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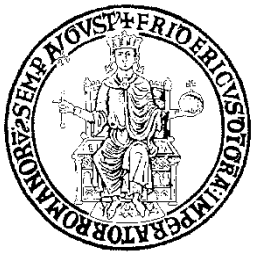

Doctorate School in
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The pluripotency transcription factor Nanog plays a potential role as a BCR/ABL independent mechanism of TKI resistance in Chronic Myeloid Leukemia.

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ABBREVATIONS

CML	Chronic Myeloid Leukemia
CP-CML	Chronic Phase Chronic of Myeloid Leukemia
Ph	Philadelphia
ABL	Abelson
BCR	Breakpoint Cluster Region
HSC	Hematopoietic Stem Cell
LSC	Leukemic stem cell
Ph+	Ph positive
CP	Chronic phase Accelerated Phase
AP	Accelerated Phase
BP	Blast Phase
FISH	Fluorescent in situ hybridization
RT-PCR	Reverse transcriptase-polymerase chain reaction
MRD	Minimal Residual Disease
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia
CMML	Chronic Neutrophilic Leukemia
SOS	Son of Sevenless
MAP	Mitogen-activated protein
TKI	Tirosin Kinase Inhibitor

INF-alpha	Interferon alpha
HU	Hydroxyurea
HSCT	Hematopoietic Stem Cells Transplantation
GVHD	Graft-versus-host-disease
PDGF	Platelet derived growth factor receptor
SCF	Stem cell factor
CHR	Complete hematological response
HR	Hematologic response
IRIS	Randomized Study of Interferon and STI571
FDA	Food and Drug Administration
CyR	Cytogenetic Response
MR	Molecular response
CCyR	Complete Cytogenetic Response
MCyR	Major Cytogenetic Response
CBA	Chromosome banding analysis
ELN	European Leukemia Net
MR	Molecular Response
OR	Optimal response (OR)
WR	Suboptimal/warning (WR)
FR	Failure response.
QRT-PCR	Quantitative Real Time Polymerase Chain Reaction

EUTOS	European Treatment and Outcome Study for CML
PFS	Progression free survival
OS	Overall Survival
KD	Kinase Domain
VEGF	Endothelial Growth Factor and tumor necrosis factor alpha α
TGFβ	Transforming Growth Factor Beta
AGP	Serum protein α1-acid glycoprotein
OCT1	Multidrug Influx Pump: The Organic Cationtransporter 1
CSC	Cancer Stem Cell
OCT4/3	Octamer-binding transcription factor 4/3
SOX2	Sex-determining region Y HMG-box 2
LT-HSC	Long-Term Subset
ST-HSC	Short-Term Subset
iPSCs	Induced Pluripotent Stem Cells
ES	Embryonic Stem Cells
EMT	Epithelial Mesenchymal Transition
NPC	Nasopharyngeal Carcinoma
HCC	Human Hepatocellular Carcinoma
MLL	Mixed Lymphocytic Leukemia
RFS	Recurrence-Free Survival

OSCC Oral Squamous Cell Carcinoma

SUMMURY

Chronic Myeloid Leukemia (CML) is a clonal myeloproliferative disease characterized by a specific chromosomal translocation t(9;22) that gives rise to a fusion gene BCR-ABL1. The oncogenic product BCR-ABL1 is a constitutively active tyrosine kinase that promotes cell proliferation and inhibits apoptosis of the leukemic clone.

In the era of target therapy, the treatment of CML patients in chronic phase (CML-CP) with tyrosine kinase inhibitors (TKIs) showed a substantially life expectancy improving. However, a large number of them develop drug intolerance or resistance and relapse after TKIs treatment.

If on one hand, BCR-ABL1 dependent Imatinib resistance can be overcome by second or third generation TKIs, on the other hand, the molecular mechanisms that underlie BCR-ABL1 independent resistance are not well clarified. It is becoming evident that persistent leukemic stem cells (LCSs) can lead to disease relapse at the time of TKI withdrawal in a relevant portion of the patients.

In this context, we sought to evaluate Nanog role in the regulation of CML cells response to TKI therapy. Nanog is an essential transcription factor involved in the regulatory networks that are responsible for stemness in embryonic pluripotent stem cells. Furthermore, functional studies have provided evidences that the expression levels of Nanog play crucial role in malignant diseases promoting tumorigenicity, invasiveness, and therapeutic resistance.

We have observed a significant level of Nanog protein expression in Philadelphia positive (Ph+) K562 cells after Imatinib treatment whether compared to untreated control since 24 hours of treatment, with a persistence

of expression at least until 72 hours. Moreover, we also have described a time-dependent up-regulation of Nanog protein in K562 Ph+ cell line treated with increasing doses not only of Imatinib (first generation TKI), but also of Nilotinib (second generation TKI), confirming the correlation between TKI treatment and Nanog overexpression. Moreover, we demonstrated that the Nanog protein overexpression is restricted to alive cells and persists after TKI withdrawal.

The RT-qPCR analysis revealed that Nanog expression is modulated at transcriptional level after exposure to first and second generation TKIs showing a correlation with Nanog protein increasing.

Furthermore, we proved that Nanog overexpression is independent from BCR-ABL1 activity; indeed, after Imatinib treatment, in K562 cell line, BCR-ABL1 expression was reduced, instead Nanog expression was increased. Finally, we evaluated Nanog expression in two cohorts of CML-CP patients at baseline treated with 1) Imatinib or 2) Nilotinib and we observed a significant up-regulation in Warning or Failure responder patients; contrariwise, Optimal responder patients showed a significant down-regulation of Nanog mRna expression.

Taken together, these findings demonstrate the involvement of Nanog in TKI resistance in K562 Ph+ cell line and identify Nanog as a potential marker of molecular response in CML patients.

1. INTRODUCTION

1.1 Chronic Myeloid Leukemia.

1.1.1 Clinical presentation

The Chronic Myeloid Leukemia (CML) is a clonal myeloproliferative disorder characterized by an accumulation of several types of myeloid precursor cells that retain the capacity to differentiate during Chronic Phase of the disease (CP-CML) [1].

CML was described for the first time 165 years ago and may be regarded as a paradigm of modern oncology. In 1960 Nowell and Hungerford described the hallmark of CML through the discovery of the Philadelphia (Ph) chromosome and its association with the development of CML. The Ph chromosome was a breakthrough in cancer biology. It is an abnormal short chromosome 22 (22der or 22q-) and was the first consistent chromosomal aberration associated with a specific type of leukemia. Indeed, it is the genetic hallmark in about 90% of CML patients at diagnosis. Ph chromosome is generated by the reciprocal translocation t(9;22)(q34;q11) of the Abelson (ABL1) proto-oncogene tyrosine-protein kinase gene located on chromosome 9 to the breakpoint cluster region (BCR) gene located on chromosome 22. The results is a novel fusion gene BCR-ABL1 that encodes a tyrosine kinase with an abnormal activity, which deregulates cell proliferation, differentiation and apoptosis [2].

It has been proposed that CML may be the result of multistep pathogenic process. Initially, the acquisition of t(9;22) occurs in a single Hematopoietic Stem Cell (HSC) that gains a proliferative advantage and/or aberrant differentiation capacity over the normal cells [3].

The Ph positive (Ph+) Leukemia stem cell (LSC) is capable of expansion in both the myeloid or lymphoid lineages. It may involves myeloid, monocytic,

erythroid, megakaryocytic, B-lymphoid and occasionally T-lymphocytic lineages, although expansion is predominantly in the granulocyte compartment of the myeloid lineages in the bone marrow. [4]

The incidence of CML is approximately 1 per 100,000 population per year with a slight male preponderance and accounts for 20% of all leukemia affecting adults. The median age at onset is from 45 to 55 years and about 50% of cases are diagnosed by physical examination or blood tests. CML can be classified into three disease phases: chronic phase (CP), accelerated phase (AP), and blast phase (BP). CML patients diagnosed in the United States are asymptomatic in about 30 to 50% of cases and more than 90% are diagnosed at a CP [5]. CML-CP occurs a relatively indolent presentation in patients, with an increase of immature and mature myeloid elements and a retention of hematopoietic differentiation. The most common features at presentation are an high white blood cell counts, splenomegaly fatigue, weight loss, abdominal fullness, bleeding, purpura, anemia, and thrombocytosis [6]. At diagnosis of CML, about 10% of patients are Ph negative; in these cases, the Ph chromosome is absent at cytogenetic analysis, but the translocation can be detected by molecular analysis. Furthermore, about 5% of patients present variant translocations involving the chromosome 22 and a chromosome other than the chromosome 9 in the simple variant, whereas one or more chromosomes are involved in addition to chromosomes 9 and 22 in the complex variant. However, patients with Ph-variants have response to therapy and prognosis similar to Ph-positive CML[5].

The AP and BP phenotypes are much more different and aggressive than CP, and these two phases are characterized by a drastic reduction of cellular differentiation with the presence of myeloid or lymphoid blast cells in peripheral

blood. Most patients evolve into AP prior to BP, but the 20% evolve into BP without AP warning signals. AP might be insidious or presenting worsening anemia, splenomegaly and organ infiltration. BP presents as an acute leukemia with worsening constitutional symptoms, bleeding, fever, and infections [2, 5]. The progress to acute leukemia is associated with secondary cytogenetic changes like additional chromosomal abnormalities include trisomy 8, isochromosome 17, trisomy 19, duplication of the Ph chromosome [7] with high levels of BCR-ABL1 expression, microsatellite instability and loss of heterozygosity [8]. Moreover, the BC induction could be promoted by mutations in p53, a tumor suppressor gene with negative regulatory function in cell cycle progression [9].

1.1.2 Diagnosis

The diagnosis of typical CML consists in the identification of Ph chromosome abnormality, the t(9;22)(q34;q11) by cytogenetic analysis or fluorescent *in situ* hybridization (FISH). In FISH analysis the fluorescent probes to BCR and ABL1 genes hybridize a specific genomic sequence. Thus, the BCR-ABL1 fusion gene is observed by a co-localization signal. Reverse transcriptase-polymerase chain reaction (RT-PCR) amplifies the region around the splice junction between BCR and ABL1. Qualitative PCR assay is useful for CML diagnosis giving information about the presence of BCR-ABL1 transcript. The above-mentioned method has been optimized for the detection of all typical BCR-ABL1 transcripts e1a2, b2a2, b3a2 and some atypical transcripts such as fusions to ABL1 exon 3. Whereas, quantitative RT-PCR is highly sensitive for the detection of minimal residual disease (MRD) providing information about the amount of BCR-ABL message [5].

1.2 The molecular biology of CML

It is widely accepted that the acquisition of the t(9;22) BCR-ABL1 traslocation is the initial event of the CML-CP. At first, this acquisition occurs in a single HSC that gains proliferative advantage ad/or aberrant differentiation capacity over normal cells, giving rise to an expansion of the myeloid compartment [2] [1].

1.2.1 ABL1 gene

The ABL1 gene encodes a member of the family of a non-receptor tyrosine kinase protein and it is the human homologue of the v-abl oncogene carried by the Abelson murine leukemia virus (A-MuLV). It is located on chromosome 9 and encodes a 145 kDa protein with a molecular structure of 12 exons and several introns. The alternative splicing of the first exons, exon 1a e 1b, gives rise two mRNA isoforms, a 6-kb and a 7-kb molecules, coding 1122 and 1142 amino acids respectively [10]. ABL1 kinase activity is regulated by a myristol group that is localized on N-terminal domain, where there are also three SCR homology domains (SH1, SH2 and SH3).The catalytic domain SH1 consists of the C-lobe and the N-lobe, with an activation loop that is tyrosine phosphorylated at 393 (Y393) when the kinase is activated. The ABL1 auto-inhibition is attributed to SH2 and SH3 domains. A proline-rich sequence is located at the center of the protein and interacts with SH3 domain of other protein.

The SH2 domain position on the N-lobe mediates allosteric activation of the kinase domain that is independent of its phosphotyrosine binding capability. The SH2-KD is most important in the oncogenic fusion BCR-ABL1, indeed it

was shown to be essential for leukemogenicity and represents an allosteric target for pharmacological intervention [11].

The C-terminal non catalytic portion is constituted by a nuclear localization sequence, a nuclear export sequence, a DNA binding site [12], a p53 binding site [13] and an actin binding region [14]. Several functions have been attributed to ABL1 protein, such as the regulation of the cell cycle in the cellular response to genotoxic stress and the transmission of information about the cellular environment through integrin signaling.[15]

1.2.2.1 BCR gene

The Breakpoint cluster region (BCR) gene is composed of 25 exons, including two alternative first (e1') and second (e2') exons. The normal BCR gene codes for two major proteins of 160 kDa and 130 kDa size, these proteins are derived from the 7.0- and 4.5-kb BCR transcripts, respectively.

The BCR gene is now known to be a complex molecule with many different functional domains. The N-terminal consists of the serine-threonine kinase domain, the SH2-binding domain characterized by three to five amino acids including a phosphotyrosine, and an oligomerization domain that is characterized by a heptad repeat of hydrophobic residues between amino acids 28 and 68. The central sequences have GEF homology (activate G proteins by exchanging), that catalyze the exchange of guanidine triphosphate (GTP) for guanidine diphosphate (GDP). At C-terminal there is a GAP domain homology (inactive G proteins) with activity for a Ras-related GTP-binding protein (p21Rac) that regulates actin polymerization and NADPH oxidase activity in phagocytic cells [16]. Moreover, BCR can be phosphorylated on

several tyrosine residues, especially tyrosine 177, which binds Grb-2, an important adapter molecule involved in the activation of the Ras pathway[15].

1.2.3 BCR/ABL1 fusion gene.

The reciprocal translocation between chromosomes 9 and 22 leads to the BCR/ABL1 fusion protein formation that occurs in 95% of CML cases. The same cytogenetic translocation also occurs in about 20% of adult Acute Lymphoblastic Leukemia (ALL), 5% of pediatric ALL and rare cases of Acute Myeloid Leukemia (AML).

In most CML patients and in one third of patients with Ph-positive ALL, the BCR breakpoints are localized within a 5.8-kb area defined as the Major Breakpoint Cluster Region (M-bcr). M-bcr consists of five exons termed M-bcr exons b1–b5. These exons are located within the central region of the BCR gene and are equivalent to exons 11–15 (e11–e15) of this gene. Most breaks occur immediately downstream of exon 2 or of the M-bcr and the alternative splicing, can produce fusion transcripts with b2a2 or b3a2 junctions. These mRNA molecules lead to a chimeric protein of 210 kDa (P210 BCR/ABL1).

