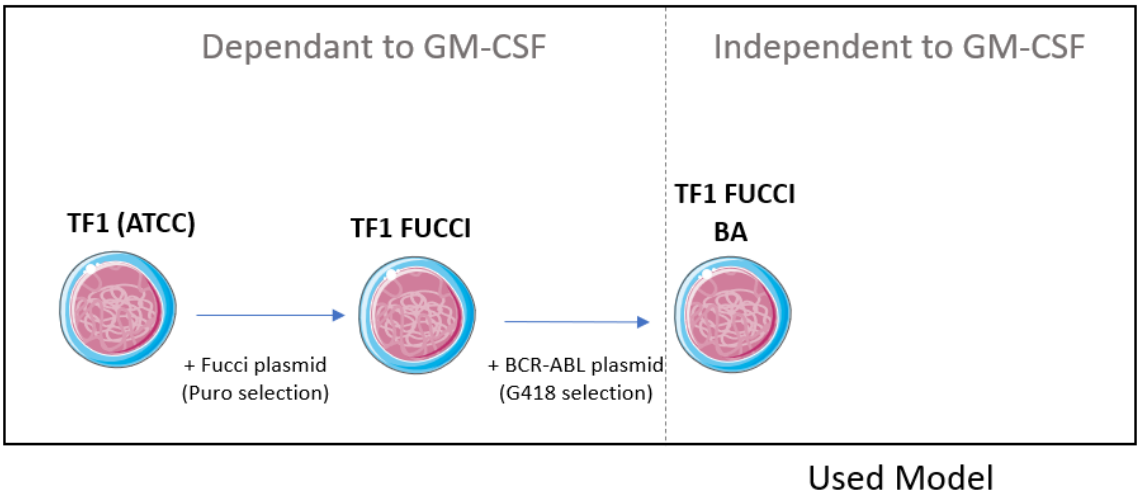


Figure 32: Hematopoietic TF1 cell lines.

3.3.3 2D experiments:

3.3.3.1 Using ECM components:

Fibronectin (FN) #F0895 2MG was used at 40µg/ml. Collagen1 (Coll) #C3867-IVI Rat was used at 50µg/ml. Coating was performed on plastic plates for at least 2 hours, then TF1 FUCCI BA cells were added on coated plates after removing the exes of FN/Coll.

3.3.3.2 Using Stromal cells:

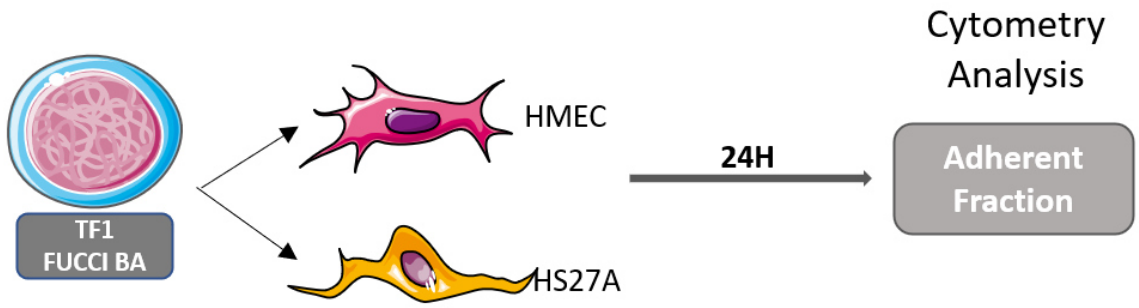


Figure 33: Co-culture of hematopoietic cells and stromal cells.

HMEC cells or HS27A cells were coated on plastic plates. After at least 1H of coating, cells were completely adherent to the bottom of the plate. Their initial media is then changed before adding TF1 FUCCI BCR-ABL+ cells.

3.3.4 3D experiments:

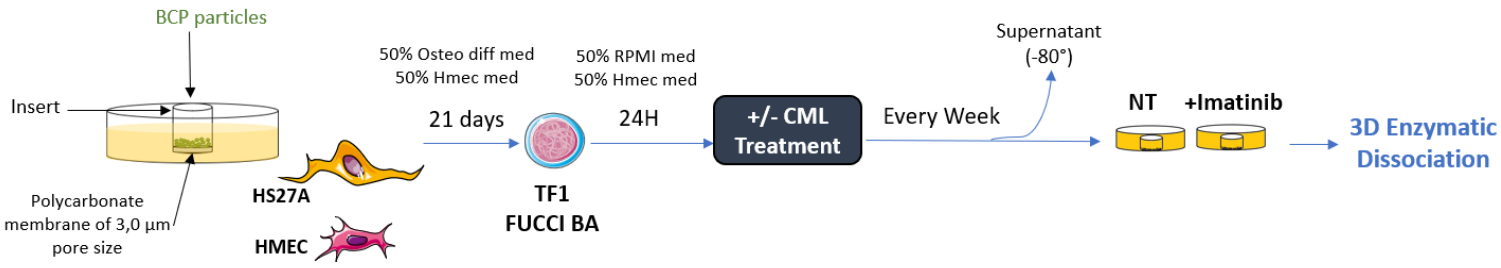


Figure 34: 3D bone marrow system mimicking the medullar niche.

Complete protocol is described in the recently published paper (*Voeltzel et al. 2020*). HS27A (2M) and HMEC(1M) cells are cultured during 3 weeks in a specific medium comprising half of HMEC cells medium described overhead. In addition to an osteoblastic- differenciation medium composed of DMEM with 10% FCS, Dexamethasone (0.1 µM; Gibco), β-

Glycerophosphate (10 mM; Sigma) and Ascorbic Acid (250 µM; Sigma) with 2 changes of complete media twice per week.

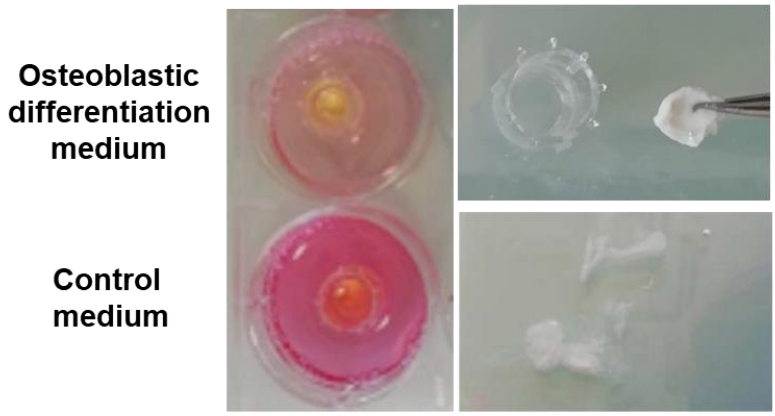


Figure 35: 3D disk rigidification.

After 3 weeks of osteo-differenciation, the system become rigid. At this step we add our cells of interest within the system (FIG). 24hours post harvesting we add the treatments in he treated systems inside of the insert as well as inside of the plate well.

We culture our hematological cells from 1 to 4 weeks then we retrieve the insert supernatant to perform ELISA tests and we retrieve the whole membrane to either fix it for a future multiplex (IF or IHC) or for an enzymatic dissociation and immune labelling to analyse it in the cytometer.

3.3.5 MTT Assay

The first day, TF1 FUCCI BA cells were seeded in a 96 well plate with a flat bottom using RPMI supplemented with 10% of serum and treatments, final volume is 100µL. All the wells at the extremity of the plate well filled with PBS1X to avoid samples dry. (8000 cell / well)

The 3rd day, 10µL of MTT reagent is added to each well except the control PBS wells (MTT previously diluted to 5mg/ml in PBS 1X and stored in darkness at -20°). Plates incubation lasts

4 hours in darkness at 37°. Following the 4 hours, we add 100µL of lyse buffer in each well (Lyse buffer: for 100 ml of buffer we mix 50 ml of 20% SDS with 50 ml of N-N-dymethylformamid. Solution is agitated until complete dissolution). At the 4th day, the read out is performed at 570 nm in the spectrophotometer (Clariostar©).

4 Results :

Our team previously described and deciphered many alterations taking place in late CML resistance stages. As well, in a recently published study, we highlighted the presence of quiescent LSCs within CML patients BM (*Arizkane et al., 2021*). These persistent cells interacted with their stroma and co activated two signalling pathways to maintain their quiescence and avoid Imatinib treatment effect (Figure 46). As well, TKI treatment of these cells resulted in a decrease of their cell proliferation enhancing thus their quiescent state (Figure 41 A).

Since we previously highlighted this unexpected treatment quiescence enhancing effect, we focused in this study on early stages of CML persistence. We look after mechanisms underlying LSCs quiescence within CML patients BM under CML treatment uptake.

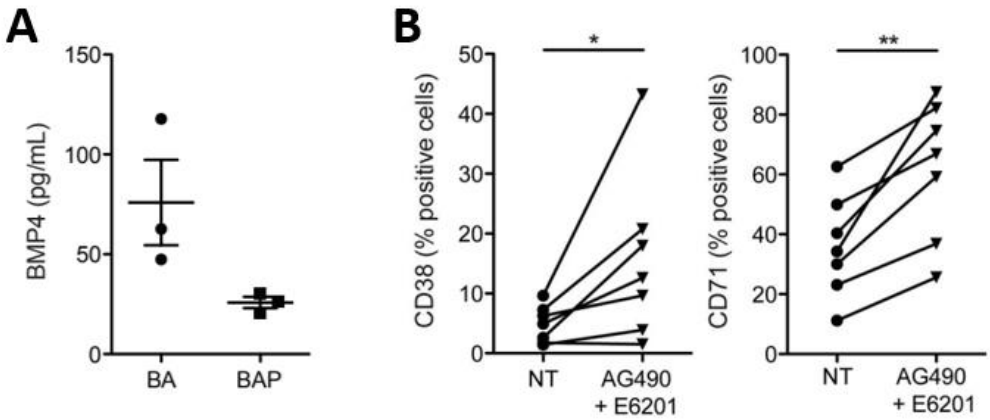


Figure 36: Dual inhibition of BMPR1B as well as JAK2 signalling impairs.
(A) Elisa test evaluating BMP4 production by CML persistent immature cells. (B) Dual inhibition of BMPR1B by E6201 and JAK2 by AG490 resulting in an increase of CML immature cells proliferation.
*p<0.05; **p<0.005; ***p<0.0005, ****p<0.00005 indicate differences between the gene expression levels in different BCR-ABL$^+$ cells sub-fraction. Statistical tests are performed with Mann Whitney unpaired test. C, D, E and F figures represent the SD mean ±.