In the case of Ph+ ALL the breakpoints are further upstream, in the 54.4-kb region between the alternative BCR exons e29 and e2, termed minor breakpoint cluster region (m-bcr). ALL is characterized clinically by pronounced monocytosis and the fusion transcript produces an oncoprotein of 190 kDa (p190 BCR/ABL). A third fusion gene (e19a2) encoding a 230 kDa (p230 BCR/ABL) protein, was associated with the rare chronic neutrophilic leukemia (CMML). Each of these onco-proteins contains the same segment of ABL, but differs for BCR segment. Sporadic cases with other junctions, such

as b2a3, b3a3, e1a3, e6a2, or e2a2,42 have been reported in patients with ALL and CML.[16]

In contrast to BCR, the breakpoints within the ABL1 gene at 9q34 can occur anywhere over a large area (greater than 300 kb), either upstream of the first alternative exon Ib, or downstream of the second alternative exon Ia, or between the two. Independently from the exact breakpoint location, splicing of the primary hybrid transcript yields an mRNA in which BCR sequences are fused to ABL exon a2 [15].

The native c-ABL tyrosine kinase is located partially in the nucleus and its kinase activity is tightly regulated. The BCR–ABL fusion results in the production of a constitutively active cytoplasmic tyrosine kinase that does not block differentiation, but enhances proliferation and viability of myeloid lineage cells. BCR–ABL is likely sufficient to cause CML, but over time other genetic events occur and the disease progresses to an acute leukemia [17].

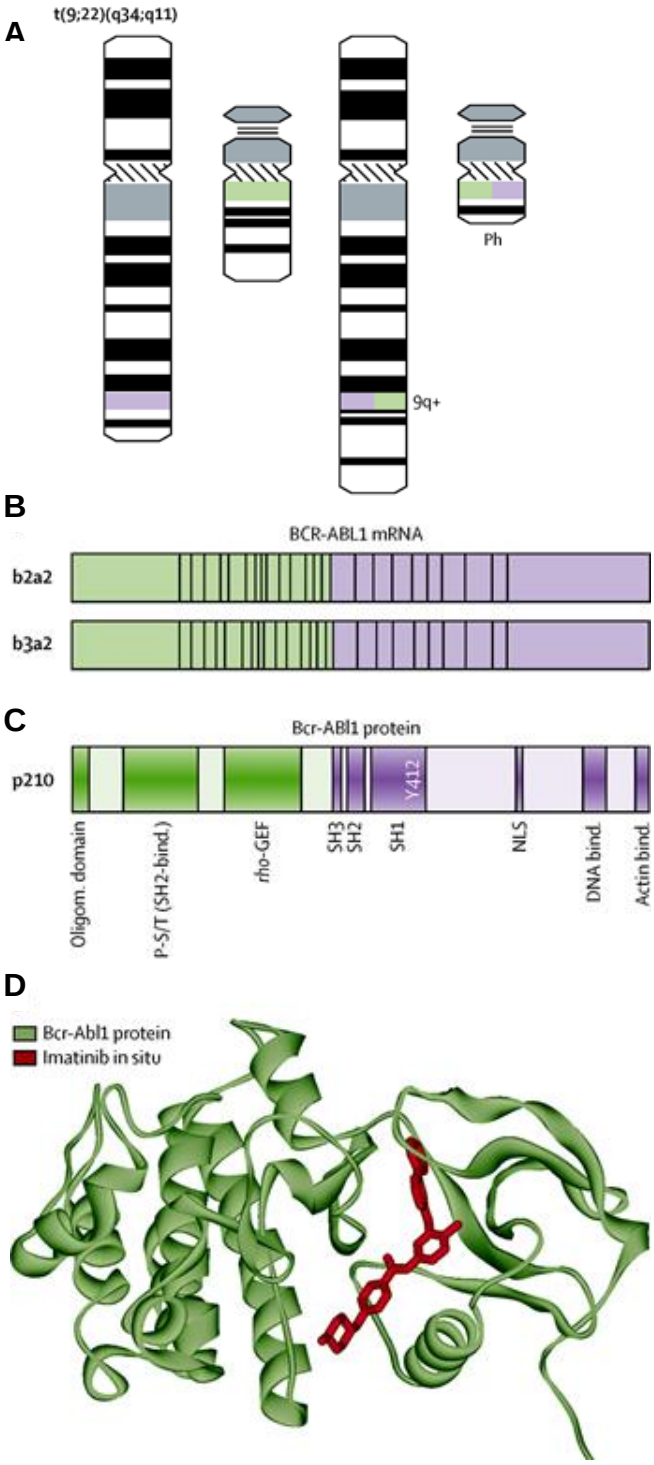


***Figure 1**. (A) The t (9;22) reciprocal translocation results in the creation of the BCR-ABL1 fusion gene which is transcribed to a BCR-ABL1 mRNA (B) and translated to a BCR-ABL protein (C). In panel D, is showed the protein interaction with Imatinib in ATP binding loop. [18]*

1.2.4 BCR/ABL1 kinase signaling pathways

In physiological conditions, ABL protein transduces signals from cell-surface growth factors and adhesion receptors to regulate cell differentiation, cell division, cell adhesion, proteasome degradation and stress response processes. It shuttles between the nucleus and cytoplasm of cells. However, when it is fused to BCR, ABL protein loses this property and is mainly retained within the cytoplasm where it interacts with the majority of proteins involved in the oncogenic signaling pathways.

The BCR/ABL leukemogenesis potential is regulated by the sequences within the first exon of BCR. The link of phosphorylated tyrosine (Y177) in BCR SH2 domain with GRB-2 protein activates the Ras signaling pathway that is most important in BCR/ABL mediated transformation. GRB-2 not only functions in normal development and mutagenesis but also plays a role in oncogenesis. When Y177 in the BCR SH2 domain is mutated, it gives rise an abolishment of GRB-2 binding with BCR-ABL1 and consequently the Ras activation was reduced.[19]

Son of Sevenless (SOS) is constitutively associated with the GRB2 SH3 domain, thereby with BCR/ABL-GRB2 it forms the GRB2/GAB2/SOS complex that stimulates constitutive activation of the RAS downstream pathway. The evolved pathways are Ras–mitogen-activated protein kinase (MAPK) leading to increased proliferation, the Janus-activated kinase (JAK)–STAT pathway leading to impaired transcriptional activity, and the phosphoinositide 3-kinase (PI3K)/AKT pathway resulting in increased apoptosis. The activation of Mitogen-activated protein (MAP) extracellular signal-regulated kinase (ERK)1/2 (MEK) lead to a G1 to S phase transition ensuing an abnormal cell proliferation[20]. The GRB2/GAB2/SOS complex triggers the PI3K/AKT

pathway, which promotes and enhances cell proliferation by inducing p27 proteosomal degradation and by mTOR upregulation blocking important cellular processes such as autophagy.

BCR-ABL1 interacts with many cytoplasmic proteins, which function as adaptor molecules, creating multi-protein signaling complexes. BCR/ABL1 activates the serine-threonine kinase AKT through PI3K, which phosphorylates downstream substrates like Bad, Caspase 9, Mdm2, and Ask1 that regulate the apoptotic machinery. Furthermore, the Akt-mTOR pathway stimulates the HIF-1α activation mimicking hypoxic conditions, and promotes the glucose transporter GLUT1 expression, which is responsible of glucose-dependent ROS production [21, 22].

All these events lead a prolonged survival and expansion of the abnormal clone. Moreover, STAT1 and STAT5 (signal transducer and activation of transcription) play a key role in BCR-ABL1 signaling. Indeed, they are constantly active in Ph+ positive cell lines and in primary cells of CML patients. STAT1 and STAT5 act in a JAK-independent manner through a direct association of their SH2 domains with phosphorylated tyrosine on BCR-ABL1 [23, 24].

The STAT5 phosphorylation gives rise the up-regulation of the anti-apoptotic molecule BCL-xL together with the inactivation of the pro-apoptotic molecule BAD by AKT [25]. Another target of the BCR/ABL activity is the proto-oncogene MYC that is expressed at a high level in CML cells. In particular, it is overexpressed in blast phase compared to the chronic phase, independently of RAS pathway. Indeed, Myc seems to be up-regulated directly by the ABL SH2 region [26]. All reported activated signaling pathways converge into a unique terminal point: loss of control of proliferation and expansion of the

leukemic clone. Defining the relative contribution of each signal transduction pathway to the leukemic process is an important area of research because the combination of a tyrosine kinase inhibitor (TKI) with a downstream inhibitor may be clinical successful strategy.

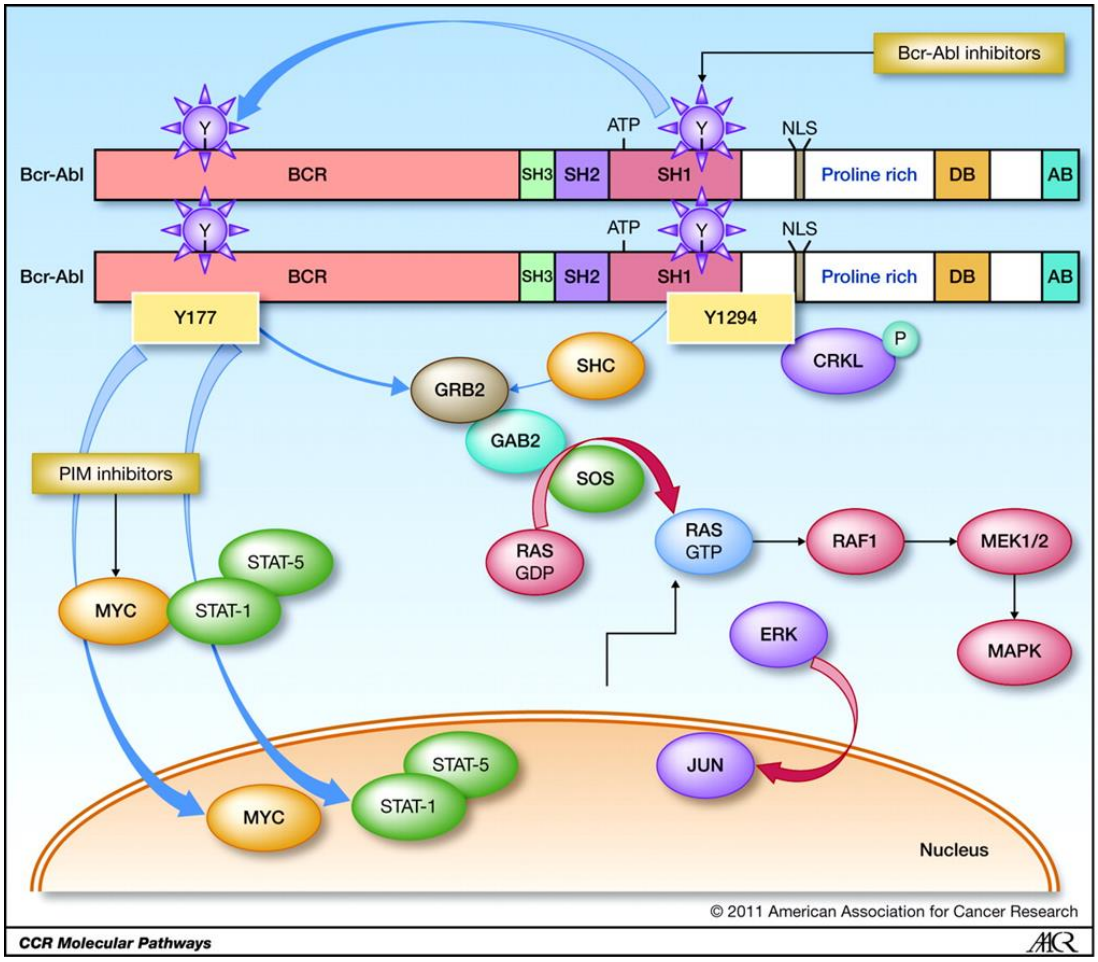


Figure 2. *Schematic representation of the molecular pathway activated by BCR-ABL1.* ***BCR-ABL1*** *phosphorylation of BCR Tyr177 is essential for BCR-ABL1–mediated leukemogenesis. The BCR-ABL/GRB2 complex recruits SOS, which is constitutively associated with the GRB2 SH3 domain. The BCR-ABL/GRB2/SOS complex stimulates conversion of the inactive GDP-bound form of Ras to its active GTP-bound state, and activation of the scaffold adapter GAB2. As a consequence, the GRB2/GAB2/SOS complex causes constitutive activation of the RAS downstream pathway, thereby activating MEK1/2 and MAPK proteins and resulting in abnormal cell proliferation.[27]*

1.3 Therapy and monitoring

1.3.1 The conventional chemotherapeutic treatment.

Before the BCR/ABL1 discovery and the introduction of Tirosin Kinase Inhibitor (TKI), CML was managed with conventional chemotherapies.

Busulfan and Hydroxyurea (HU) was given in low doses and rarely produced cytogenetic remissions and an improving overall survival. In a clinical trial was possible to induce cytogenetic remissions in CML-CP patients with intensive chemotherapy and splenectomy in a significant fraction of patients. However, the patients overall survival was modest and the blastic crisis transformation was observed within 5-6 years [28]. On the other hand, patients treated with Interferon alpha (INF-alpha) showed a survival increase compared to those that received HU and Busulfan. In particular, INF-alpha induced a persistence of complete remission still after stopping treatment. However, INF-alpha showed a high toxicity that is not tolerate by most patients. In addition, using both PCR and FISH analysis, small quantity of Ph+ cells could be detected in the majority of Bone Marrow (BM) patients with long-term cytogenetic remissions [29]. The curative therapy in majority of CML patients remained allogeneic bone marrow CD34+ hematopoietic stem cells transplantation (HSCT). Nevertheless, allogeneic HSCT is still a controversial treatment due to of the early mortality and the relatively high incidence of complications, including graft-versus-host-disease (GVHD), especially in older patients who are less able to tolerate the intensive treatment.[30]

1.3.2 The Target Therapy

1.3.2.1 Imatinib (Gleevec)

Imatinib mesylate, an example of rational drug design, was the first Tyrosine Kinase Inhibitor (TKI) used in a clinical setting with excellent results. It is a 2-phenylaminopyrimidine that acts as a specific inhibitor of several tyrosine kinase enzymes. STI571 or Imatinib was identified in the late 1990, when Druker and colleagues demonstrated the high selectivity to ABL1 kinase in cell BCR/ABL positive [31]. Imatinib is an ATP competitive inhibitor; that binds ABL1 inactive conformation of BCR-ABL1 kinase and blocks the ATP binding site. In this way, Imatinib avoids the transfer of a phosphate group to tyrosine on the protein substrate and subsequent conformational switch to the active form [32]. As the result, this drug inhibits proliferation and induces apoptosis in BCR-ABL1 positive cell lines as well as fresh in Ph+ leukemic cells of CML patients. Imatinib also inhibits the receptor tyrosine kinases for platelet derived growth factor receptor (PDGF) and stem cell factor (SCF) called c-kit and ARG but not the Src family kinases. [31, 33]

In phase I and II clinical trials, Imatinib showed a great efficacy with more than 90% complete hematological response (CHR) and 30-40% complete cytogenetic responses (CCyr) of enrolled CML patients.