1) Dormant state of persistent CML LSCs is reversible

In our previous paper, we highlighted the evidence that persistent CML LSCs do not set an autocrine loop of BMP4 production as in resistance stage[93] (Figure 36 A). Furthermore, the inhibition of two pathways that are the canonical BMPR1B/SMADs and the non canonical JAJ2/STAT3 pathways result in a quiescence impair as well as a differenciation fostering (Figure 36 B).

a) Quiescent CML LSCs do highly express IL3-R at their cell surface

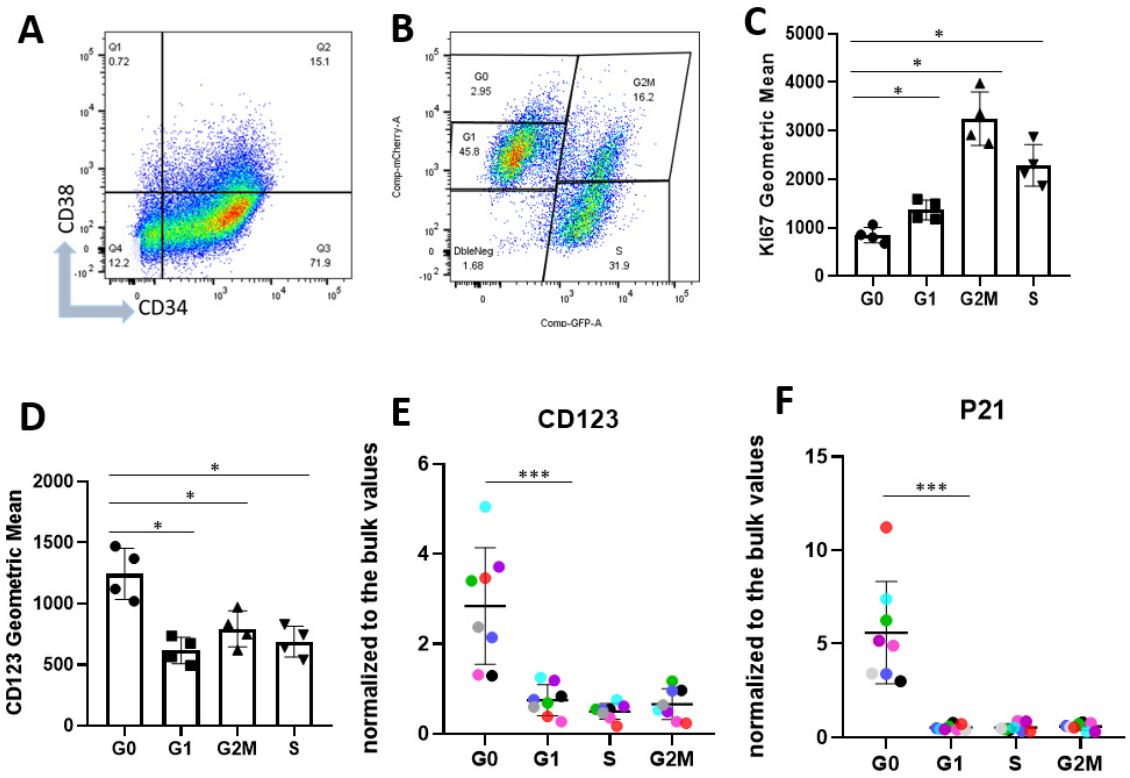


Figure 37: a sub-fraction of immature BCR-ABL+ immature cells display high levels of P21 and CD123. Cytometry analysis of BCR-ABL$^+$ FUCCI cells shows in (A) CD34 and CD38 expression in these cells as well as the FUCCI pattern (B). (C) Cytometry analysis compares KI67 as well as CD123 expression in different BCR-ABL$^+$ cell populations that are cycling differently. After cell sorting of different BCR-ABL$^+$ cells sub-fractions, transcriptional analysis of CD123 and P21 genes in G0, G1, S and G2M sorted cells in performed (E) and (F). Transcriptional analysis is reported normalized on the bulk values (unsorted heterogeneous BCR-ABL$^+$). *p<0.05; **p<0.005; ***p<0.0005, ****p<0.00005 indicate differences between the gene expression levels in different BCR-ABL+ cells sub-fraction. Statistical tests are performed with Mann Whitney unpaired test. C, D, E and F figures represent the SD mean ±.

In this study we focus on CML persistent LSCs that face CML treatment within newly diagnosed CML patients BM without being completely eradicated and persist through remission until it causes patients' relapse. To this end we choose an immature BCR-ABL+ model that we also transfected with the FUCCI cell cycle reporter enabling a dynamic cell quiescence assessment in our study. We first evaluate our model immaturity as it serves to study CML LSCs (Figure 37 A). We examined the FUCCI pattern in this immature CML population (Figure 37 B) as well as the nuclear KI67 and the membrane CD123 expression in all CML cells sub-factions that the dynamic FUCCI system helps to delimit (Fig 37 B, C, D). Furthermore, we examined the transcriptional level of a described cell cycle inhibitor (Figure 37 F) as well as a stem cell marker (Figure 37 E).

These results demonstrate that our BCR-ABL^{+} model is highly immature as much as 87% of CML cells do express CD34+ and 71.9% of total cells are CD34+ CD38- (Figure 37 A). As well, the used FUCCI plasmid enables a dynamic tracking of CML LSCs at different cell cycle stages such as G1, S, G2M and G0 through m-Cherry and m-Venus differential expression (Figure 37 B). Furthermore, KI67 nuclear protein appears to be downregulated in the G0 LSCs sub-fraction compared to the G1, G2M and S sub-populations which harmonies with the FUCCI gating pattern and suggests that G0 gated cells (Figure 37 B,C) are not, or low cycling compared to the other sub-fractions.

The stem cell marker CD123 which is the IL-3α receptor appears to be significantly more expressed at quiescent CML LSC cell surface compared to the cycling CML LSCs (Figure 37 D). Interestingly CD123 is also overexpressed in G0 LSCs at the mRNA level (Figure 37 E) as well as the cell cycle inhibitor P21 (Figure 37 F).

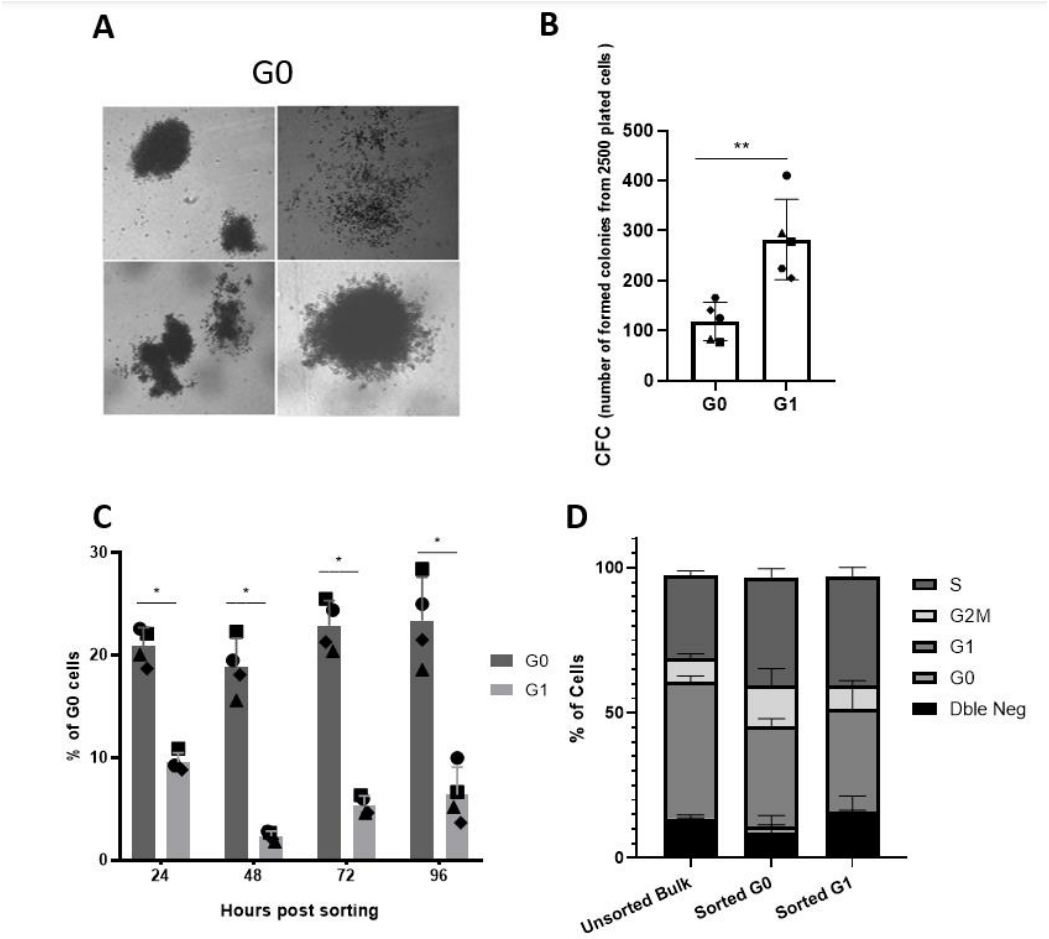


Figure 38: Quiescent BCR-ABL+ immature cells are heterogeneous and able to re-enter cell cycle
Colony Forming Assay using sorted G0 BCR-ABL+ immature cells displays different types of myeloid and erythroid colonies (A). Sorted G0 and G1 BCR-ABL+ cells do: (B) form colonies on methylcellulose in significantly different speed. (C) maintain their quiescence after 4 days of sorting (G0) or induce their quiescence over time culturing (G1). (D) Sorted G0 and G1 are reported to progress in all cell cycle stages 6 days after sorting. *p<0.05; **p<0.005; ***p<0.0005, ****p<0.00005 indicate differences between the gene expression levels in different BCR-ABL+ cells sub-fraction. Statistical tests are performed with Mann Whitney unpaired test. B and C figures represent the SD mean ±.

Importantly, LSCs present within CML patients BM are known to be heterogeneous. As well, in our model, all immature CML LSCs are not cycling homogeneously and do not express IL-3Rα similarly (Figure 37 D,E). These elements underly the fact that CML patients at remission do not relapse at the same time. Furthermore, IL3-R appears to be a cell surface and intracellular marker for quiescent CML LSCs.

b) Persistent LSC sub-fraction is heterogeneous, and its dormancy state is reversible with a high and low dormancy states.

To evaluate and confirm CML LSCs heterogeneity and quiescence, further tests including functional assays were performed. We first performed CFC assay using sorted G0 cells (Figure 38 A). Then, after 13 days, colonies number originating from initial G0 and G1 sorted and seeded LSCs was compared (Figure 38 B). Subsequently, sorted G0 and G1 LSCs were maintained in normal cell culturing conditions for four days and at 24, 48, 72 and 96 hours post sorting, G0 LSCs percentage was reported in the two G0 and G1 originally seeded LSCs (Figure 38 C).

Finally, a later time point reported cycling activity of initial seeded G0 versus G1 LSCs 6 days post sorting (Figure 38 D).

G0 and G1 LSC sub-fractions were sorted with cell sorting machine (BIORAD S3E) and same number from each sub-fraction was plated on separate methylcellulose semi-rigid dishes. After 13 days we can notice the presence of several colonies that grew from initial sorted quiescent LSCs (Figure 38 A). Even more, the formed colonies are morphologically different as we can distinguish myeloid colony in the first right square and two erythroid colonies in the first left square. In addition, a report of colonies number originating from initial G0 and G1 seeded LSCs shows a significant difference (Figure 38 B). A high number of G1 LSCs formed colonies is compared to a lower number of G0 LSCs forming colonies.

This suggests that G1 LSCs are enriched in progenitors while G0 LSCs are less forming colonies and subsequently less enriched in progenitors while more enriched in highly immature LSCs as the same cell number was initially seeded from both samples.

In addition, sorted G0 and G1 LSCs were maintained in normal culturing conditions for up to 4 days towards quiescence assessment in this originally no cycling and cycling seeded LSCs (Figure 38 C). The outcome showed that at 24, 48 72 and 96 hours of culture post sorting the tendency remains the same. Indeed, small percentage (less than 10%) of initial G1 LSCs can stop their cycling and enter in a quiescent state. As well, a fraction of initially dormant LSCs can undergo cell cycling by losing their dormant state. Nonetheless, after 4 days of culturing

initial dormant LSCs still maintain a high percentage of quiescence as at least 20% of these initial G0 LSCs remain dormant. A significant difference in quiescence entrance/maintenance between the initial G0 and G1 populations suggests that dormant LSCs can gradually re-enter active cell cycle. Additionally, cycling LSCs can reverse their cycling activity and enter in a dormant state. Finally, sorted G0 and G1 LSCs as well as unsorted bulk (heterogeneous LSC population) were maintained in normal culturing conditions for 6 days(Figure 38 D). Following the 6 days, each cell cycle stage frequency was reported withing the 3 initially seeded populations. Interestingly, after 6 days of culture both initially sorted G0 and G1 LSCs do re-renter active cell cycle undergoing the whole cell cycle stages. Nonetheless, compared to the bulk and the G1 original LSCs, G0 LSCs do still maintain a higher G0 LSCs percentage even after 6 days on culture in a rich FBS supplemented media.

This outcome suggests that initial G0 LSCs do contain dormant LSCs with different degrees of dormancy. in other words, a dormant LSCs may re-enter cell cycle in a quick manner while a deeply dormant LSC can take much more time or needs specific intrinsic or extrinsic signals to re-enter cell cycle.

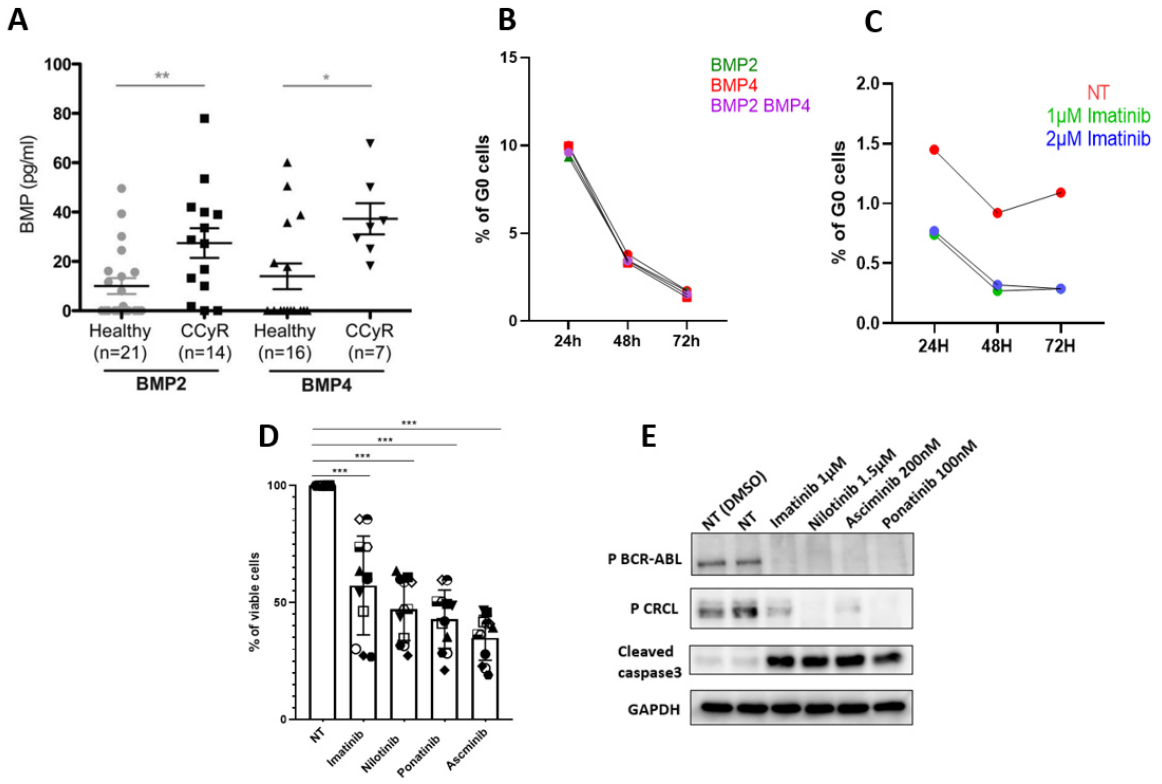


Figure 39: Treatment of BCR-ABL+ cells in suspension with CML treatments decreases CML cells viability but does not impact CML LSCs quiescence.

(A) CCyR CML patients still do present a significant increase of BMP2 as well as BMP4 production within their BM compartment. (B) BCR-ABL+CML cells were treated with BMP2, BMP4 or the combination of both during 24, 48 and 72hours to assess variation of G0 fraction. (C) BCR-ABL+CML cells were treated with two doses of Imatinib during 24, 48 and 72 hours to evaluate G0 cells. BCR-ABL+CML cells treatment with imatinib, nilotinib, ponatinib and asciminib esults in a decrease of their cell viability assessed with an MTT assay (n=12) (D) It also results in a decrease of BCR-ABL oncoprotein activation as well as an increase apoptosis (n=4). *p<0.05; **p<0.005; ***p<0.0005, ****p<0.00005 indicate differences between the treatments effect in BCR-ABL+ cells. Statistical tests are performed with Wilcoxon paired t test. A figure represents the SD mean ±.

Reported to clinical CML remission cases, these results highlight the fact that in CML patients, heterogeneous fraction of quiescent LSCs do persist within the BM. Thus, dormancy state is reversible as a dormant LSC can undergo active cycling if necessary and a cycling LSCs can enter in a dormant state. A dormancy gradient does certainly exist as low dormant LSCs will undergo a fast waking up cycling while high dormant LSCs will maintain their dormancy for long time persisting in CML patient's niche. This may explain the fact that CML remission patients do not relapse similarly.

1) In presence of BMP4 releasing stroma, CML treatment increases LSCs persistence

As our lab highlighted the importance as well as the involvement of BMP4 in CML resistance[93,494], our previous study highlighted as well the importance of this cytokine for CML

LSCs quiescence maintenance *in vitro.* Of note, we reported a high level of BMP2 and BMP4 in CML patients at remission plasma compared to healthy donors' plasma (Figure 39 A). This underlines the importance and eventual involvement of this cytokine in LSC quiescence and dormancy maintenance and proves that even if CCyR is achieved, CML patients still do not have a normal cytokine production within their BM.

a) BMPs & TKIs do not modulate LSCs quiescence in absence of stroma

Firstly, we treated LSCs in suspension with BMP2 and BMP4 cytokines separately and together during 1, 2 or 3 days to evaluate BMPs effects on CML LSCs' quiescence induction (Figure 39 B). We did not assess any enhancing or inhibiting effect of BMPs on CML LSC quiescence while in suspension. As well, considering imatinib quiescence induction assessed in our previous study (Figure 41 A), we treated CML suspension LSC with 1 and 2µM of imatinib (Figure 39 C). Similarly to BMP treatment, imatinib did not affect CML LSC quiescence in suspension.

CML treatment is efficient on suspension immature LSCs

Considering the involvement of imatinib in quiescence induction in our previous study (Figure 41 A), the use of this first line TKI treatment in our study permits to link the observed effects to the CML patients at remission that still uptake imatinib. Furthermore, as described in the introduction, numerous TKIs do exist and do act on the same target BCR-ABL ATP pocket, nonetheless, their efficiency on BCR-ABL1 oncoprotein as well as their induced side effects do change. To this end, we chose to focus on first generation (imatinib), second generation (nilotinib) as well as the third generation (ponatinib) of TKIs. In addition, the recent discovery and clinical use beginning of a new CML drug that targets ABL Myristoyl pocket drawn our interest to include it in our study and compare its effects with TKIs effects.

We firstly used imatinib (1µM), nilotinib (1,5µM), ponatinib (100nM) and asciminib (200nM) to treat CML LSCs in suspension for 72 hours. Following the 72 hours the MTT viability assay plate was read in the Clariostar© machine (Figure 39 D). Then we treated CML LSCs in suspension for 24 hours before protein extraction from the dry pellets. Western blot analysis

revealed the expression of active BCR-ABL, active, P-CRCL, cleaved caspase 3 and the housekeeping gene GAPDH (Figure 39 E). The DMSO use serves as a control for nilotinib effects as this drug is diluted in pure DMSO.