In phase III, newly diagnosed patients with CML-CP were enrolled in an International Randomized Study of Interferon and STI571 *(IRIS)* in which Imatinib at single daily dose (400 mg) and IFN-alpha and cytarabine was compared.

The IRIS highlights an Imatinib superiority in rate of CHR, MCyR and CCyR respect to IFN alpha plus cytarabine. Moreover, was demonstrated that Imatinib treatment significantly reduced the disease progression to AP or BC.

Therefore, Food and Drug Administration (FDA) approved Imatinib as first-choice treatment for newly diagnosed CML in December 2002 [33]. Unfortunately, after one decade of clinical trial applying Imatinib as front line therapy for patients with CML, it is well known that TKI is not able to eradicate leukemia and primary or secondary drug resistance eventually occurring in the first two years of treatment. Hence, new drugs with ever-increasing specificity and anti-leukemia power have been developed.

1.3.2.2 Second generation TKIs

The identification of Imatinib resistance led to a focused effort to develop additional TKIs with more efficacy against kinase specific mutations.

Dasatinib (BMS-354825, Sprycel) is a second-generation BCR-ABL1 TKI indicated for Imatinib resistant or intolerant CML patients. It is an ATP-competitive inhibitor non-phenylaminopyrimidine-based drug and inhibits BCR-ABL1 tyrosine kinase both in the active and in inactive conformation of ABL1 ATP-binding domain [34]. Dasatinib is a dual Src-Abl inhibitor. In particular, inhibits Src-family kinases, ABL1 and other tyrosine kinases like PDGFR and c-Kit. It is more potent inhibitor in comparison with Imatinib mesylate and shows activity against most of the well-characterized BCR-ABL1 mutants except T315I [35].

Nilotinib (AMN-107, Tasigna) as well as Dasatinib is a second generation TKI and it was developed from Imatinib by crystallographic analysis. It is an ATP-competitive phenylaminopyrimidine that, similar to Imatinib, binds the same inactive conformation of ABL1 kinase. Nilotinib blocks the substrate binding site proximal to the activation loop and causes the inhibition of the ATPase catalytic activity by the disruption of the ATP–phosphate binding site [36].

Nilotinib has a higher affinity for ABL1 kinase domain than Imatinib, resulting in a greater potency and selectivity, whereby CyR and MR are significantly faster. In addition, it inhibits the activity of Arg, Kit, and PDGFR, but not Src family kinases [37]. Nilotinib is indicated for the treatment of CML patients with Imatinib resistance or intolerance, is effective against 32 out of 33 Imatinib-resistant point mutations, except T315I mutation [35]. Nevertheless, patients continually encountered with some hematological and non-hematological toxicity during the course of study. [18] ENESTnd and DASSION studies assessed the efficacy of Nilotinib (400 mg twice daily) and Dasatinib (140 mg once daily) versus 400mg of Imatinib in newly diagnosed CML-CP patients. The studies highlighted that Dasatinib and Nilotinib are superior in terms of achieving faster CCyR, MMR and lower progression rates than 400mg of Imatinib. They are well-tolerated therapeutic option for patients with CML-CP resistant or intolerant to Imatinib therapy [38, 39].

Finally, **Bosutinib SKI-606** is an alternative second generation TKI; originally, it has been proposed as a Src tyrosine kinase inhibitor, but was subsequently found as ABL1 tyrosine kinase inhibitor.

1.3.2.3 Third generation TKI

Ponatinib is a third generation TKI active against unmutated and mutated BCR-ABL1. It has efficacy for the threonine-to-isoleucine mutation at position 315 (T315I), which is present in up to 20% of patients with TKI resistance. A complete cytogenetic response and clinically significant activity was observed in CML and Ph+ ALL patients including those with T315I mutation [40].

1.4 Molecular monitoring in CML

As results of the TKI success, it was necessary to introduce guidelines to monitor the treatment response in CML patients. The monitoring of the response to TKIs therapy allows to detect early relapse of disease, thus a good management strategies in CML patients

There are three different types of therapy response: hematologic response (HR), cytogenetic response (CyR), and molecular response (MR) [41]. Complete hematological response (CHR) consists in a normalization of peripheral blood counts without immature blood cells and normal spleen size. The CyR is quantified by determining of the number of Ph+ metaphase cells. Using chromosome banding analysis (CBA) on BM cells and counting at least 20 metaphases may be observe: 1) the CCyR in the absence of Ph+ metaphase cells; 2) the major cytogenetic response (MCyR) when are present 0-35% Ph+ metaphase cells; 3) the partial cytogenetic response when the Ph+ cells are 1% to 34%.

The European Leukemia Net (ELN) recommendations suggest cytogenetic testing at 3 and 6 months, then every 6 months until a CCyR is achieved and subsequently every 12 months whether regular molecular monitoring cannot be assured. CBA, used to assess the degree of CyR, can be substituted by FISH of blood interphase cell nuclei only for the assessment of CCyR, which is then defined by <1% BCR-ABL1 positive nuclei of at least 200 nuclei.

The MR is determined by a decrease of the amount of BCR-ABL1 mRNA in the peripheral blood by quantitative real time polymerase chain reaction (qRT-PCR). Modern qRT-PCR can be detect residual disease to a sensitivity of 0,01% and often to 0.001. MR is assessed, according to the International Scale

(IS), as the ratio of BCR-ABL1 to control transcript (usually ABL1 and GUS beta). It is expressed and reported as percent of BCR-ABL1 on a log scale, where 10%, 1%, 0.1%, 0.01%, 0.0032%, 0.001% correspond to a decrease in tumor load of 1, 2, 3, 4, 4.5, and 5 logs respectively, below the standard baseline that was used in IRIS study [18, 42].

Since the inter-laboratory results cannot be compared, a methodological standardizing was necessary between laboratories. After many considerations, several genes are widely accepted as suitable controls, including ABL1, GUS, and BCR. The expression of the control genes is critically important because describes the sensitivity of the BCRABL1 detection assay. At this time, it is recommended that a sample should have at least 10,000 ABL1 or 24,000 GUS copies to pass minimum quality standards [42].

In 2006 a group of CML experts by means of the European Leukemia Net began a project with the aim to develop recommendations for disease management. In this regard, both cytogenetic and RT-qPCR dates was include for disease monitoring.

In the recommendation of 2009 Baccarani and colleagues, formally defined the management of CML patients treated with Imatinib in front-line. In 2013 an in the latest 2015, after the introduction of second and third generation TKI, CML therapy guidelines was revised.

Regardless of the TKI is used an optimal (OR), suboptimal (WR) or failure (FR) response. The optimal response is defined when BCR-ABL1 transcript levels is <10% at 3 months, <1% at 6 months and $\leq$0.1% from 12 months. The OR is associated with the best long-term outcome, with a duration of life comparable with that of the general population, indicating that there is not suggestion for a change in the treatment. A BCR-ABL1 transcript levels >10% at 6 months and

>1% from 12 months define failure response. The failure indicates that the patient should receive a different treatment to limit the risk of progression and death. Between OR and FR there is an intermediate warning zone that requires more frequent monitoring, known as suboptimal. BCR-ABL1 transcript levels is >1-10% at 6 months and >0.1-1 % at 12 months. Suboptimal responders may be eligible for alternative approaches, although the condition of suboptimal response may be only transitory [43, 44].

The proportion of blasts in the blood and bone marrow together with age and spleen size are used in scoring system for the prediction of survival. The Sokal score was developed for patients treated with Busulfan while the Hasford score for patients treated with INF-α and either continue to have value in the TKI era. Recently, European Treatment and Outcome Study for CML (EUTOS) developed a new formula to predict prognosis, known as the EUTOS score. It is most simple and asses the progression free survival (PFS) and overall survival (OS). In particular, patients with low EUTOS score had significantly better 5-year PFS than patients with high EUTOS score. EUTOS, Sokal or Hasford scores are used with MR to describe the response achieved after a definite duration of TKI therapy [18, 45, 46].

Definition of the response to TKIs (any TKI) as first-line treatment

	Optimal	Warning	Failure
Baseline	NA	High risk or CCA/Ph+, major route	NA
3 mo	BCR-ABL1 ≤10% and/or Ph+ ≤35%	BCR-ABL1 >10% and/or Ph+ 36-95%	Non-CHR and/or Ph+ >95%
6 mo	BCR-ABL1 <1% and/or Ph+ 0	BCR-ABL1 1-10% and/or Ph+ 1-35%	BCR-ABL1 >10% and/or Ph+ >35%
12 mo	BCR-ABL1 ≤0.1%	BCR-ABL1 >0.1-1%	BCR-ABL1 >1% and/or Ph+ >0
Then, and at any time	BCR-ABL1 ≤0.1%	CCA/Ph– (–7, or 7q–)	Loss of CHR
			Loss of CCyR
			Confirmed loss of MMR*
			Mutations
			CCA/Ph+

Figure 3 .*The definitions of response are the same for patients in CP, AP, and BP and apply to second-line treatment, when first-line treatment was changed for intolerance. The response can be assessed with either a molecular or a cytogenetic test, but both are recommended whenever possible.*
After 12 months, if an MMR is achieved, the response can be assessed by RT-PCR every 3 to 6 months, and cytogenetic is required only in case of failure or if standardized molecular testing is not available. [42]

1.5 Mechanism of TKI resistance

Despite the majority of CML-CP patients obtained an optimal clinical response, a large number of them develop drug intolerance or resistance and relapse after TKIs treatment. Several studies are focusing on the mechanisms of resistance by which the leukemic cells survived to TKIs treatment. It is widely shown that ABL kinase domain mutations are implicated in the pathogenesis of TKI resistance. Moreover, is evident that the presence of mutations does not clarify all cases of resistance in CML patients. Thus, BCR-ABL1 independent mechanism may contributes to resistance to TKIs.

1.5.1 BCR-ABL1 dependent resistance

The mechanism of TKI resistance can be divided in primary or secondary. Primary or intrinsic resistance occurs when in a defined time point has not been achieved a drug response. On the other hand, secondary or acquired resistance is defined as loss of an established response to TKI treatment. In addition, secondary resistance is characterized by the loss of complete hematologic remission, of a complete cytogenetic response and of MMR and the detection of kinase mutations and clonal evolution.

The criteria to define the failure response of first-line TKI therapy in CML patients has been summarized in ELN [44, 47].

Soon after the Imatinib introduction, several *in vitro* studies described some derived Ph+ cell line that developed resistance to TKI. BCR-ABL1 genomic amplification and above all BCR-ABL1 KD mutations are the best-characterized mechanisms conferring resistance to TKI therapy [48].

Firstly, le Coutre and Weisberg observed that the Imatinib resistance was the result of elevated ABL1 kinase activity due to a genetic amplification of the BCR-ABL1 sequence [49, 50].

However, all of these samples were derived *in vitro*, thus could not described exhaustively the clinical TKI resistance. Until, Gorre et al identified by means FISH analysis genetic duplication of the BCR-ABL1 gene in cells of Imatinib-resistant patients [51]. Actually, about 40-90% of Imatinib resistant patients carry a mutation in BCR-ABL1 that influence the oncogenic kinase properties[47]. The mutation frequency in patients with Imatinib resistance changed in the different phases of CML: from 25% to 30% in early CP patients on first-line Imatinib to approximately 70% to 80% of BC patients [52].

Mutations are located in several structural subunits of KD and can be divided into several groups: 1) mutations in the binding site of TKI; 2) mutations in the ATP binding site; 3) mutations in activate loop and 4) mutations that involve the catalytic domain. However, not all mutations give rise to Imatinib clinical resistance. Mutation analysis with Sanger sequencing, is usually performed in non-responder patients after TKI therapy and the results obtained may guide to the selection of subsequent TKIs.

In the same BCR-ABL1 mRNA molecule can be find two or more codon changes know as compound mutations that characterized a single leukemic clone. Whereas polyclonal mutations are defined as two or more codon changes across different BCR-ABL1 mRNA molecules, and therefore presumably belonging to different mutant clones [53].

T315I represents a particularly critical mutation since it is rather frequent (about 15-20%) and induces not only Imatinib resistance, but also resistance to second-generation TKI, Dasatinib and Nilotinib. The Threonine with

Isoleucine substitution is located in ATP-binding site of ABL kinase. This mutation leads to a missing of binding site and a consequent structural hindrance that blocks the access for Imatinib, Nilotinib, Dasatinib and Bosutinib [47].

Moreover, few mutations are known to confer clinical resistance to Nilotinib (Y253H, E255K/V, and F359V/C/I) or Dasatinib (V299L, T315A, and F317L/I/V/C) [54].

The introduction of newer technologies with greater sensitivity allowed the identification of low-level mutations, but their specificity are limited for definite spectrum of mutations [55]. These mutations are below the detection limit of conventional direct sequencing and their clinical significance in CML patients has long been debated and remains unclear. However, retrospective studies have suggested that mutations found in rare Ph+ cells may fail to expand and their detection does not consistently predict relapse [56].