The MTT assay outcome reported a significant CML cell viability decrease for all the used drugs (Figure 39 D). Indeed, imatinib treatment decreases cell viability by 40%, nilotinib treatment decreases it by 55%, ponatinib decreases it by 58% and finally asciminib showed the best effect with 65% of viability decrease. To confirm these effects, a western blot revealed a complete inactivation of BCR-ABL oncoprotein after 24 hours of treatment whatever the used drug (Figure 39 E). Furthermore, BCR-ABL P-CRKL specific target is also completely inactivated with nilotinib and ponatinib treatments while a slight protein band still appear after imatinib or asciminib treatments. The cleavage of caspase 3 demonstrates that the four used CML drugs are effective and do induce cell apoptosis in CML LSCs.

Interestingly, considering the previous highlight of heterogeneity within our CML LSCs, persistent percentage of viable cells as well as residual P-CRKL bands post CML treatment may reflect the sensitivity of a subset of CML LSCs but also a non-response of some dormant LSCs that persist within CML patients BM.

C ECM presence allows preferential 2nd TKI effect on LSC quiescence

In CML BM niche, FN and collagen are widely present and even increased compared to non-leukemic BM niche. As no effect was observed on treated LSCs suspension cells with TKI and

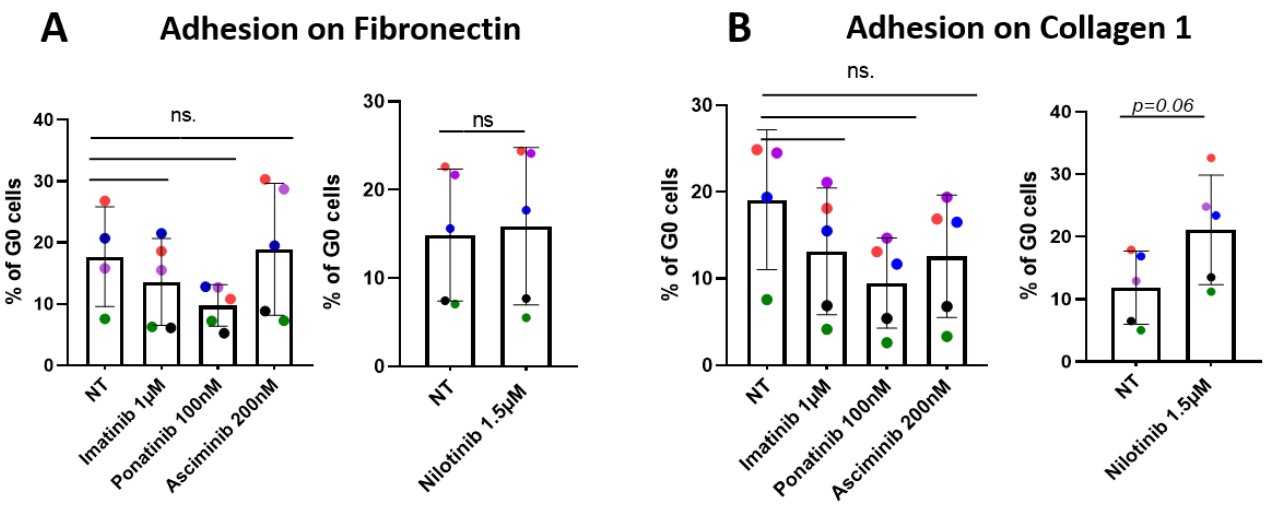


Figure 40: Treatment of adherent BCR-ABL+ cells on collagen with nilotinib increases BCR-ABL+ cells quiescence.
BCR-ABL+CML cells coating on fibronectin (FN) (A) or collagen (B) layers then treating with imatinib, nilotinib, ponatinib and asciminib were performed and the adherent BCR-ABL+ cells were analysed in the cytometer to report their quiescence levels (n=5). *p<0.05; **p<0.005; ***p<0.0005, ****p<0.00005 indicate differences between the treatments effect BCR-ABL+ cells quiescence. Statistical tests are performed with Wilcoxon paired t test. Figures A and B represents the SD mean ±.

BMPs, we added extracellular matrix (ECM) components to permit an attach for LSCs that appears to be mandatory for a quiescence induction.

After at lest 2 hours of FN (Figure40 A) and collagen 1 (Figure 40 B) separate coating we added CML LSCs on the formed ECM then we treated using imatinib (1µM), nilotinib (1,5µM), ponatinib (100nM), asciminib (200nM) and DMSO (NT serving as a control for the nilotinib). After 24 hours of treatment, we retrieved adherent CML LSCs for a cytometry analysis of the quiescent fraction in each condition.

The outcome resulting from a treatment on FN did not reveal any significant results (Figure40 A). Nonetheless, the graph tendency (Figure 40 A) shows a slight decrease of G0 percentage when cells are treated with imatinib or ponatinib. This tendency is not observed for asciminib nor nilotinib treatments. As well, when we used collagen coating the results for imatinib, ponatinib and asciminib treatments were not statistically significant (Figure 40 B). Nonetheless, using these treatments we note a slight tendency for the whole graph points showing a slight decrease of CML LSCs quiescence. However, nilotinib treatment outcome appears to be different (Figure40 B). Indeed, this 2nd TKI generation increases by 2 folds LSC CML quiescence in presence of collagen support.

These results suggest that in presence of ECM CML treatment will affect and modulate CML LSCs quiescence. The fact that imatinib, ponatinib and asciminib slightly decrease LSCs quiescence in presence of FN or collagen while nilotinib increases LSC quiescence on collagen suggest that TKIs do not impact likewise CML LSCs quiescence within leukemic niche. Furthermore, the presence of other components in the leukemic niche may enhance or hinder LSC persistence treatment induction. This may explain the difference in CML patients relapse or treatment escape dissimilarly according to the administrated CML drug.

D) BMP4 seems to increase LSC quiescence & may synergize with specific treatment to promote LSC dormancy

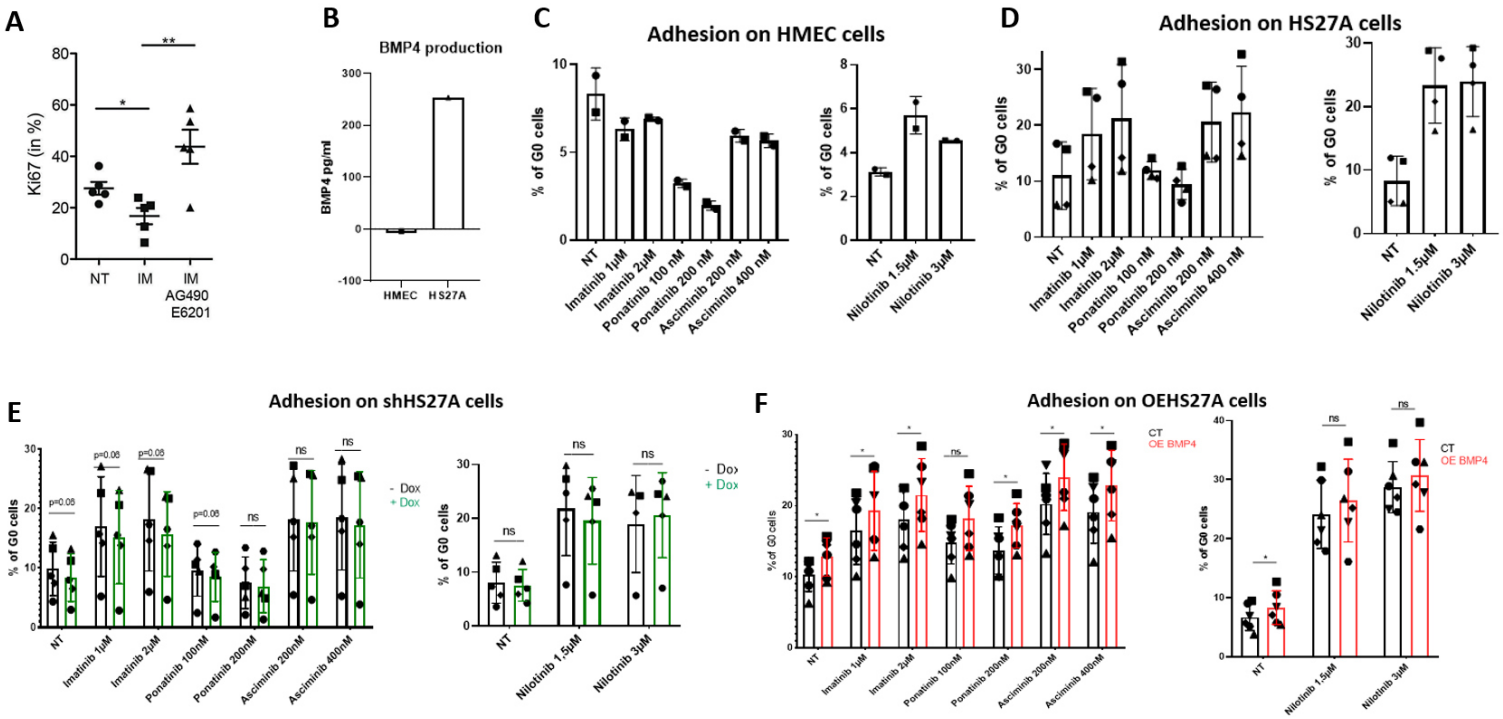


Figure 41: dual presence of BMP4 and CML treatment do increase BCR-ABL+ quiescence *in vitro*: (A) Imatinib treatment enhances persistent CML LSCs while BMPR1B and JAK2 inhibition enhances quiescent cells proliferation. Elisa test shows an absence of BMP4 production by HMEC endothelial cells and a high BMP4 production by HSC27A (B). BCR-ABL+CML cells coated on HMEC (C), HS27A (D), HS27A shBMP4 (E) or HS27A OEBMP4 (F) feeding layers are then treated with imatinib, nilotinib, ponatinib and asciminib. 24 hours post treatment, adherent hematological cells are retrieved and analysed in a cytometer (C,D,E and F) to report variation of G0 % of *p<0.05; **p<0.005; ***p<0.0005, ****p<0.00005 indicate differences between the BMP4 and treatments effects on BCR-ABL+ cells quiescence. Statistical tests are performed with Wilcoxon paired t test. Figures B,C,D and E display the SD mean ±.