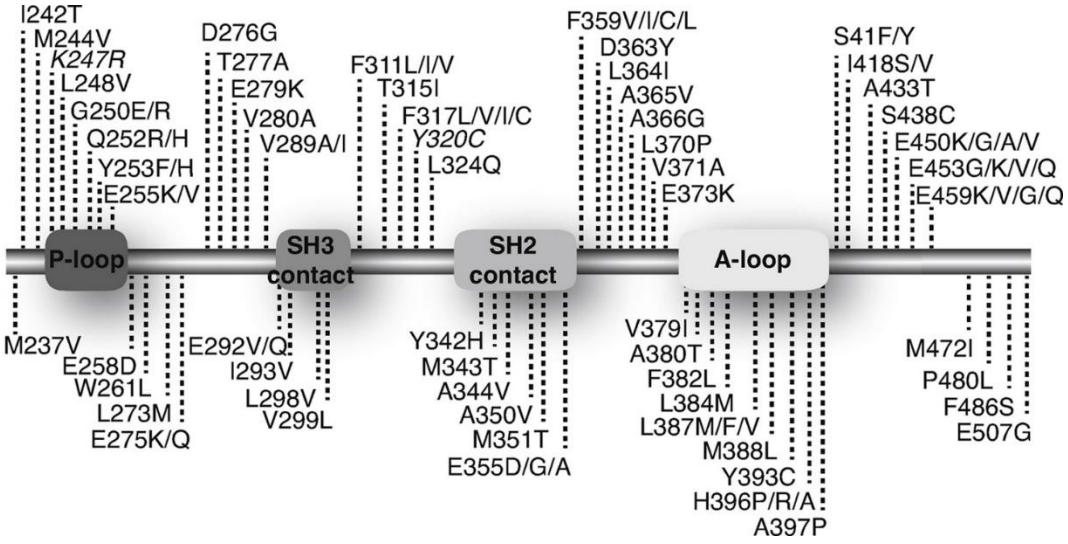


Figure 4. *Map of mutations in the BCR-ABL1 KD identified in clinical samples from patients resistant to Imatinib. Key structural motifs within the KD are indicated: P-loop indicates phosphate binding loop, SH2 contact and SH3 contact represent the contact regions with SH2 and SH3 domain-containing proteins, and A-loop indicates the activation loop. K247R and Y320C are in italics because they have been reported to be single-nucleotide polymorphisms. Numbering of residues is according to ABL1a isoform.[52]*

1.5.2 BCR-ABL1 independent resistance

In mutation-negative patients with CML, other resistance mechanisms have been investigated. Often, it is possible that more than one factor may cooperate to determine the resistance phenotype [52]. It is widely known that both in CML and in other malignancies the compensatory activation of intracellular signaling pathways may contribute to survival of Ph+ cells.

Regarding cell-extrinsic factors, several studies have shown the importance of BM microenvironment for LCSs survival, and thus for TKI resistance. The BM microenvironment consists of a heterogeneous population of cells that provide the structural and physiological support for hematopoietic cells. It contains BM stromal cells (such as extracellular matrix and mesenchymal-derived cells) and promotes self-renewal, quiescence, differentiation, survival, proliferation of hematopoietic cells. These cells are supported by fibroblast-like bone marrow stromal cells, osteoblasts, and osteoclasts which secrete soluble factors and extracellular matrix proteins that mediate these functions [57]. This rich environment provides as a safe haven not only for normal and malignant hematopoietic cells, but also for epithelial tumor cells that metastasize to bone, offering protection from chemotherapeutic agents by common mechanisms. Environment-mediated drug resistance includes a combination of soluble factor mediated drug resistance and cell adhesion mediated drug resistance.

Growth factors such as vascular endothelial growth factor (VEGF), transforming growth factor beta (TGFβ) and tumor necrosis factor alpha (TNFα) are required for the establishment of the metastatic microenvironment [58]. While BCR-ABL1 induces VEGF expression through a PI3K-mTOR dependent pathway [21], IL-6 supported myeloid differentiation in CML [59].

Signals derived from the BM stroma can effectively reconstitute the downstream signaling pathway of BCR-ABL1 protein, such that CML cells can achieve BCR-ABL1 independent growth in the BM, making them resistant to BCR-ABL1 TKI. Several studies have been investigated BM niche role in the modulation of TKI effects on CML cells, confirming stroma-mediated drug resistance mechanisms. Moreover, a growing number of evidence have demonstrated that numerous intracellular pathways are responsible of BCR-ABL1 independent resistance in CML cells. However, the nature of intrinsic resistance still needs to be clarified.

It is widely accepted that pharmacological mechanisms are involved in drug resistance bringing on a significant variability of Imatinib plasma level among, in CML patients treated with standard daily dose of 400 mg [60]. The increase of Imatinib plasma levels is due to the serum protein α1-acid glycoprotein (AGP) that is able to bind Imatinib in the plasma causing its intracellular concentration reduction [17]. The expression levels of MDR-1 gene is implicated in the resistance to various chemotherapeutic drugs. Both breast cancer resistance protein ABCG2 (BCRP) and ABCB1, a multidrug efflux pump, are correlated to the BCR-ABL1 resistance. Indeed, they regulate the intracellular uptake of Imatinib and are functionally overexpressed in CML stem cells of CML-BC patients, giving rise Imatinib resistance [61, 62]. A novel mechanism of acquired pharmacokinetic drug resistance in cancer patients that are chronically treated with Imatinib, involves a multidrug influx pump: the organic cation transporter 1 (OCT1). It mediates the active transport of Imatinib into the cells causing a decrease of the intracellular TKI. CML patients who have a suboptimal response to Imatinib have low OCT-1 activity, thus may predict for a less favorable molecular response. [63, 64]

Constitutive activation of Src kinases family is an example of BCR-ABL1 independent signaling. SFKs have been demonstrated to regulate cell proliferation and survival and they have also been implicated in the development of late-stage CML [65].

Last but not least, our group described a novel BCR-ABL1–independent mechanism of resistance to IMA therapy in patients with CP-CML. In the study was suggested the phosphatase SHP-1 role in CML transformation and progression. SHP1 seems to be physically associated with BCR-ABL1 [66] being able both to block BCR-ABL1–dependent transformation and to mediate PP2A-induced BCR-ABL1 proteasome degradation. It is expressed at a low level in an Imatinib resistant CML cell line and in CML-CP patients that did not achieve MMR at 18 months. SHP-1 interacting with SHP-2 regulates the activation status of this latter phosphatase in CML cells, thus Imatinib resistant cell line with a low SHP- 1 expression shows a sustained activated status of SHP-2 after Imatinib treatment [67]. TKIs failed to kill BCR-ABL1-expressing CML stem cells because these cells are not addicted to BCR-ABL1 oncoprotein for their survival. Quiescent CML stem cells account for approximately 0.5% of the CD34+ population and are characterized by the aberrant activation of pro-survival and self-renewal pathways regulated by cell-intrinsic and cell-extrinsic factors. It has been shown that BCR-ABL1 is overexpressed in primitive CML cells. Despite Imatinib resistant stem cells CD34+CD38– carried a single copy of BCR-ABL1, they expressed significantly higher BCR-ABL1 transcript. Moreover, in the CML precursor cells there is the upregulation of CXCR4 (cell surface adhesion molecule), an important molecule for stromal interaction regulating cell homing to the BM microenvironment [68].

In light of the fact that a small subpopulation of quiescent CML cells exhibit an intrinsic resistance, the deep eradication of Ph+ cells may be precluded and then, the final cure of the disease too.[69]

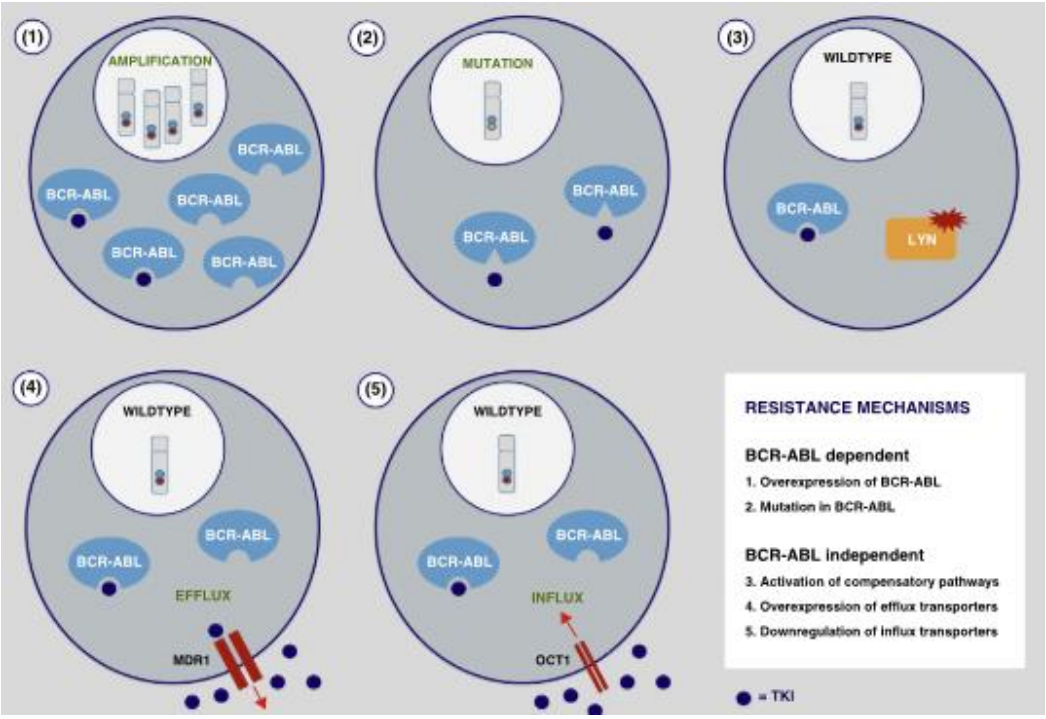


Figure 5. *Schematic representation of BCR-ABL1-dependent and -independent mechanisms of TKI resistance*: *(1) Amplification leads to the overexpression of the BCR-ABL1 kinase. (2) mutations in BCR-ABL1 lead to a conformation change in ABL and the ineffective binding of the TKI. (3) activation of other compensatory pathways (e.g. LYN). (4) overexpression of efflux transporters leads to low TKI levels in the cell. (5) downregulation of influx transporter inhibits effective TKI shuttling in the cell.*

1.5.3 The contribution of Leukemic stem cells (LSCs) in drug resistance

In the contest of the BCR-ABL1 independent mechanisms, many studies are needed to understand the upstream mechanisms that make Leukemic stem cells insensitive to TKIs therapy. Consolidated evidence from stem cell biology have been provided the relationship between stem cells and tumor cells formalizing the notion that some tumors are composed of Cancer stem cells (CSCs): a cells subset with both self-renewal proprieties and propagation potentials that sustain tumor growth and remain in patients after convention cancer therapy.

Most studies have highlighted that many pathways classically associated with normal stem cell development also regulate cancer progression. While, for most cancers, the target cell of the transformation events is unknown, some types of leukemia derive from typical mutations that accumulate in hematopoietic stem cells HSCs, a subpopulation of cancer cells within the tumor with common phenotypic properties. However, it is unclear whether there is a predisposing event to the acquisition of known mutations in leukemia. Leukemia are blood cancers, and the hematopoietic system is one of the best tissues to study the notion of the cancer or LSCs. Since the 1970s, the concept of tumorigenic LSCs has emerged and the small subset of leukemic cells was well characterized as capable of extensive proliferation *in vitro* and *in vivo*.

In hematopoietic system, HSCs can be divided into a long-term subset (LT-HSC), capable of undefined self-renewal, and a short-term subset (ST-HSC) that self-renew for a well-defined interval. HSCs give rise to non-self-renewing oligolineage progenitors, which in turn lead to a progeny with a more restricted differentiation potential, and finally to mature cells.

Since normal stem cells and LSCs share the ability to self-renew, as well as various developmental pathways, it is possible that LSCs derived from HSCs that have accumulated mutations.

In CML it is well known the pathogenesis hallmark and the life expectancy of patients has improved significantly with target therapy, but remains unclear why many of them resistant or relapse after stopping treatment. Therefore, many studies have focused their attention on LSC.

The BCR-ABL1 fusion protein can be found in myeloid, erythroid, B lymphoid, and occasionally T lymphoid cells in the majority of CML patients, suggesting that the original translocation takes place in LT-HSCs. However, as the BCR-ABL1 gene was detected in endothelial cells of CML patients [70], it has been suggested that the BCR-ABL1 fusion gene may be present in an earlier stage such as putative hemangioblast cells, a very primitive cell population with both hematopoietic and endothelial differentiation potential. Nevertheless it has yet to be clarified [71]. A very interesting study has shown that primitive non cycling BCR-ABL1 positive cells escape the cytotoxic effects of Imatinib suggesting that the mechanisms governing induction of cycling of CML stem cells are complex and at least in part, independent of BCR-ABL1 signaling [72, 73]. To confirm this, recent studies have shown that induced pluripotent stem cells (iPSCs) derived from CD34+ blood cells isolated from CML patients (CML-iPSCs) resisted to TKI treatment, whereas hematopoietic progenitors obtained from iPSCs partially recovered TKI sensitivity, after induction of hematopoietic differentiation. Thus, their survival did not depend on BCR-ABL11 [74].

Tumor cell population with stem cell-like properties often express pluripotency related gene such as Nanog, octamer-binding transcription factor 4/3 (Oct4/3) and sex-determining region Y HMG-box 2 (Sox2) which are essential

transcription factors in embryonic stem cells (ESCs). These transcription factors are involved in various somatic cancers and drive tumor development [75]. Indeed, a particular feature of CSCs has been described in Lucena cell line resistant to chemotherapy (MDR) derived from the parental Ph+ cell line K562. These cells expressed the phenotypic profile CD34+ CD38- that is the hallmark of the early stage hematopoietic stem cells [76]. Furthermore, high levels of stem cell markers Sox2, Oct4 and Nanog have been observed in another MDR cell line selected with doxorubicin compared to the parental K562 cells [77]. These data indicate that the stem cell markers contribute to the high malignant potential of LSCs and may be responsible for drug resistance in CML patients too.

1.6 Homeobox protein Nanog

The Nanog gene is a member of the homeobox family of DNA binding transcription factors.

The name Nanog derives from Tır nan Og, the mythical Celtic land of youth and was first time identified in 2003 in a screen for pluripotency promoting genes in mouse ES cells [78, 79]. Nanog is known as a master transcription factor essential for maintaining cell stemness but the precise mechanism involved in the regulation of stem cell self-renewal and pluripotency are still poorly understood. Many studies have shown that Nanog with Oct4 and Sox2 is involved in the maintenance of pluripotency and self-renewal in undifferentiated ES cells [80]. Interestingly, recent evidence has revealed that Nanog is also one of key transcription factors that could reprogram a human somatic fibroblast into an embryonic stem cell-like pluripotent cell, termed inducible pluripotent stem cell (iPS) [81].

The human Nanog gene is located on chromosome 12 at 12p13.31 spans approximately 7 kb and consists of four exons [82]. In this region, Nanog gene can undergo tandem duplication, which generates two copies (97% identical), but their transcripts are often differentially spliced.

The second copy, known as Nanogp1 or Nanog2, is a pseudogene and has regions with high homology to Nanog introns and exons. To date are known 11 Nanog pseudogenes (NanogP2-NanogP11) located on different chromosome. They are the results of mRNA retrotransposition characterized by the absence of introns 5’ promoters sequences [83]. Nanog is a 305 amino acids protein with three functional domains: the N-terminal domain, which contains 94 amino acids (amino acid 1-95); the homeobox domain (amino acid

96–155), which contains 60 amino acids; the C-terminal domain with 151 amino acids (amino acid 156–305). The N-terminal is rich in serine, threonine, and proline, providing a structural motif for the transcriptional activity of Nanog1. This region is tightly regulated through phosphorylation or other post-translational modifications. The C-terminal region contains two potent transactivation subdomains [84, 85]. The homeobox domain, in the central region, contains a DNA-binding motif; its N- and C-terminal regions are shown to contain nuclear localization sequences and its middle region is reported to harbor potent nuclear export motif, allowing the Nanog1 protein to transport in and out of the nucleus [86]. Nanog1 and Nanog2 are expressed in pluripotent stem cells and have a length of 232 amino acids, then pseudogene NanogP7 and P8

The isoform P2, P4, P5, P9 and P10 of Nanog protein are truncated proteins due to premature stop codon, while Nanogp7 and P8 do not contain stop codons and are able to encode full-length proteins. In particular, NanogP8 encodes a 305 amino acids protein that differs from the Nanog1 gene by only three amino acids. Since Nanog is also found in derivative ES cells and in the developing germ line of mammals, it is essential for early embryonic development [87]. Nanog has been shown to maintain the pluripotency of ES cells even in the absence of the LIF/Stat3 pathway.

Leukemia inhibitory factor (LIF) is a member of the IL-6 cytokine family and it is responsible for maintaining the cells self-renewal in ES.

The presence of LIF leads to the activation of the JAK and Stat signaling, in particular Stat3 activation is sufficient to prevent ES cells differentiation in the presence of serum [88]. In the absence of LIF, Oct3/4 is unable to prevent the ES cells differentiation into the trophoectoderm lineage and elevating Oct3/4

levels does not rescue pluripotent ES cells from reverting back to a differentiated state.

Nanog overexpression is sufficient: 1) to drive cytokine-independent self-renewal of undifferentiated ES cells; 2) to avoid the need for LIF/Stat3 expression to block ES cells differentiation into the primitive endoderm; 3) to support ESC self-renewal. These evidences suggest that Nanog acts orchestrating the molecular switch to a purely undifferentiated state [87]. Although the mechanisms through which Nanog regulates stem cell pluripotency are still unclear, it has been proposed that Nanog regulates pluripotency mainly with two mechanisms. It acts as a transcription repressor for downstream genes that are important for cell differentiation, such as Gata4 and Gata6. On the other hand, it promotes the activation of positive self-renewal genes, such as Rex1 and Oct4. Recent study has been reported that Nanog may be regulated by Stat3 and interacts with Wnt and BMP4 signaling pathways, too malignancy [88]. Suzuki A and colleagues demonstrated that phosphorylated Stat3 can bind-the promoter region of Nanog and activates its transcription. On the other hand, Bourguignon reported that Nanog forms a complex with Stat3 in the nucleus leading to Stat3 specific transcriptional activation and multidrug transporter, MDR1 (P-glycoprotein) gene expression, which are associated with cell proliferation [89]. Several findings suggest that Stat3-Nanog interaction plays an important role in cancer.

1.6.1 The role of Nanog in malignant phenotype of cancer stem cells

Functional studies suggested the role of Nanog in malignant disease, with implications in cancer prognosis and anticancer therapeutics. Therefore, its expression correlates with several oncogenic signal transduction pathways involved in cell proliferation, clonogenic growth, tumorigenicity, invasiveness, and therapeutic resistance. CSCs and ESCs are characterized by a very similar proprieties, such as fast proliferation and poor differentiation state. As well as in ESC also in CSCs, Nanog appears to function as a vital transcription factor of cell cycle progression through the positively regulation of CDK6 and CDC25A genes [90]. On the other hand, Nanog negatively regulates Bcl-2 expression, suggesting that it could be involved in drug resistance of CSCs by blocking the induction of apoptosis [91]. Generally, Nanog mRna is not observed in the stem cells of adult organism, but is heterogeneously expressed in both the nucleus and cytoplasm of several type of human cancer: such as embryonic carcinoma breast cancer, glioma, retinoblastoma, colon-rectal and ovarian cancer, prostate cancer and hepatocellular carcinoma [92]. These observations suggest the functional role of Nanog in tumor development, disease progression and Epithelial Mesenchymal Transition (EMT) [86].

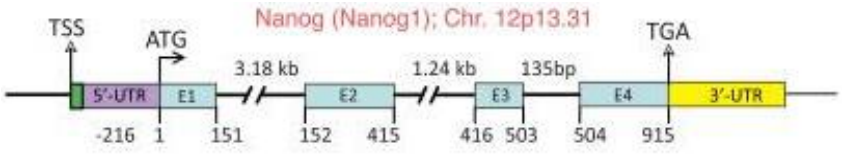
Furthermore, many studies have shown that Nanog overexpression correlates with a poor prognosis of patients with several malignancies. The Nanog expression promotes tumor cell growth, anti-apoptosis proprieties and metastasis in Nasopharyngeal Carcinoma (NPC) as well as in Human Hepatocellular Carcinoma (HCC) cancer cells. Due to the Nanog expression, HCC cells exhibit a high capacity to metastasize showing a chemotherapy resistance to sorafenib and cisplatin too [93]. Ovarian cancer is the most lethal

in all gynecological malignancies and high levels of Nanog mRNA and Nanog protein were observed in ovarian cancer cells. They expressed resistance properties such as sphere-forming and tumor regeneration ability and chemotherapy resistance [94]. It has been shown that the expression of Nanog correlates with drug resistance to cisplatin in oral squamous carcinoma (OSCC) cells [88]. Oct4 and Nanog expression may be a key factor in the resistance to chemotherapy and tumor growth of breast CSCs. Thus, down regulation of Oct4 or Nanog expression may reduce chemotherapeutic drug resistance and tumorigenicity in breast CSCs [95]. Furthermore, it was also observed that the Nanog and Oct4 expression was significantly correlated with larger tumor sizes and vascular invasion, likewise the median recurrence-free survival (RFS) was significantly shorter than that of patients with Nanog-negative tumors. Furthermore, Nanog expression levels correlate with stage and prognosis of cervical cancer in patients, suggesting that Nanog may support the development and progression of cervical cancer. It facilitates immune evasion capabilities among CSCs through T cell leukemia/lymphoma 1A/Akt (Tcl1a/Akt): a signaling axis potentially conserved in some of other cancer types [96].

A correlation with poor prognosis was also described for Nanog in leukemia field: in mixed lymphocytic leukemia (MLL) Nanog2 is involved in regulating leukemic stem cell functions [97]. Instead, in acute T cell lymphoblastic leukemia (T-ALL) NanogP8 is associated with gain of proliferation, increased self-renewal, and reduced apoptosis via blocking cell cycle progression through p53 [98]. These findings demonstrated that Nanog is a pro-tumorigenic factor that may assist in the clinic as a biomarker for cancer diagnosis, prognosis and predictor of anticancer therapeutic efficacy.

In the past, our group has carried out microarray experiments on gene groups selected through the bioinformatics algorithm of “Di Bernardo” team. The gene expression of Nanog was significantly increased in a Ph+ cell lines resistant to Imatinib. These results have a particular interest in context of TKI resistance because it was observed that the expressions of Oct4, Sox2, and Nanog, were elevated in a Ph+ doxorubicin resistant cell lines compared to parental cell lines. This resistant cell line exhibited more potent *in vitro* and *in vivo* tumor-initiating properties, as revealed by sphere assay, self-renewal assay, soft agar assay, and animal studies [77]. Thanks to consolidate experience of our research group in the study of resistance mechanism of CML, it will be interesting to assess whether Nanog has a role in the TKI resistance observed in patients with CML-CP.

A



B

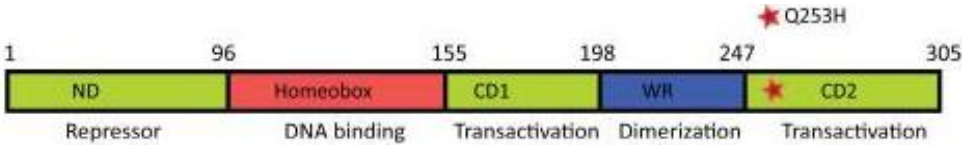


Figure 6. *Graphical illustration of the NANOG gene sequence (A) and structure of Nanog protein (B). NANOG mRNA variants NANOG-001 and -002 encode for a protein with a length of 305 amino acid (aa) and 289 aa, respectively. NANOG-001, the 305 aa long protein with a molecular weight of 34.6 kDa is usually analyzed to study the role of NANOG (B). It consists of a Serine-, Threonine- and Proline-rich N-terminal region as well as eight W-repeats at its C-terminus (aa 104-151). The DNA-binding facilitating homeodomain spans from aa 95-155. Formation of secondary structures (helix, strand and turn) occurs mainly within the homeobox-coding region.[99]*

2. AIMS

2. Aims of work

The introduction of BCR-ABL1 tyrosine kinase inhibitors has revolutionized the therapy of CML patients; indeed, nowadays, patients diagnosed with CML and treated with TKIs are expected to have a substantially longer survival.

However, despite the success of target therapy, the majority of CML patients develop resistance to TKI therapy and, in particular, to first generation TKI Imatinib, which still represents the first-line therapy of CML.

If on one hand, BCR-ABL1 dependent Imatinib resistance can be overcome by second or third generation TKIs, on the other hand, the molecular mechanisms that underlie BCR-ABL1 independent Imatinib resistance are not well characterized.

Different studies have demonstrated the role of Nanog in promoting tumorigenesis and chemoresistance through the regulation of cancer stem cells in solid tumors; indeed, elevated Nanog levels correlate with a poor disease-free and overall survival in patients with breast, prostatic, ovarian, gastric, lung or hepatocellular carcinoma.

Nanog is transcriptional factor and a stem cell marker required for maintaining pluripotency of embryonic stem cells and preventing cell differentiation. These findings highlight that it is certainly interesting to investigate also the potential role of Nanog in controlling leukemic stem cells (LSC) population, in drug resistance and poor prognosis of CML patients.

The aim of this work is to evaluate the involvement of Nanog in the BCR-ABL1 independent TKI resistance of leukemic cell line. Finally, we will corroborate our in vitro results on CML patients in order to understand whether Nanog may

be considered as an early marker of molecular response in CML-patients treated with first and second generation TKIs.

3. RESULTS

3.1 Nanog protein expression is modulate in K625 cell line after Imatinib exposure.

To determinate whether pluripotent stem cell marker Nanog may have a potential role on resistance to TKI treatment on BCR-ABL1 positive cell lines, we first examined the expression of Nanog in Ph+ K562 cells, derived from a patient affected by CML-BP.

In particular, we treated K562 cell line with a clinical relevant concentration of Imatinib (5uM Ima), and analyzed the expression level of Nanog after 72hrs. Densitometric quantification showed that the band at 42 kDa of Nanog was significantly more intense in K562 treated with 5uM Ima than in untreated K562 (i.e. K562 cell line cultured in the presence of regular media – RM – and mock drug vehicle represented by PBS).

Data were shown in a single exemplificative experiment (**Fig. 7, Panel A**), or as average of the ratio observed between Nanog and β-Actin housekeeping protein levels in three independent experiments (**Fig. 7, Panel B**).

In order to understand whether Nanog modulation was related to the time of Imatinib exposure, we performed a time course experiment in which K562 cells were treated with 5uM Ima for 24, 48 or 72 total hours. Nanog protein expression is significantly increased (p=0.05) already after 24 hours of treatment (**Fig. 8**), but also during the considered time course (72hrs).

Data were shown in a single exemplificative time course experiment (**Fig. 8 – Panel A),** or as average of the ratio observed between Nanog and β-Actin housekeeping protein levels in three independent experiments (**Fig. 8– Panel B**).

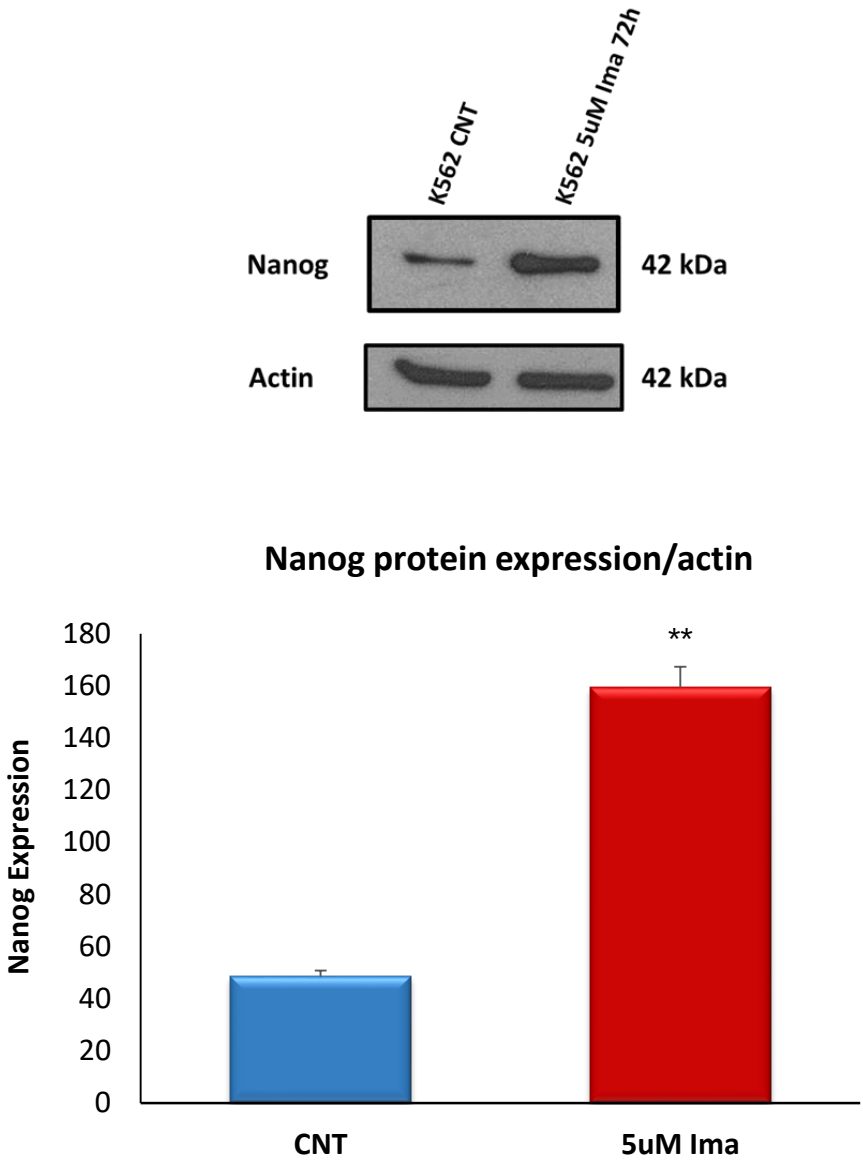


***Figure 7**. Western blotting analysis of Nanog protein expression in K562 cell line treated with 5uM Imatinib, compared to β-Actin housekeeping protein. Nanog protein expression is shown in a single exemplificative experiment (Panel A), or as average of the ratio observed between Nanog and β-Actin housekeeping protein levels in three independent experiments (Panel B).*