After assessing the involvement of BMP4 receptor (BMPR1B) in quiescence induction (Figure 41 A), in addition to the nilotinib positive effect on LSCs quiescence on collagen layer, we performed 2D co-cultures to furnish a stroma for LSCs before treating them with CML treatments.

We firstly used two different cell types. An endothelial HMEC cell and HS27A. The latter overproduces BMP4 while HMEC cells does not (Figure 41 B). When we coated a layer of HMEC (Figure 41 C) or HS27A (Figure 41 D) stroma, we waited until the stroma adhered and formed a uniform adherent layer, then we added our CML LSCs and we treated for 24 hours using imatinib (1µM), nilotinib (1,5µM), ponatinib (100nM) and asciminib (200nM) or double of each concentration in addition to DMSO (nilotinib control). After 24 hours of culturing, we retrieved the whole adherent cells, we stained the samples with CD45 antibody to distinguish haematological cells and we performed our analysis in the cytometer machine reporting the variation of G0 population in each condition.

Furthermore, to modulate BMP4 supply in the system we established 2 cell models, a stromal inducible model (HS27A shBMP4) that decreased BMP4 production after doxycycline adding and an overexpressed model (HS27A OEBMP4) that overexpressed BMP4 cytokine. We used HS27A shBMP4 (Figure 41 E) and HS27A OE BMP4 (Figure 41 F) as a layer for LSC culturing and treatment with same drug doses as Figures 41 C and D, then after 24H the retrieved cells were stained with CD45 and analysed with the cytometer.

BMP4 appears to be overexpressed by stromal HS27A while endothelial HMEC cells do not express this cytokine (Figure 41 B). A use of HMEC layer for CML LSC treatment was done to see if a contact cell to cell without BMP4 cytokine supply will have an impact of LSC quiescence induction. Experiment using HMEC as a layer was only performed twice, the reason why no statistical significancy can be retrieved from it. Nonetheless a general tendency shows that imatinib, ponatinib and asciminib treatments decrease LSC quiescence compared to the control condition. Interestingly nilotinib treatment induces the opposite effect as its use generates an increased tendency for LSC quiescence induction (Figure 41 C). Afterwards we

tested LSC adhesion on a BMP4 releasing stroma (Figure 41 D). The outcome results in a slightly higher quiescence induction in the DMSO control (Figure 41 D) compared to the same condition in the culture with HMEC cells (Figure 41 B). Thus, this suggests that the toxic DMSO effect is counterbalanced by BMP4 cytokine. Moreover, Imatinib and asciminib treatments do increase by 2 folds CML LSC quiescence in presence of BMP4 while ponatinib does not appear to induce any effect on the LSCs quiescence. Nilotinib treatment effect remains the most remarked with the highest LSC quiescence induction that goes up to 23% (Figure 41 D). We note that no dose effect was observed for the drugs we used except for imatinib that in presence of BMP4, increases CML LSCs quiescence in a treatment dose dependant manner.

After remarking this synergy between BMP4 stromal production supply and Nilotinib treatment in CML LSCs quiescence increase, we used the inducible and the overexpressed BMP4 HS27A stromal cells to better evaluate BMP4 involvement in CML LSCs dormancy.

In the control condition using HS27A shBMP4 without doxycycline (Figure 41 E) as well as CT HS27A OEBMP4 (Figure 41 F) the graphs pattern is similar as the resulted outcome from HS27A use (Figure 41 D). We note a LSC quiescence increase for all the used drugs except the ponatinib that does not seem to impact LSC quiescence (Figure 41 D,E and F). As well, the outcome does not show any remarkable Drug dose effect on LSC quiescence except for Imatinib (Figure 41 F). This suggest that a plateau may be reached at the first drug dose. Figure 35 D results are not statistically significant regarding the large error bars, nonetheless a general tendency can be retrieved. The decrease in BMP4 supply slightly decreases LSCs quiescence in imatinib, ponatinib, nilotinib and NT condition. For the asciminib condition the slight BMP4 decrease does not appear to interact with the drug quiescence effect as the drug appears to enhance LSC quiescence with a normal or slightly decreased BMP4 provision.

On the other hand, the use of HS27A OE BMP4 (Figure 41 F) should bring a mirror outcome of HS27A shBMP4 use (Figure 41 E).

In presence of a high BMP4 production, CML LSC quiescence is significantly fostered in all our conditions except 100nM ponatinib treatment as well as nilotinib treatment for which no statistically significance was obtained nut the graph increased quiescence pattern is observed (Figure 41 F).

As CML treatments supplemented with less BMP4 results in a slight LSC dormancy decrease and a higher BMP4 supply results in a LSC quiescence fostering, BMP4 supply by MSCs seems to affect LSCs quiescence by increasing it. Nonetheless, Considering the outcome differences between the non-BMP4 producing stroma and the BMP4 producing stroma (Figure 41 E,F) a synergy effect between BMP4 and CML treatment appears to exist and enhance CML LSC quiescence.

Furthermore, as high BMP4 levels were reported in CCyR CML patients in our previous study (Figure 39 A). This underlines the fact that the non-restored cytokine levels at remission achievement is a consequence of the modified CCyR CML patients BM niche and these alterations do certainly support LSCs quiescence within patients BM. In addition, as these patients continue CML treatment daily uptake, this second LSC quiescence enhancer will foster CML LSCs dormancy that is already settled within the CCyR patients BM.

To further investigate BMP4 and CML treatments involvement in CML LSCs quiescence, we used a BM-like model that was settled in our lab and serves to mimic hematopoietic medullar niche.

3) CML LSC dormancy is enhanced and maintained within a BM like structure

CML leukemic niche has largely been described as a modified microenvironment supporting LSCs survival, maintenance and quiescence at the expense of the present normal HSCs. For this purpose, we used a BM-like model to decipher the mechanisms settled within human leukemic BM and supporting CML LSCs dormancy during treatment exposure. This BM model comprises two different cells. Endothelial cells (HMEC) that mimics vasculature presence within Hematological niche (Vascular niche) and stromal MSCs (HS27A) that differentiate into osteoblasts mimicking thus the endosteal niche.

a) Number of Hematological CML cells decreases after four weeks of efficient treatment exposure:

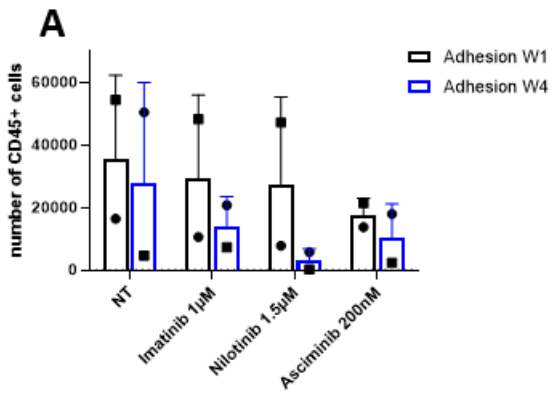


Figure 42: CML treatment affects CML LSCs cell proliferation within 3D system.
(A) After one week and 4 weeks of different treatment exposure, cytometry analysis was performed on 3D retrieved cells. An antibody staining of the CD45 helped to report CD45+ left cells within the BM-like system.

In our BM-like system, we harvest HMEC endothelial cells and HS27A stromal cells on BCP particles with a specific osteoblastic differentiating media for 3 weeks. Following the three weeks, a bone like rigid structure is formed and allows CML LSCs harvest on it. After adding CML LSCs, we wait for 24 hours then we treat the system using different ABL TKI and a non-TKI ABL drugs: imatinib (1µM), nilotinib (1,5µM) and asciminib (200nM) (Figures 42, 43 and 44). The cells of interest were retrieved after 1 and 4 weeks of harvesting. We choose a short time treatment to be able to compare it to a longer treatment time (4 weeks). The choice of 4 weeks was based on a pilot 3D experiment where we cultured CML LSCs for 6 weeks. The outcome showed an effect at week4 and after, at week 5 and week 6 a plateau effect was observed helping us to readjust long treatment exposure to 4 weeks.

This experiment was repeated 2 times and others are ongoing. No statistical significance could be retrieved but a tendency is displayed on the graph.

Following one week of imatinib and nilotinib treatment, LSC number slightly decreases within the system. After asciminib treatment the cells proliferation decrease is clearly more pronounced (Figure 42). After 4 weeks of treatment, LSCs proliferation is much more affected suggesting that over time, CML treatments eradicates proliferating CML cells.

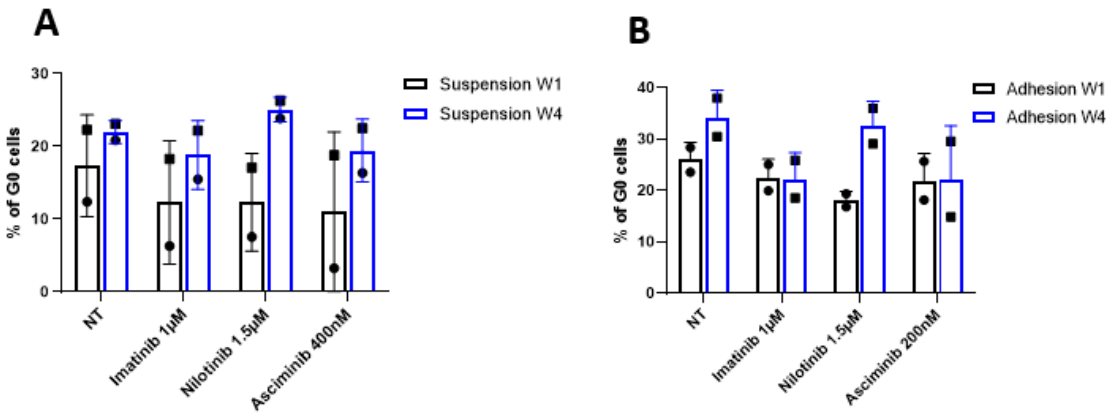


Figure 43: CML BCR-ABL+ cells that adhere to BM- like structure are more quiescent compared to non-adherent cells

After one week and 4 weeks of different treatment exposure, cytometry analysis was performed on 3D retrieved cells to report LCSs quiescence variability in the suspension (A) and adherent (B) compartments (n=2).

b) Adhesion to BM-like stroma seems to enhance LSC dormancy

From 1 to 4 weeks CML LSCs were treated using Imatinib, Nilotinib and asciminib within the BM like structure. A comparison between the quiescence in suspension fraction and adherent fraction revealed that the adherence to the BM-like structure enhances CML LSCs dormancy whatever the used treatment. Concerning the treatment effects, it appears to decrease CML LSCs quiescence after a short treatment time with imatinib, nilotinib and asciminib in the suspension fraction (Figure 43 A). This result suggests that when CML LSCs detach from the BM niche they are more efficiently targeted by CML treatments. Interestingly alongside 4 weeks of treatment exposure CML treatments do not decrease LSCs quiescence in the suspension compartment (Figure 43 A). This suggest that with medium change, the cycling

cells are removed systematically from the system, and at W4, the suspension fraction that we analysed was quiescent few days before collecting it and just left the niche compartment before analysis. Even if these LSCs are in the suspension fraction they still have a certain degree of its quiescence "memory" rending them persistent to CML treatment.

In the adherent compartment reported quiescence was higher either at W1 or W4 compared to the suspension compartment assessed quiescence (Figure 43 B). At one week of treatment, adherent LSCs are slightly affected by the three treatments regarding the slight quiescence decrease (Figure 43 B). But at W4, the CL LSCs present in the CM-like niche are highly dormant as they persisted several weeks in this compartment. We report that Imatinib as well as asciminib treatments decrease CML LSCs persistence in interaction with the stroma. Interestingly, Nilotinib results are similar to the non-treated condition as at W4 the nilotinib treatment is not decreasing CML LSCs quiescence anymore. This may be the same biological phenomenon that happens in CCyR CML patients that even with a daily treatment uptake, CML LSCs undergo their quiescence maintenance as if no treatment was uptake. The fact that this treatment induces a slight effect at W1 suggests that alongside treatment time CML LSCs become insensitive to treatment or the treatment itself begins to contribute to the CML LSCs quiescence maintenance or induction.Nonetheless this experiment was only repeated twice and other ongoing experiments should be analysed to obtain a better clue on CML treatments effects on LSCs dormancy.

c)BM-like microenvironment seems to enhance IL3-R expression on LSCs while CML treatment

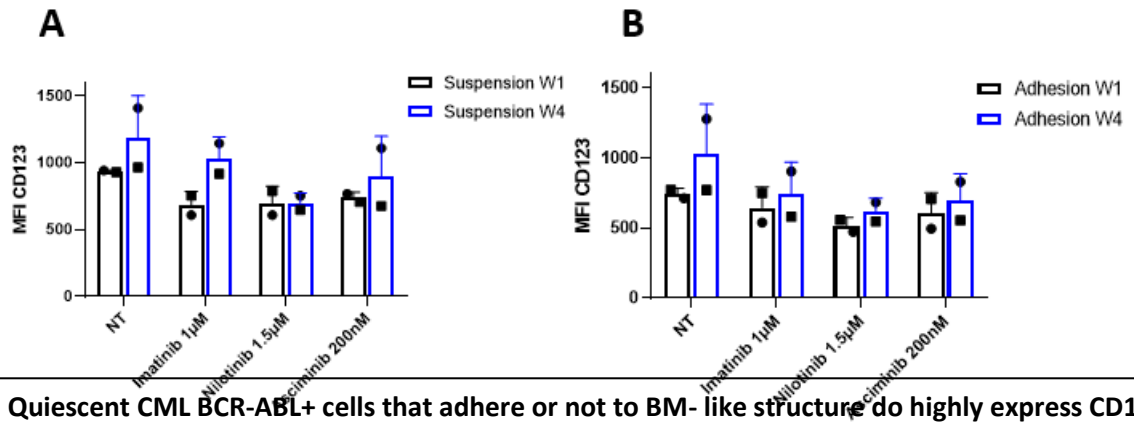


Figure 44: Quiescent CML BCR-ABL+ cells that adhere or not to BM- like structure do highly express CD123 at their cell surface

After one week and 4 weeks of different treatment exposure, cytometry analysis was performed on retrieved G0 persistent LSCs to report LCSs CD123 intensity in suspension (A) as well as in adherent (B) fractions (n=2).

slightly decreases this receptor overexpression:

Considering the high expression of IL-3R that we highlighted at the beginning of our study in the quiescent CML LSCs, we kept focused on this receptor during our study. In both suspension and adherent compartments, CML treatments slightly decreased IL-3 receptor expression on quiescent LSCs (Figure 44 A and B). Nonetheless, the culturing time within the BM-like niche slightly increases IL-3 expression at persistent LSCs cell surface. This effect is slightly hindered by imatinib and asciminib while better inhibited by nilotinib as this treatment decreases the best CD123 expression at dormant LSCs cell surface.

d)TKI long exposure treatment dramatically increases BMP4 production by BM-like niche

As no dramatical effect was observed with TKIs and Asciminib treatments on CML LSCs quiescence. We wondered if this treatment changes the medullar niche itself. For this, we performed an Elisa test to quantify BMP4 production within the system after 1 and 4 weeks of treatment (Figure 45).

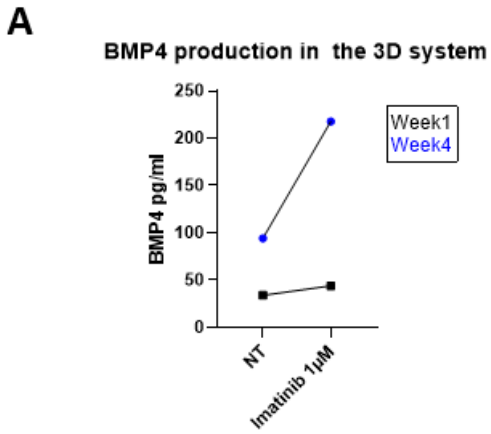


Figure 45: BMP4 cytokine is overproduced by the 3D system after 4 weeks of culture imatinib treatment fosters this increase.

Elisa test was performed on the BM-like system supernatant to evaluate BM-syetm and treatment impact on BMP4 cytokine production.

This experiment results in the discovery of an BMP4 production increase by the BM-like system supplemented by a dramatic BMP4 production within the medullar niche after imatinib treatment.

Precedent results demonstrated that imatinib acts directly on CML LSCs (Figure 41 A) and this result (Figure 45) suggests that Imatinib treatment may also affect the medullar niche and remodulate cytokines production by surrounding BCR-ABL negative cells in order to supply CML LSCs with specific cytokines or growth factors that will impact its dormancy within the niche.

As we demonstrated that BMP4 production level is high in CML CCyR patients plasma (Figure 39 A), this result suggest that TKI treatment uptake may be responsible of this altered cytokines production that results from microenvironment stimulation.

At remission, persistent chronic myeloid leukemia cells are enriched in bone morphogenetic protein and quiescence signalling pathways.

5 Major conclusion :

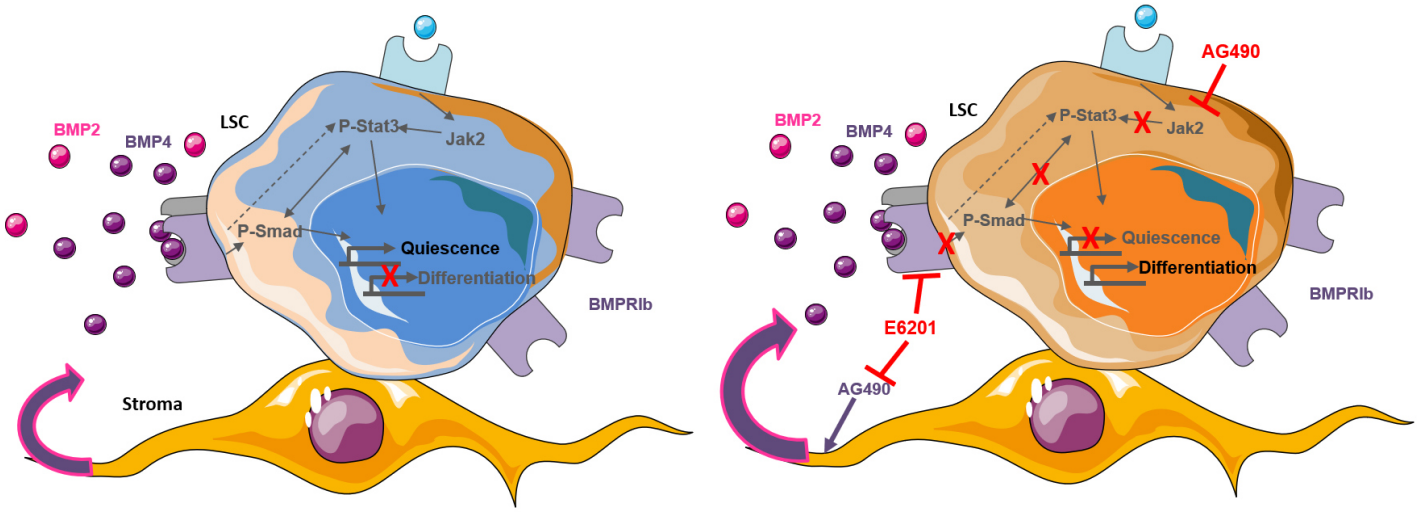


Figure 46: Dual targeting of BMPR1B & JAK2/STAT3 impairs LSC quiescence:

Manque description

6 Discussion :

Compared to the last decades, the overall survival of patients suffering from cancer has increased thanks to new therapies, patient care and early diagnosis for some cancers. Even though, Cancer Stem Cells (CSCs) remain a key point to understand the persistence as well as resistance phenomenon in cancers as they are considered as a subpopulation of primary tumor initiated cells [303]. This bulk of tumor cells has been described for the first time in Acute Myeloid Leukemia (AML) by Bonnet and Dick, 1997.

As normal SCs, CSCs do self-renew and differentiate to ensure a pool of tumor cells. The authors showed that between 0,1 – 1% of CSC 34+ can generate AML disease *in vivo*. Several studies have demonstrated that CSCs are mainly responsible of the resistance to therapies in several cancers to enhance the pathogenesis and tumorigenesis. These specific cells are also responsible of the metastatic increase potential through several inducer signals like WNT, Notch or TGFb[303,305]. Especially after conventional therapies, CSCs will turn dormant and after gaining several abilities to upregulate drug efflux pumps as well as several cytokines production and modulating important signaling involving TGFb, Notch and in addition to enhancing their telomerase activity, protection ability against ROS and DNA repair system, this very small subpopulation will be able to cause a Tumor relapse and re-generate the disease [305].