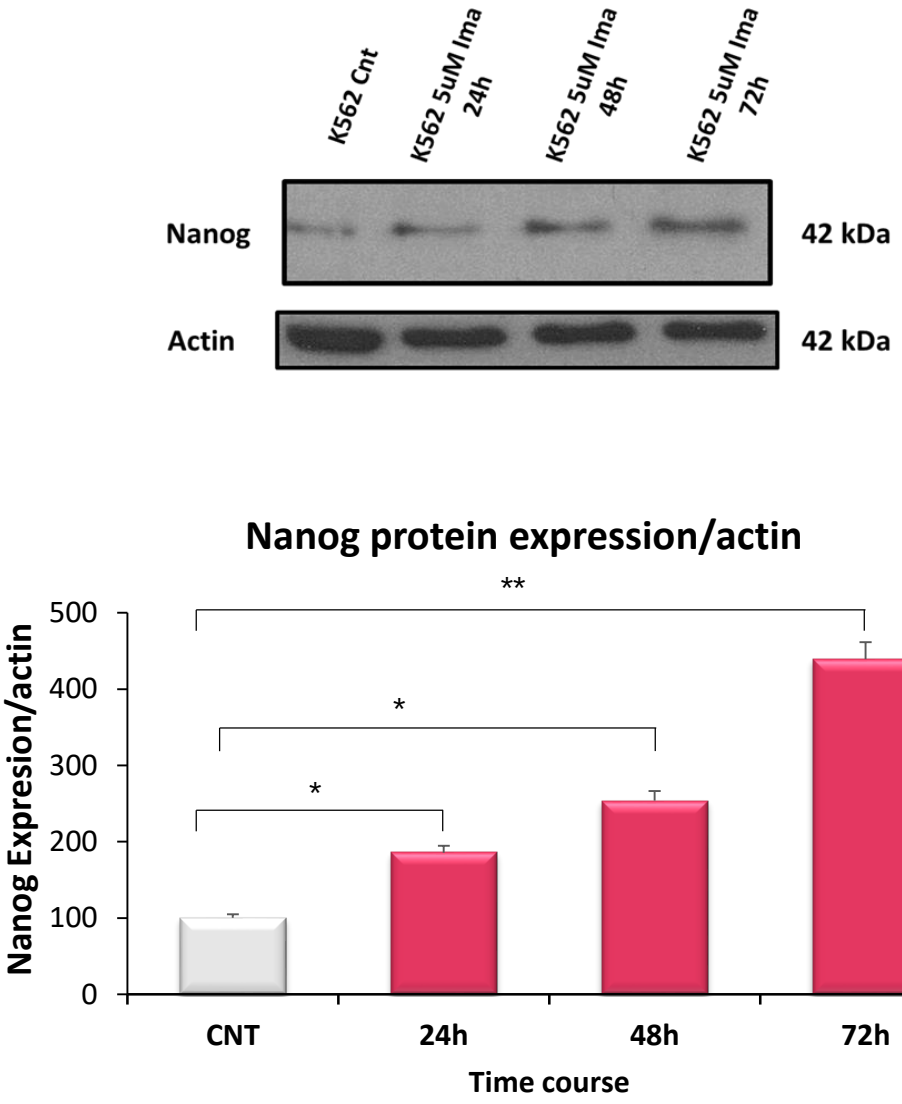
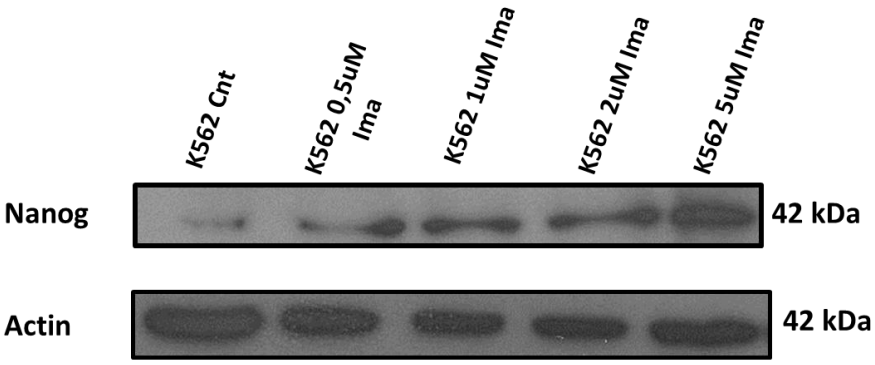


Figure 8. *Western blotting analysis of Nanog protein expression in K562 cell line treated for 24, 48 and 72 hours with 5uM Imatinib, compared to β-Actin housekeeping protein. Nanog protein expression is shown in a single exemplificative experiment (Panel A), or as average of the ratio observed between Nanog and β-Actin housekeeping protein levels in three independent experiments (Panel B).*

3.2 Nanog protein expression increased in a dose dependent manner after Imatinib treatment

To evaluate whether Nanog expression is modulated by Imatinib in a dose related manner, we exposed K562 cells to different Imatinib concentrations (0.5 uM, 1uM, 2uM and 5uM Ima) for 24h. Although Nanog protein is detectable at low level by Western blotting (WB) in untreated K562 cells (Cnt), densitometric band quantification showed a significant up-regulation of the protein expression even by the lowest Ima concentration of 1uM after 24h of treatment.

Increasing the Imatinib concentration to 5 uM, we observed a significant up-regulation of Nanog protein ($p=0.022$). Data were shown in a single exemplificative experiment (**Fig. 9**, **Panel A**), or as average of the ratio observed between Nanog and β-Actin housekeeping protein levels in three independent experiments (**Fig.9 – Panel B**).



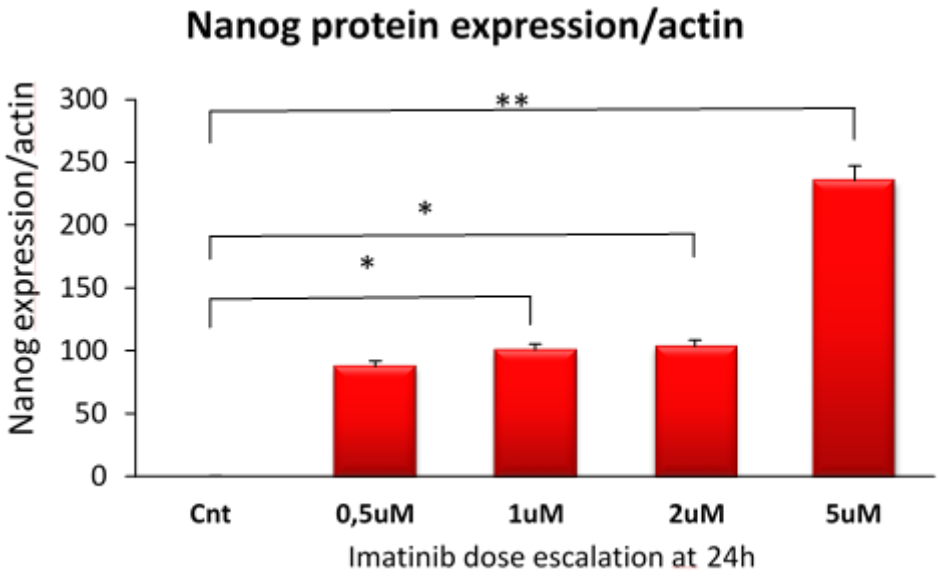


Figure 9. *Western blotting analysis of Nanog protein expression in K562 cell line treated for 24 hours with a dose escalation of 0.5 uM, 1uM, 2uM and 5uM Imatinib, compared to β-Actin housekeeping protein. K562 cells treated with 1-5uM Ima dose escalation show a significant dose dependent increase of Nanog protein expression at 24h. Nanog protein expression is shown in a single exemplificative experiment (Panel A), or as average of the ratio observed between Nanog and β-Actin housekeeping protein levels in three independent experiments (Panel B).*

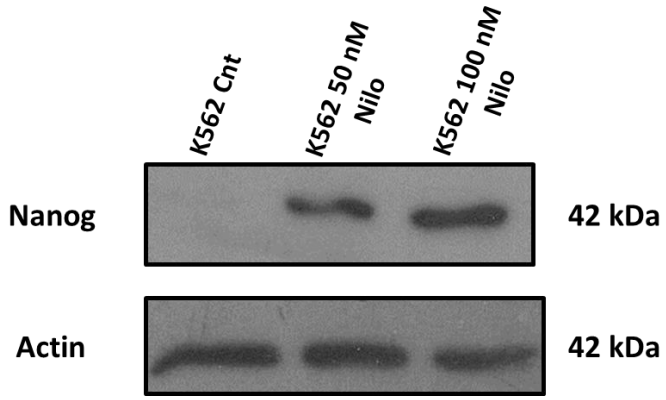
3.3 Second generation TKI induce increasing expression of Nanog protein.

In order to prove that the modulation of Nanog by TKI treatment were independent from first generation TKI, we treated K562 cells with second generation TKI Nilotinib (Nilo), that is not sharing with Imatinib any off target protein signaling down-regulation. In particular, we treated K562 Ph+ cell line with 50nM, 100nM of Nilotinib.

The data of WB densitometric band quantification showed that Nanog protein levels increase in K562 cell line when treated with either 50nM or 100nM of Nilotinib, but only after 72 hours (p value < 0.05). Data were presented in a single exemplificative experiment (**Fig. 10 – Panel A**), or as average of the ratio observed between Nanog and β-Actin housekeeping protein levels in three independent experiments (**Fig.10 – Panel B**).

These finding supports the idea that Nanog expression may related to BCR-ABL1 inhibition in CML Ph+ cell line when exposed to first and second generation TKI used in clinical practice.

Panel A



Panel B

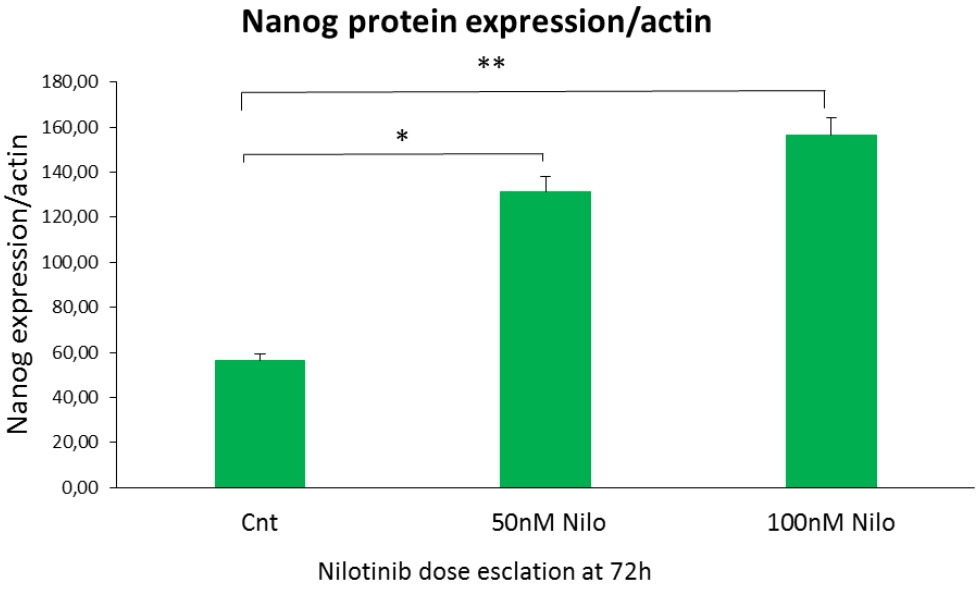


Figure 10. *Western blotting analysis of Nanog protein expression in K562 cell line treated for 72 hours with a dose escalation of 50nM or 100nM Nilotinib, compared to β-Actin housekeeping protein. K562 cells treated for 72h with 50nM and 100nM Nilo show a significant dose dependent increase of Nanog protein expression. Nanog protein expression is shown in a single exemplificative experiment (Panel A), or as average of the ratio observed between Nanog and β-Actin housekeeping protein levels in three independent experiments (Panel B).*

3.4 Nanog expression is modulated at a transcriptional level in K562 cell line after exposure to first and second generation TKIs.

In our study, we attempted to evaluate whether exist any correlation between Nanog expression at protein or mRNA level after TKIs treatment in Ph+ K562 cell line.

Thus, RT-qPCR for Nanog mRNA expression was carried-out on K562 cell line treated with increasing doses of Imatinib (0.1uM, 0.5uM, 1uM, 2uM and 5uM) or Nilotinib (5nM, 10nM, 50nM, 100nM and 500nM). Total mRNA was isolated from K562 cell line at 24, 48 and 72 hours after TKI treatment.

As shown in Figure 11, Nanog mRNA expression was significantly increased in K562 cell line treated with Imatinib during the time course of 72 hours. Moreover, we confirmed that Nanog expression is also modulated in a dose dependent manner at the transcriptional level.

Furthermore, the evaluation of Nanog mRNA in K562 cells treated with Nilotinib, show to be a more sensitive test than WB, since able to highlight a significant modulation of the transcript during the time course and also at the very low concentration of 10nM of Nilotinib (**Fig. 12**).

Moreover, we demonstrated that Nanog mRNA expression is inversely correlated to BCR-ABL1 mRNA expression. Indeed, RT-qPCR was conducted to analyze p210 mRNA expression in K562 cell line after 5uM Ima exposure.

As we expected, p210 mRNA levels were significantly reduced after Ima treatment, while Nanog mRNA expression significantly increased (**Fig.13**).