CML disease remains an example in which CSCs that are leukemic stem cells (LSCs) are involved in CML patients relapses as well as their resistance to treatment or their initial bad response to therapy an no CCyR achievement[495–497]. This life threatening blood cancer was revolutionized during last two decades thanks to Tyrosine Kinases discovery[498]. Nowadays, most people suffering from CML disease will have a very good disease prognosis, specially if they are diagnosed in the early chronic phase (CP) and do respond well to TKIs their life expectancy will be similar to a healthy person one.

As the CML disease is very well treated and the majority of CML patients do respond well to the disease treatment, currently the major topic in CML is the treatment arret in CCyR CML patients that achieved at least 5 years of disease remission. Considering the TKIs treatments side effects, enormous cost for the society or the patient itself (depending on the country) as well as treatment noncompliance, several clinical studies tried a TKI treatment arrest in CCyR CML patients[398,499,500].

Among these studies, the STIM (STop IMatinib) TKI treatment arrest study was performed by *Mahon et al*. French group[499]. Initially included 100 CML patients had no *BCR-ABL1* detection at the mRNA level for at least 24 months, and they were followed up between July 2007 to December 2009 in the study. Among the enrolled CML patients, 69 had at least one year follow up. In the 6 months following the Imatinib uptake cessation, 42 among the 69 CML patients relapsed indicating that at 12 months, the probability of persistent complete molecular remission for these 69 patients was 41%. A reintroduce of Imatinib uptake was proposed for the 42 relapsed patients and 26 CML patients among them achieved a complete molecular

remission after imatinib rechallenge while the other 16 patients responded less nut showed a significant decrease in their *BCR-ABL1* transcript level.

The outcome of this study highlighted the fact that when the treatment pressure is maintained, CCyR CML patients will maintain their remission achieved state whereas after TKI drug pressure removal patients' remission will drop. At the cellular level, a small sub fraction of CML LSCs that are initially sensitive to CML treatment do emerge and re-initiate CML disease after drug discontinuation. This sub fraction of CML LSCs persist in CML patients Bone Marrow (BM) surviving within a modified leukemic niche (Figure 29) and is responsible of disease persistence forming a reservoir of residual disease in these CML patients BM. Since then, several studies focused on CML LSC persistence and extrinsic niche signals supporting this persistence from the beginning of TKI exposure to the disease relapse after treatment arrest.

In our lab, we focused for several years on Bone Morphogenetic Proteins (BMPs) considering their deep involvement several cancer diseases including CML[92] . Indeed, we highlighted BM microenvironment modification during CML indicating that an overproduction of BMP2 and BMP4 by microenvironment cells in addition to LSC BMPR1B cell surface increase, the response to these cytokines results in an abnormal expansion of the myeloid progenitors and BMP4 will foster BCR-ABL+ LSC self-renewal[93]. Furthermore, in another study our lab demonstrated that in addition to the furnished BMPs by stromal cells, CML LSCs do set an autocrine loop of BMP4 production as well as an exocytosis of BMPR1B responsible of the up-regulation of TWIST1 leading to TKI treatment resistance and an abnormal increase of leukemic progenitors expansion[494]. In a recent study our lab showed the deep involvement of BMP4 in CML persistence as CML LSCs were co-activating canonical BMPR1B/SMADs and a non-canonical JAK2/STAT3 pathways to maintain their quiescence in CML niche thanks to the stromal BMP4 mediated supply[70]. Following this path, we looked after the mechanisms underlining CML persistence by involving TKIs treatments trying to decipher how does TKI treatment impact LSC dormancy within CCyR CML patients BM.

I. Role of BMPs alterations in CML persistence

a) At disease diagnosis

From the disease diagnosis BMP pathway seems to be dysregulated. Indeed, in our lab, a previous analysis showed a high expression of BMPR1B in CML newly diagnosed patients [93]. Furthermore, another study from our lab showed that BMPR1B transcript expression in 71 newly diagnosed CML patients revealed an elevated BMPR1B expression in around 40% of the diagnosed patients[494]. CML patients in CP did also display an overexpression of BMPs target genes RUNX1 and ID1. Furthermore, a decrease in BMP2, BMP4, ID2, ID3 and RUNX2 genes was reported[494]. Another group also demonstrated that in 30 CP-CML CD34+ cells a downregulation of BMP2 as well as RUNX1 was reported with a highly expressed BMPR1B[501]. In a recent study we performed BMPs quantification in newly diagnosed CML patients plasma.

We found that for 34 patients a high BMP2 production was reported compared to 23 healthy donor samples. Also 20 diagnosis CML patients showed a significant production increase of BMP4 compared to 17 healthy donors[70]. This outcome confirms that from the diagnosis stage at CML CP, patients do already present a significant dysregulation of BMPs pathway in their BM compared with normal donors and this dysregulation contributes to disease tumorigenesis and may be involved in the persistence phenomenon as well as the resistance to TKI treatment.

b) In CML patients at remission under CML treatment uptake

Under treatment uptake, CML LSC intrinsic signalling as well as its surrounding cells behaviour do change in response to the treatment exposure[93]. In our lab, selected $CD34^+$ BM cells from CML patients that reached cytogenetic response presented a low expression of BMPR1B thanks to the TKI drug exposure[494]. As well, in our recent study we did not report any overexpression of BMPR1B or BMP4 at the mRNA levels in CML LSCs coming from CCyR patients[70]. Nonetheless, we recently discovered that in CCyR CML patients plasma, significant increased amounts of BMP2 and BMP4 cytokines are reported[70]. These outcomes emphasize the fact that even by achieving cytogenetic remission, normal BMPs levels are not restored and CCyR CML patients do still have a significant deregulation of BMPs production within their BM microenvironment. This BMP supply may play a role in LSCs protection and persistence alongside treatment exposure.

II. Involvement of BMPR1B in LSC persistence

a) BMPR1B expression in CML LSCs

BMPR1B is widely overexpressed in AML LSC as well as in resistant CML LSCs[494,502]. Nonetheless, in our recent study we did not report any overexpression of BMPR1B in persistent LSC (group A) coming from CCyR CML patients[70]. However when we specifically blocked BMPR1B receptor using E6201 in our persistent LSC model, we significantly downregulated its canonical P-SMADs downstream signalling. This suggest that even if BMPR1B is not necessarily overexpressed at persistent LSC cell surface, it plays a key role in signalling transmission during CML persistence. Thus the assumption that BMPRB internalisation within LSCs cytoplasm and its membrane delocalisation when needed can be given and it would be interesting to assess BMPR1B internalization as well as membrane stabilisation during in CCyR CML LSCs.

b) JAK/STAT3 & CML persistence

JAK/STAT pathway dysregulation have been reported in several myeloproliferative neoplasms. It has been demonstrated that BCR-ABL can directly activate JAK2 protein. STAT3 role has also been highlighted in CML TKI resistance as well as STAT5 that can be constitutively activated independently from JAK2 by BCR-ABL 190kDa and 210kDa isoforms[503–505] . This cytoplasmic STAT5 activation is linked to cell cycle progression through increased cycline D1 and anto-apoptotic Bcl-XL protein expression [64]. Furthermore, STAT5 deficient mice fail to develop a CML like disease suggesting the key role of this protein expression in CML leukemogenesis[506]. In our recent study, we highlighted JAK2/STAT3 deep involvement in CML LSCs dormancy without any activation of STAT5. Indeed, we demonstrated that a correlation do exist between BMPR1B and JAK/STAT3 in a highly quiescent CML LSC sub-fraction that persist in CCyR CML patients[70]. Furthermore, we showed that persistent CML LSCs do co activate *in vitro* the canonical SMAD1/5/8 pathway and a non-canonical JAK2/STAT3 pathway to maintain their dormancy within CML niche[70].

III. LSC population heterogeneity and CML treatment

a) Different LSCs subsets do exist

As long as TKI treatment dramatically improves CML disease healthcare, it shows a significant effect on BCR-ABL+ CML cells which enables CML patients deep molecular response (MMR) achievement[369,382,388,405]. Nonetheless, TKI treatment remains not yet curative as it fails to eradicate persistent LSCs that continuously threaten disease response in CCyR CML patients[507,508]. This suggests the existence of heterogeneous LSC sub-populations within CML patients BM that do respond differently to TKI treatment and do subsequently vary in terms of intrinsic regulation. Indeed, several studies highlights different kind of LSCs within CML bone marrow (Figure 29). As well, Gustaccini et al. demonstrated the existence of two CML LSC sub-groups within CCyR CML patients that were followed during 12months[509]. The LSCs Group B presented a highly proliferative behaviour and decreased during treatment exposure while the LSCs Group A presented quiescence signatures and persisted along treatment exposure[509]. In addition, in our recently published study we highlighted differences between these two LSC sub-groups through an RNA sequencing showing that Group A presented an enriched BMP pathway signatures as well as a correlation between BMPR1B and JAK/STAT3 rending it intrinsically different from the sensitive and highly proliferative Group B. As other studies highlighted the importance of STAT3 and BMPR1B in CML treatment escape independently to BCR-ABL[494,510], we confirmed this non canonical pathway as being involved in CML quiescence maintenance as a dual targeting f BMPR1B and JAK2 signalling results in quiescent LSC disturbing and proliferation initiation[70].

b) Dormancy heterogeneity

Stem cell dormancy is a normal state in which many SCs do enter to preserve their stem potential in regenerating progenitors. As well, CSCs do also divide rarely and do enter a state of deep quiescence also called "dormancy"[511]. This acquired state may rend them insensitive to cytotoxic treatments as they stay non cycling consuming very low nutrients and oxygen to ensure a minimal metabolism to survive.

B 1) P 21 regulation

P21 also called p21Cip1 has a heterogeneous and multifaced functionality. This broader regulator is deeply involved in cell senescence, cell quiescence as well as tumour suppression[512]. Furthermore, this regulator has a key role in DNA repair, pluripotent stem cell reprogramming, cell differenciation, apoptosis and migration[513]. Nonetheless, its main role has largely been described in cell cycle regulation as it controls cycling checkpoints ensuring a proposer cell division by directly binding to Cycline Dependent Kinases (CDKs). Its expression is also mandatory to maintain HSC quiescence in vivo[194]. A basal level of p21 is necessary for normal stem cell homeostasis maintenance but increased p21 levels result in SC differenciation and self-renewal inhibition[514]. In leukemia, P21 is indispensable for maintaining LSCs self-renewal as well as it prevents DNA Damage and exhaustion of LSCs[515]. Its lack can result in a depletion of LSCs[516].

In our finding, we noticed an overexpression of P21 in a quiescent BCR-ABL+ LSC group (figure 30 F). This sub population presented low KI67 nuclear expression compared to cycling BCR-ABL+ LSC(figure 30 F). Furthermore, this same quiescent sub fraction displayed an overexpression of the IL3-R at the mRNA level as well as at its cell surface (Figure 30 E and D). As this LSC sub-fraction do persist to CML treatment in presence of a stroma (Figure 35) its highly expression for p21 nuclear protein may help as an additional target it to eradicate these residual persisting LSCs that escape to CML treatment through dormancy.

CD123

Several groups described CD123(= IL3-Ra chain) as relevant marker for the identification and targeting of LSCs for refractory or relapsed leukemia. Indeed, giving its very low or absent expression at HSC cell surface[517], this receptor can be a more or less specific target to eliminate leukemic cells[517]. Given the facility to target this cell membrane receptor, a drug anti CD123 was developed for a clinical trial. IMGN632 CD123 inhibitor undergoes currently a clinical development to treat several hematological malignancies in which CD123 is overexpressed such as AML or ALL [70,518]. First trials already began using CD123 inhibitor as a monotherapy for BPDCN patients but also in relapsed AML by combining it to venetoclax and azacitidine[519]. Interestingly, CD123 appears to be overexpressed in our persistent CML LSC model at the mRNA level[70]. Furthermore, recent results confirm the overexpression of this marker at both mRNA and protein levels in a quiescent sub-fraction of CML LSCs (Figure 30 D,E). Considering its already described overexpression in leukemic cells, its upregulation in our study underlines

its importance in LSC dormancy. To better assess its role, an experiment blocking this receptor in our study should be performed to evaluate its impact on LSC dormancy. Also, considering the autocrine loop of IL-3 that some CML LSCs do set up (Figure 29). An Elisa assay should be performed to complete our results and see if this IL-3 autocrine loop production is performed by our quiescent CML LSCs which will better explain the persistent cells increased sensitivity for this cytokine.

IV. Niche role in persistence maintenance

a) Physical support & ECM

Hematopoietic niche forms a physical homing zone for quiescent and cycling hematopoietic cells[520] (Figure 11). In leukemic diseases, this Bone marrow Microenvironment (BMM) that initially protects and maintains HSC homeostasis is changed and contributes to leukemogenesis by supplying protection as well as paracrine signalling to support LSC maintenance[521] (Figure 29). In CML, abnormal deposition of Fibronectin FN as well as collagen is detected within the niche and is reported to be enhancing BCR-ABL+ cells resistance to TKI treatments (Figure 29). Our recent outcomes showed that a culture of CML LSCs on collagen will enrich this population quiescence (10% of G0 LSCs) given that without a stoma quiescent LSC sub-fraction is around 1.5% (figure 32 B) without any effect of the TKI treatment in absence of ECM. As well, nilotinib treatment of these CML LSCs coated on collagen will result in an increase of their quiescence state that doubles from 10 to 20% of quiescent LSCs with 1,5µM or nilotinib treatment (Figure 34 B). It is known that the one of the most abundant ECM components within BM is collagens (Types: I, II, III, IV, and X)[522]. Nonetheless, its exact role in HSC and LSC quiescence is not yet deciphered. Our findings suggest that this ECM component support LSC quiescence and synergizes with TKI treatment to enhance LSC dormancy as in its absence, TKI treatment do not modulate LSC quiescence (Figure 32 B). To better understand Collagen involvement in LSC quiescence, current 3D experiments are ongoing to complete Figures 36, 37 & 38 outcomes and stain collagen component within our 3D BM system that maintains LSC dormancy. This future result will give us a better clue on collagen localisation and formation within the system as well as its interaction with different used CML treatments (Imatinib, Nilotinib and Asciminib) to impact LSC dormancy in a minimized BM structure. Furthermore, Leukemic niche presents a support with its numerous cells such as osteoblasts, endothelial cells, fibroblasts and MSCs that represent a physical maintain for quiescent LSC that attach via integrins, cadherins, jagged1 and selectin proteins to these cells (summarized in Figure 29) . As a study highlighted, The N-cadherin and b1 integrin as anchoring molecules for stem cells but also as HSC cell cycle regulators [211] . Indeed, the cell to cell interaction within BM niche serves to maintain long term dormancy of HSCs[523]. Additionally, N-Cadherin that activated intracellular βcatenin will activate a downstream WNT pathway responsible of quiescence maintenance[524] . Similarly to normal HSC, LSC do certainly need a cell to cell contact to ensure a deep dormancy within leukemic niche.

b) BMP supply:

Leukemic niche involvement in LSC support has been reported in several studies including studies conducted in our lab that underline an abnormal production of BMP2 and BMP4 cytokines by the LSC surrounding cells within CML patients BM[93,494]. In persistence case, our resent article highlights the increased BMP2 and BMP4 cytokines within CCyR CML patients' plasma showing that CML treatment permits MMR achievement but does not restore normal BMPs levels within CCyR CML patients BM. In addition we showed that the stromal BMP4 supply is necessary for CML LSCs quiescence maintenance[70]. Furthermore, in recent outcomes we discovered That when CML LSCs are cultures in a non BMP4 releasing stroma, LSC quiescence was not modulated (Figure 35B). However, when we culture CML LSCs on a BMP4-relasing stroma (Figure 35 C), percentage of quiescent LSC was the same (NT condition in Figure 35 B and C). But when we treated LSC with various CML inhibitors, the outcome changes depending on the stromal BMP release. Indeed, CML treatments of LSC that adhere to a BMP4 producing stroma will dramatically increase LSC dormancy as it goes from10% for the non-treated condition to 20% for the Imatinib or Asciminib treated condition and 23% for the Nilotinib treated condition (Figure 35 B, C). Furthermore, a designed HS27A shBMP4 and OEBMP4 cell lines confirmed our outcomes by showing a modulation of LSC quiescence that follows the BMP4 supply. The summary of these experiments confirmed the effect and involvement of BMP4 in LSC dormancy maintenance but also highlights the synergy between BMP4 and CML treatments resulting in a high increase of LSCs quiescence instead of eradicating these cells.

Through our recent findings, in presence of a BM produced BMP4, CML treatments will foster CML LSC dormancy within BM niche.

c) General remodelling over treatment.

CML treatment do affect CML LSC as well as the surrounding cells even if stromal and endothelial cells do not express the BCR-ABL oncoprotein. CML niche remodelling over treatment involves several actors for which their initial function will change making them behave to support LSC leukemogenesis within BMM. Endothelial cells within the CML niche do produce BMP2 as well as BMP4[525]. As well, osteoblasts do also produce numerous cytokines including BMP2 and BMP4[526]. Present HSCs do also produce BMP2 and BMP4 but also the I-SMADS BMP6 and BMP7[527,528].LSC itself will produce BMP2, BMP4 and will increase its BMPRs at cell surface to increase cytokines uptake and drives tumorigenesis[93,494,529,530]. To better understand LSC dormancy maintenance inside of CML BM, we used a BM-like model that was developed in the laboratory[531]. This standardized small system mimics medullar niche and enables LSC culture and maintenance. It comprises HMEC endothelial cell line mimicking vascular niche as well as a HS27A stromal cell line that will differentiate into osteoblasts mimicking thus endosteal niche. The goal of using it is to decipher CML treatments impact on LSC dormancy. Our outcomes were assessed at 1 week on CML LSC culturing versus a long culturing time of 4 weeks.

The outcome showed that from W1 to W4 number of CD45+ cells decrease in the system and decreases more when Imatinib, Nilotinib and asciminib were used (Figure 36). This suggest that cycling CML LSCs are efficiently eliminated alongside treatment. Furthermore, we noticed that CML LSCs that adhere to the system have an increased quiescence compared to the non adherent LSCs (Figure 37). As well, CML treatment may affect LSC quiescence as it decreases it at 1W of treatment. After 4W of treatment, Imatinib and Aciminib decrease LSC quiescence even if supported by a stroma. Interestingly, Nilotinib treatment does not affect LSC quiescence within the niche and gives same outcome as NT condition. This may reflect the LSC dormancy in CML patients or a beginning of treatment escape where LSC do not respond to TKI treatment. But we remarked that TKI treatment, even if not driving major effect on LSC persistence, is dramatically modifying LSC niche by increasing BMP4 production within the system (Figure 39).

Furthermore, as we highlighted CD123 outcomes in this study, the 3D retrieved cells were analysed to evaluate this IL-3R expression. We remarked that LSC culturing within BM-like microenvironment increases CD123 expression at LSC cell surface (Figure 39). Nonetheless, CML treatment will decrease CD123 expression on CML LSCs. This suggest that CML treatment is still efficient against LSCs by acting on it and modifying their intrinsic signals leading to CD123 expression. Nonetheless, LSC heterogeneity may result in a general CD123 decrease by CML drugs but small LSC clusters may not respond to this treatment effect and cannot be distinguished in our result.

For this reason, we performed a 3D experiment seeding primary CML CD34+ cells coming from CCyR CML patient. Ongoing RNA sequencing of 3D retrieved cells will give us a better clue on our LSC heterogeneity as well as the increased signatures in quiescent LSCs. Furthermore, in parallel an optimisation of IHC multiplex is ongoing. The latter will serve to stain 3D-BM like structure slices with different markers sur as BCR-ABL to determine LSC from HSC sub fraction, CD73 to differentiate endosteal compartment, CD31 to differentiate vascular compartment, P21 and KI67 to delimit cell quiescence and BMPR1B to assess receptor localization within the system. This future but already ongoing result will give us a clue on persistent LSC localisation within the system, on ECM such as collagen expression in the system as well as the variation of these markers alongside TKI first (Imatinib) and second (Nilotinib) generation as well as Asciminib treatment for which, these outcomes can predict mechanisms that will occur in CML patients treated with this new drug.

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