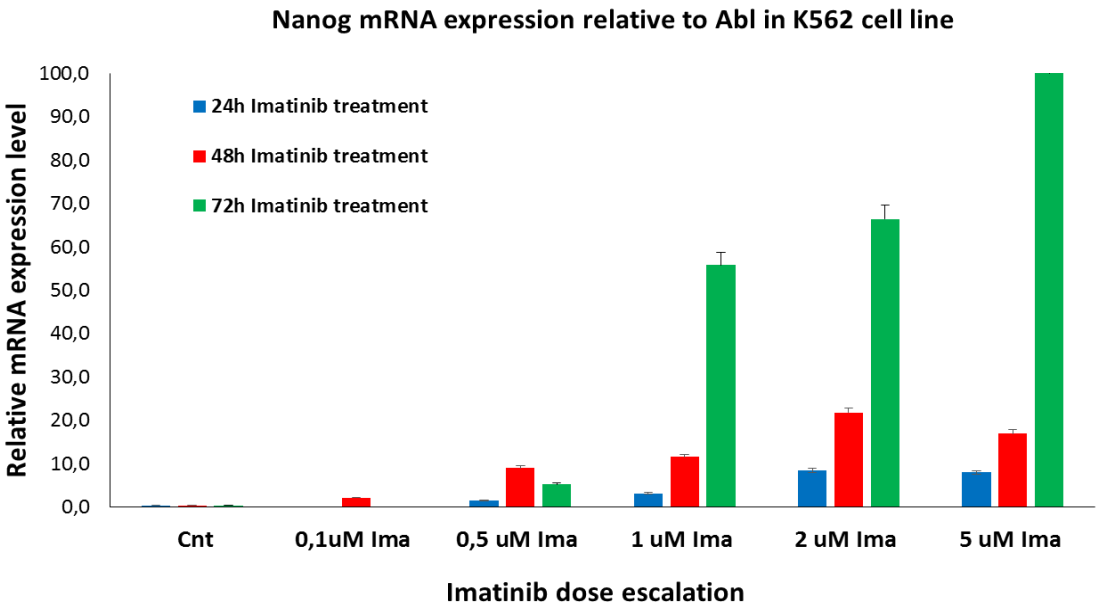


Figure 11. *Gene expression analysis by RT-qPCR of Nanog mRNA expression in K562 cells treated with 0.1uM, 0.5uM, 1uM, 2uM or 5uM Imatinib dose escalation for 24, 48 and 72 hours, normalized by ABL control gene. Nanog mRNA expression shows a dose dependent increase in K562 cells treated with increasing doses of Ima; the increase is particularly significant at 72 h.*

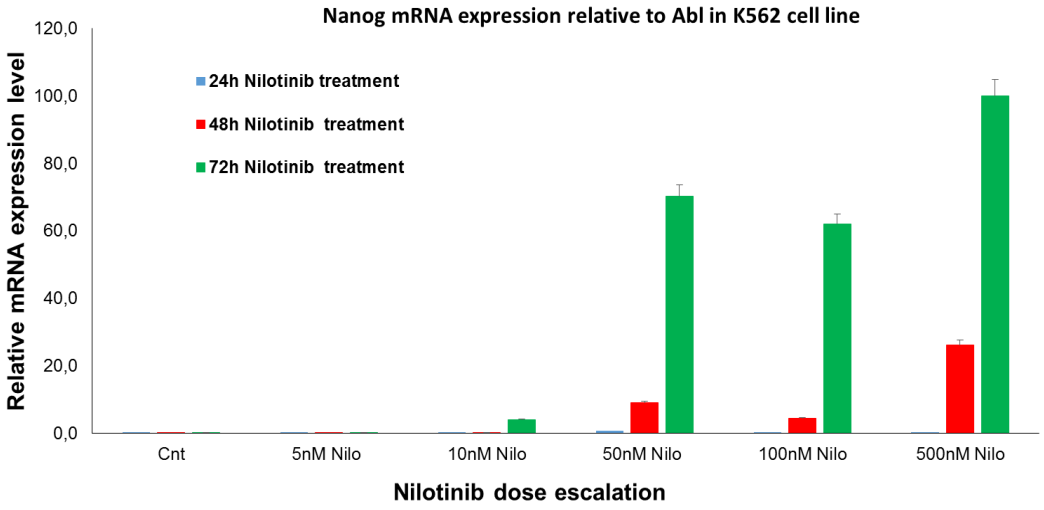


Figure 12. *Gene expression analysis by RT-qPCR of Nanog mRNA expression in K562 cells treated with 5nM, 10nM, 50nM, 100nM or 500nM Nilotinib dose escalation for 24, 48 and 72 hours, normalized by ABL control gene. Nanog mRNA expression shows a dose dependent increase in K562 cells treated with increasing doses of Nilo; the increase is particularly significant at 72 h.*

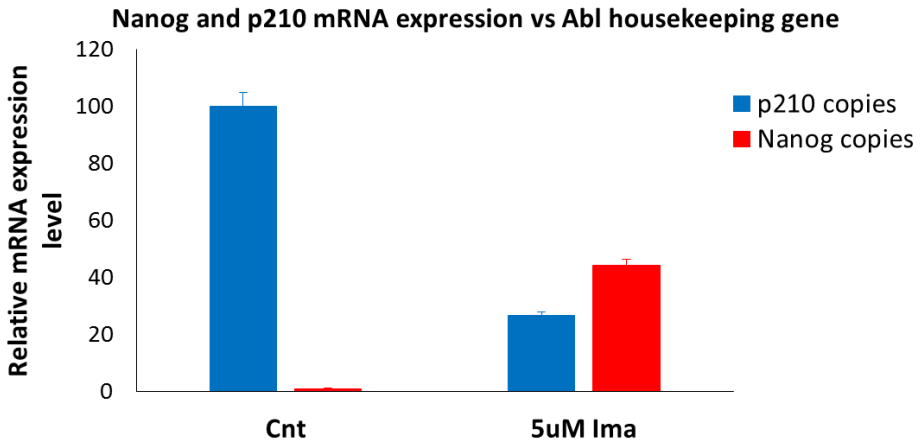


***Figure 13**. Gene expression analysis by RT-qPCR of Nanog mRNA and p210 mRNA expression in K562 cells treated with 5uM Imatinib, normalized by ABL control gene. p210 mRNA levels (blue) were significantly reduced in K562 treated with Ima, compared to control; instead, Nanog mRNA expression (red) is significantly increased in K562 treated with Ima, compared to control.*

3.5 K562 cells survived after TKI exposure express high levels of Nanog protein.

It has been reported that Ph+ staminal cells are not sensible to TKI-induced apoptosis.

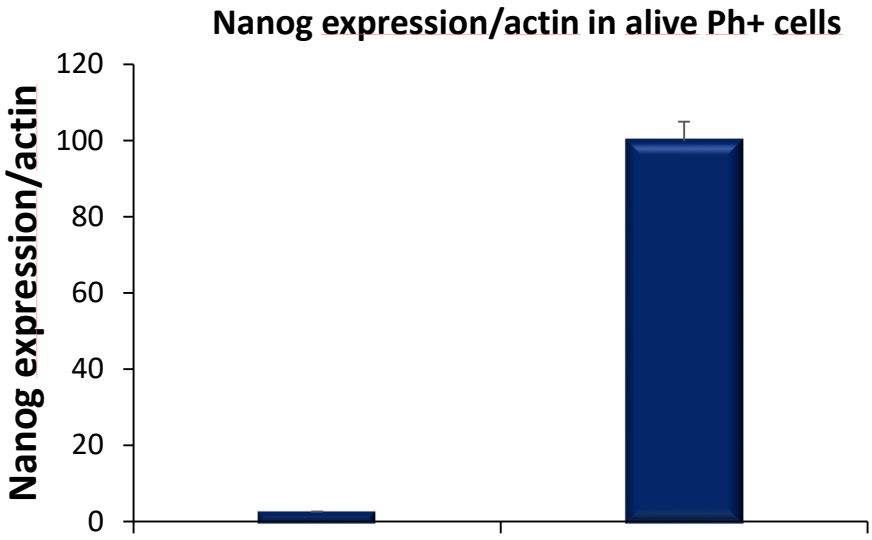
In particular several studies have shown that the iPS Ph+ cells are refractory to the pharmacological action of TKIs, as a result their survival did not depend on BCR-ABL1.

Thus, we sought to evaluate the correlation between Nanog overexpression and cellular viability after TKI treatment. In particular, we exposed K562 cell line to 5uM Ima for 72 hours. We selected vital K562 cells through the elimination of no vital cells by Dead Cell Removal Kit (Miltenyi) and we confirmed cell viability by Trypan blu exclusion.

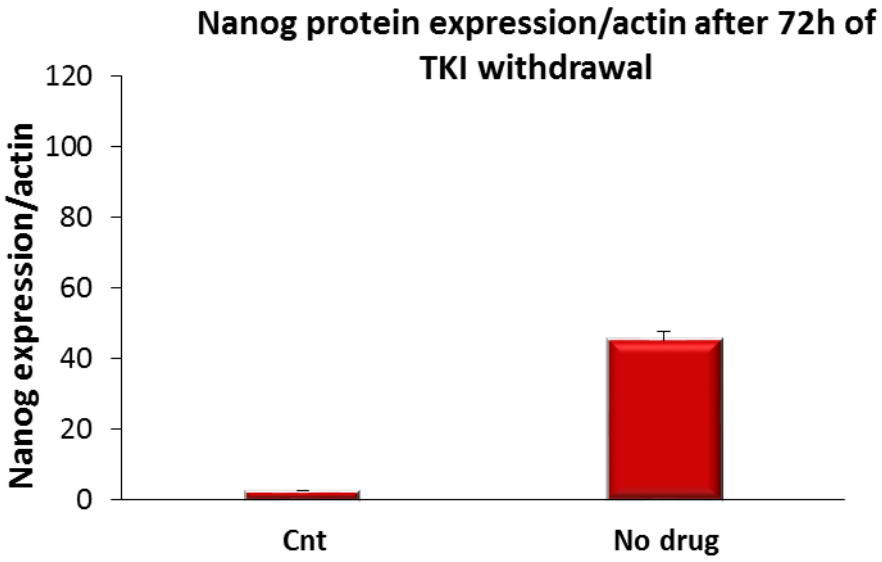
After the exclusion of the dead cells, we evaluated Nanog protein expression in the selected viable K562 cells survived after Imatinib treatment with densitometric band quantification analysis, showing that the Nanog overexpression is present in this cell fraction (**Fig**. **14 A, C**).

Moreover, we sought to evaluate whether this overexpression could persist overtime. Thus, the selected alive Ima-treated K562 cells were re-plated in a fresh medium without Imatinib for 72 hours. WB analysis show a significant persistence of up-regulation of Nanog protein expression (**Fig.14 B, C**).

Panel A



Panel B



Panel C

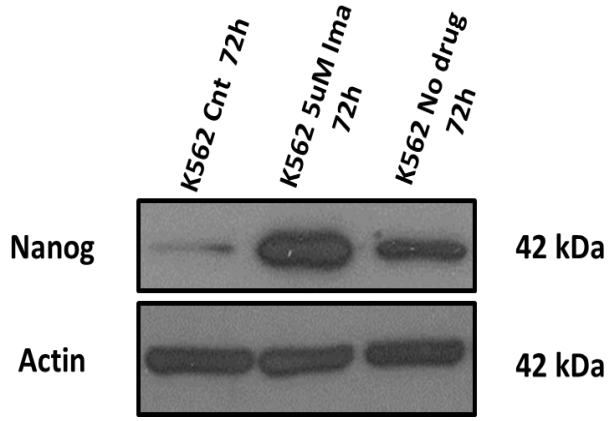


Figure 14. *Western blotting analysis of Nanog protein expression in K562 cells subjected to viability selection after 72 hours of 5uM Imatinib treatment, compared to β-Actin housekeeping protein. Nanog protein expression analysis in alive K562 cells after 72h of Ima treatment withdrawal.*

A,C) Nanog protein expression in viable selected K562 cells survived after 72h of Ima treatment, compared to untreated control; B,C) Nanog protein expression in viable selected K562 cells survived after 72h of Ima treatment, and re-plated in the absence of Imatinib.

3.6 Transcription levels of Nanog mRNA are up-regulated with warning/failure response in CML patients treated with Imatinib as front line therapy.

In order to evaluate whether Nanog expression could have a role in the regulation of CML patients response to TKIs therapy, we performed a RT-qPCR for the expression of Nanog mRNA in 60 CML patients at diagnosis enrolled in Hematological Clinical Unit of Federico II University.

First, we distributed the enrolled CML patients based on drug treatment; in particular 25 patients received Imatinib as *front-line* therapy, while 35 received Nilotinib as front-line therapy. Thereafter, we stratify CML patients in three distinct response categories based on the molecular BCR/ABL1 analysis performed at 12 months from the beginning of Imatinib and Nilotinib therapy: patients achieved optimal response (OR), patients with a warning response (WR) and patients with a failure response (FR) (Baccarani M et al, Ann Hematol 2016).

The Nanog expression was evaluated in cells of BM and peripheral blood (PB) samples of the enrolled CML patients at diagnosis.

We observed a significant correlation between patients outcome and the expression of Nanog in pre-treatment patient's samples. The Nanog gene expression was normalized by the expression of ABL1 mRNA (normalized copy number, NCN).

In PB samples at diagnosis of WR/FR patients treated with Imatinib, the normalized copy number of Nanog was 25.65 ± 18, whereas patients with OR showed 1.12 ± 0.36 (**Fig. 14**). Moreover, as shown in Figure 14 transcription levels of Nanog mRNA were also significantly increased in BM samples of patients treated with Imatinib.

The same analysis was conducted in a cohort of 35 CML patients at diagnosis treated with Nilotinib as first line therapy. Both the PB samples that BM sample of WR/FR patients showed a significantly higher expression of Nanog mRNA (NCN 3.941± 1.951 vs 0.13 ± 0.048, $p<0.05$).n

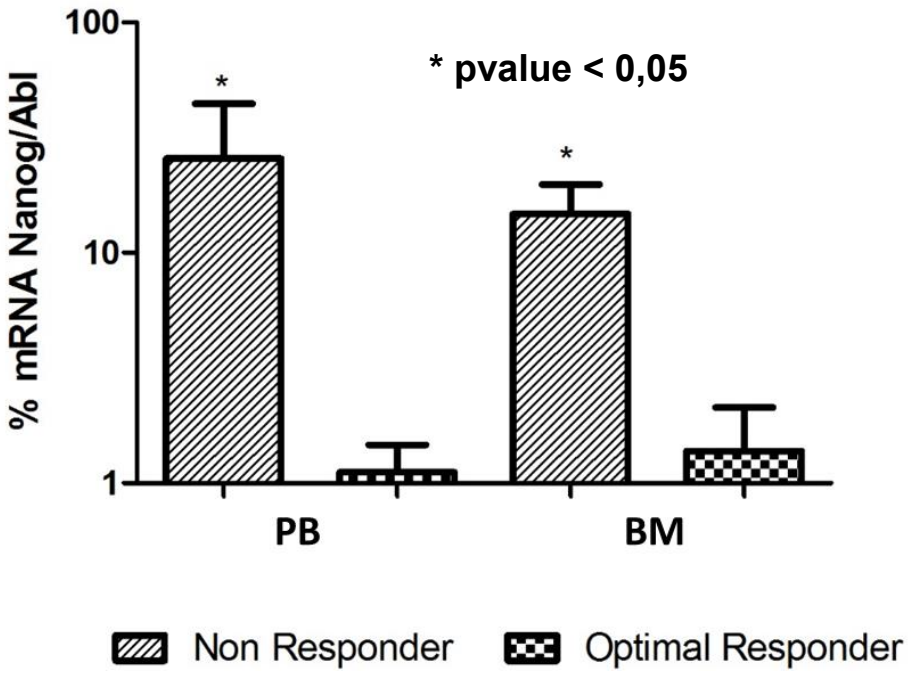


Figure 15. *Gene expression analysis by RT-qPCR of Nanog mRNA expression in cells from bone marrow (BM) and peripheral blood (PB) samples of 25 CML patients at diagnosis treated with Imatinib normalized to Abl control gene. Nanog mRNA expression is higher in warning/failure responders than in optimal responders both in PB (pvalue 0.05) and in BM (pvalue 0.03).*

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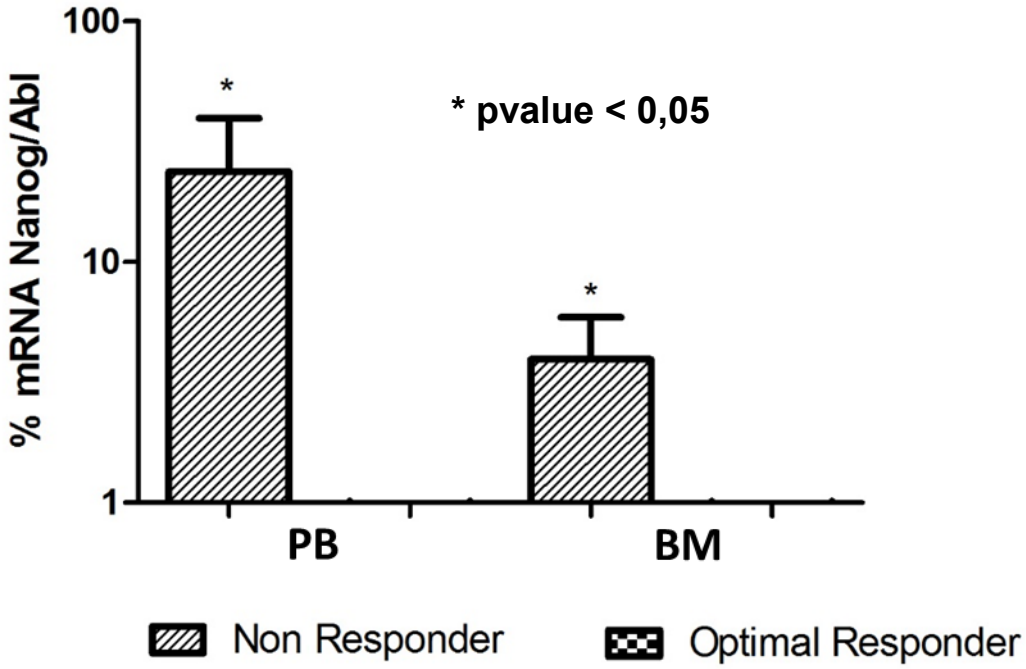

Figure 16. *Gene expression analysis by RT-qPCR of Nanog mRNA expression in cells from bone marrow (BM) and peripheral blood (PB) samples of 35 CML patients treated with Nilotinib, normalized to Abl control gene. Nanog mRNA expression is higher in warning/failure responders than in optimal responders both in PB (pvalue 0.05) and in BM (pvalue 0.03).*

4. DISCUSSION

4.1 Conclusion e future prospective.

The emergence of drug resistance continues to limit the success in finding a cure for hematologic malignancies. CML represents a disease that is initially driven by the well-established oncogenic event resulting in the expression of the BCR-ABL1 fusion oncogene. The onset of drug resistance in CML patients during tyrosine kinase inhibitors therapy leads to a failure in complete eradication of CML disease and, consequently, to the persistence of minimal residual disease, responsible of disease relapse.

To date, the most clear and common mechanisms of drug resistance, in particular to first generation TKI Imatinib, are those due to the acquisition of point mutations within the kinase domain of BCR-ABL1; these mutations have become druggable targets since the introduction of second and third generation TKIs. Otherwise, the molecular pathways activated in cases of resistance phenomena not dependent from BCR-ABL1 mutations are still not clearly described.

In this work, we evaluated the contribution of the transcription factor Nanog in BCR-ABL1 independent TKI resistance mechanisms.

Nanog is known as a transcription factor required for maintaining the pluripotency of embryonic stem cells, but several correlation studies have demonstrated that also CSCs express high levels of Nanog. Indeed, Nanog is overexpressed in malignant, high grade, poorly differentiated cancer cells. In particular, Nanog overexpression is found in many solid tumors, such as breast, ovarian, colorectal, gastric, lung, hepatocellular and prostate carcinoma as well as in head and neck squamous cell carcinoma. Moreover, Nanog expression is been detected not only in solid tumors, but also in a blood

tumor, the acute lymphoblastic leukemia (ALL), that is a disease associated with a very poor outcome [92]. Several studies have highlight an increased expression of stem pluripotent cells markers such as Oct4, Sox2 and Nanog, in Ph+ cell line that shown a drug resistance.[77]

Consequently, in this work we investigated the possible role of Nanog in TKIs resistance in CML.

Our results have demonstrated that Nanog protein and mRNA are expressed at very low level in K562 Ph+ cell line, but their up-regulation is observed in Ph+ cell line treated with increasing doses of Imatinib. This data underline for the first time that Nanog protein is up-regulated after inhibition of the oncogenic pathway of BCR-ABL1 in CML cells.

In addition, to corroborate our data, we also proved that the up-regulation of Nanog is not related to Imatinib treatment, but also to the BCR-ABL1 inhibition obtained through the treatment with a second TKI generation represented by Nilotinib.

Furthermore, we investigated whether Nanog mRNA correlates with the expression at the protein level. Thus, we optimized a RT-qPCR as a validated and standardized molecular test to evaluate Nanog expression in either cell line or primary human cells derived from BM or PB samples of patients with leukemia.

The expression of Nanog mRNA in CML cell line increases overtime after Imatinib or Nilotinib treatment, in a dose-dependent fashion, corroborating our results obtained at the protein level.

Moreover, in order to investigate a possible correlation between Nanog expression and BCR-ABL1 oncogene, we conducted a gene expression analysis on CML cell line treated with Imatinib to evaluate both BCR-ABL1 and

Nanog transcript levels. The analysis has highlighted that Nanog expression inversely correlates with BCR-ABL1 expression. Indeed, after Imatinib treatment, BCR-ABL1 expression was reduced, while Nanog expression was significantly increased.

We could not exclude that Nanog could be a marker of apoptotic or dying cells in our experimental setting. Thus, we selected all the viable cells survived after Imatinib treatment showing that Nanog overexpression was a prerogative of this subpopulation. Moreover, keeping the cells in culture for an additional 72hrs in the absence of TKI, we also proved that the selected cells were real survived cells, capable of proliferation, and in which Nanog was still up-regulated respect to the control. Finally, we evaluated Nanog expression levels in a cohort of CML patients treated with first and second generation TKI. The patients have been enrolled in the Division of Hematology at University of Naples Federico II and was stratify in two experimental groups according to the treatment received. Nanog expression evaluated in hematopoietic cells in patients with warning or failure response to Imatinib therapy resulted up-regulated respect to that observed in optimal responder patients. The same analysis was carried out in the second group of 34 patients receiving Nilotinib. The Nanog expression was significantly unregulated both in peripheral blood and bone marrow samples warning/failure responders.

All these data strongly suggest that Nanog could have a role in Ph+ cells resistant to first or second generation TKI treatment. Its expression could be evaluated not only as a drug resistance marker in CML disease, but also as a prognostic and predictive biomarker at diagnosis for unfavorable patient outcome.

Our future perspectives are basically aimed to the development of in vitro functional studies in order to clarify Nanog function in CML disease, through gene expression assays that could allow us to determine the intracellular pathogenic pathways that regulate Nanog upstream and downstream signal transduction.

Recently, Songna Yin at al described that Nanog expression was down regulated by the action of phosphatase SHP1 in blastocyst cells. In particular, SHP-1 regulates the expression of Nanog by dephosphorylation of STAT3 [100].

Since, our group previously shown that SHP-1 expression is lower in BM or PB samples of patients affected by CML resistance to Imatinib than in patients with Optimal response.

In a future prospective, it will be interesting to assess whether Nanog has a role in the TKI resistance observed in patients with CML-CP and whether there is a relationship with modulation of SHP1.

In addition, we intend to extend the number of CML patients enrolled in our study and to evaluate Nanog expression at different follow-up time points during TKI in order to evaluate the possible role of Nanog as early prognostic marker of patient response in CML disease.

The emerging role of Nanog in CML will lead to a deeper understanding of molecular dynamics involving pathways related to TKI resistance. Moreover, Nanog could represent a novel and un-discovered druggable targets in order to reduce the emergence of TKI resistance.

5. Methods

5.1 Cell cultures

5.1.1. K562 cell line

K-562 human BCR-ABL1 positive cell line (DSMZ) were maintained in culture at a density of 0.5 x 10^6 cells/ml in RPMI 1640 medium (Aurogene) supplemented with 10% fetal bovine serum (FBS; Aurogene), 1% L-glutamine (L-Glu, Aurogene), and 1% penicillin/streptomycin (P/S, Lonza) at 37°C in 5% CO2 in a humidified incubator. Cell line viability was assessed by Trypan Blue exclusion using the Burker chamber.

5.2 Viable cell isolation

Dead Cell Removal Kit (Miltenyi) was used to separate dead cells from cell cultures. Briefly, K562 cells was collected and centrifuged at 1000 rpm for 10 min; the supernatant was completely removed and cell pellet was resupended in 100 µL of Dead Cell Removal MicroBeads. After incubation of 15 minutes at room temperature, a positive selection column type MS has been chosen and placed in the magnetic field of a MACS® Separator. The column has prepared by rinsing with 1X Binding Buffer. The cell suspension was applied in 500 µL of 1X Binding Buffer into the column, so that the negative cells passed through. Live cell fraction was collected and rinsed with 1X Binding Buffer.

5.3 Drug and Reagents

Imatinib mesylate was supplied by Novartis Pharma. It was diluited in DMSO as a 10 mmol/L stock solution and stored in aliquots at −20°C.Nilotinib was supplied by Novartis Pharma. It was diluited in DMSO as a 10 mmol/L stock solution and stored in aliquots at −20°C. Dasatinib was supplied by Bristol-

Myers Squibb. It was diluited in DMSO as a 10 mmol/L stock solution and stored in aliquots at −20°C.

5.4 Western blotting analysis

To obtain total lysates for immunoblotting analysis, K562 cells were washed in PBS and collected by gentle scraping in ice-cold RIPA buffer supplemented with protease inhibitor cocktail II (Roche Diagnostic, Monza, Italy). After sonication and incubation for 1 hour on ice, we centrifuged at 12,000 rpm at 4 °C for 30 minutes and collected the supernatants.

The protein content of resulting supernatant was determined using the Bradford reagent. 30 µg of proteins were mixed with a Laemmli sample buffer; then, they are applied and resolved on SDS-PAGE 10% polyacrylamide gels. Following transfer onto PVDF membranes, non-specific binding sites were blocked by incubation for 2 hrs at 4°C with 5% non-fat dry milk (Bio-Rad Laboratories, Milan, Italy) in PBS-Tween buffer; subsequently, incubated with primary antibodies overnight at 4°C. After three 10-min washes with PBS-T, the membranes were incubated 1h with the appropriate secondary antibody. Excessive antibodies were then washed away three times (10 min) with PBS-T. Immunoblots were visualized by enhanced chemiluminescence (ECL) Western Blotting Substrate (Thermo Scientific). Films were developed using a standard photographic procedure and the relative levels of immunoreactivity were determined by densitometry using ImageJ Software (NIH, Bethesda, MA, USA).Primary antibodies used were: rabbit polyclonal anti-Nanog (1:1000 Cell Signaling), mouse polyclonal β-actin (1:15.0000 Sigma Aldrich) Secondary antibodies were: goat anti rabbit (1:10000 Santa Cruz Biotechnology), goat anti mouse (1:10000 Santa Cruz Biotechnology).

5.5 Real-time quantitative Reverse Transcription - Polymerase Chain Reaction (RT-qPCR)

K562 and CML patients cells total RNA was extracted using RNeasy Mini Kit (Qiagen), following the manufacturer's instructions, and quantized using Nanodrop 8000 (Thermo Scientific). Nanog mRNA expression was evaluated by One-step RT-qPCR kit (Life Technologies gene expression assay), starting from 1 µg of RNA and following the manufacturer's instructions; one-step RT-PCR kit combines the first-strand cDNA synthesis (reverse transcription) reaction and PCR reaction in the same tube, simplifying reaction setup and reducing the possibility of contamination. The reverse transcription reaction consists of 15 min at 48°C; the activation of AmpliTaq Gold® DNA Polymerase of 10 min at 95°C. The qPCR was performed using ABI PRISM 7900HT Sequence Detection System (Applied Biosystems); amplifications were carried out by 35 cycles of the following parameters: denaturation step at 95°C for 15 sec, annealing and extension steps at 60°C for 1 min. ABL was used as housekeeping control gene.

5.6 Statistical Analysis

All data are presented as mean ± 1 SD. The student's t test was used to evaluate the statistical significance of Nanog expression levels after TKIs treatment compared to untreated control, with a p value <.05 indicating a significant difference.

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