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Role of Telomeres in Onco-Hematology

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Presentata da:	Dott.ssa Federica Orrù
Coordinatore Dottorato	Prof. Roberto Orrù
Tutor	Prof. Giorgio La Nasa Dott. Giovanni Caocci

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A mio marito Marco

Summary

Abstract

Telomeres are protective structure located on the endings of human chromosomes, that prevent translocation and progressive shortening of DNA coding sequences. Several studies showed that telomeres are involved in regulation of cell lifespan and proliferative potential. The role of telomeres in oncogenesis is still a matter of active investigation. Recently, some papers identified telomere length alterations in patients affected by Chronic Myeloid Leukemia and Myelofibrosis. My research activity focused on the relation between telomere length and the outcome of patients with Chronic Myeloid Leukemia and Myelofibrosis. My aim was to investigate new therapeutic targets and outcome predictive factors. In particular, I pursued two research lines: first, I tried to evaluate the role of telomeres as biomarker to discriminate patients with Chronic Myeloid Leukemia that could safely stop tyrosine kinase therapy. Next, I investigated the relation between telomere length and response to Ruxolitinib, a recently approved JAK-STAT inhibitor, who proved to be highly beneficial to high-risk patients with Myelofibrosis.

I enrolled a population of 32 patients affected by Chronic Myeloid Leukemia who discontinued imatinib after achieving complete molecular remission and 32 age-sex-matched controls, for whom was possible to evaluate telomere length. I performed the clinical management of these patients and the data analysis. The relative telomere length (RTL) was determined by q-PCR as the telomere to single copy gene (36B4) ratio normalized to a reference sample (K-562 DNA). Age-corrected RTL (acRTL) was also obtained. The 36-month probability of treatment-free remission (TFR) was 59.4 %. TFR patients showed shorter acRTL compared to relapsed (mean $\pm$ SD = 0.01 ± 0.14 vs 0.20 ± 0.21; $p = 0.01$). TFR was significantly higher in CML patients with acRTL ≤ 0.09 (78.9 vs 30.8 %, $p = 0.002$). These findings indicate that telomere length seems to be related to treatment free remission in CML patients, and may be a useful biomarker to select patients candidate to treatment interruption.

Next, I enrolled a population of 11 patients affected by Myelofibrosis, eligible to Ruxolitinib treatment, for whom was possible to evaluate telomere length before and after a median of 1000 days of Ruxolitinib therapy. I performed the clinical management of this cohort, and the data analysis. The RTL was determined as stated before. Related samples Wilcoxon signed-rank test performed before treatment with Ruxolitinib showed that the mean RTL was shorter in patients compared with age-and sex-matched healthy controls (1.08 vs 1.26, respectively; P = 0.09). The most interesting finding was that Mann-Whitney Utest showed shorter acRTL in MF patients with high IPSS compared to patients with intermediate-2 IPSS (mean ± SD = 1.016 ± 0.22 vs 1.34 ± 0.14; p= 0.03); furthermore, median RTL increased significantly (1.30 vs 1.08; p = 0.018), showing overlapping values with the healthy controls. Median RTL elongation from baseline was 15%. These findings seem to indicate that telomeres are involved in the dynamics of Myelofibrosis evolution and treatment response, and may be a possible therapeutic target in these patients.

In conclusion, this research highlights some interesting points of the complex relation between telomere length and outcome of patients treated for Chronic Myeloid Leukemia and Myelofibrosis. If confirmed, these findings will be a useful advance in understanding response to treatment in these populations.

Introduction

Most of circulating peripheral blood cells has a determined life duration. To equilibrate continual loss of differentiated cells, it has been estimated that an adult human being will need to produce daily 100-200 billions of mature hematic cells. Moreover, in addition to physiological requirements, hematopoietic system has to be able to rapidly react to pathological conditions. All blood cells derive from a relatively small population of hematopoietic stem cells (HSC) and progenitor cells [1]. While fetal liver and cord blood are a rich source of HSC, after delivery most of hematopoietic stem cells reside on bone marrow. HSC need to be able to generate all hematopoietic cell lines, without losing self-renewal potential. Instead, progenitor cells have the potential to differentiate towards different cell lines, however losing self-renewal potential. While actually we know many of the properties of this population, several features are not clear: particularly, the self-renewal machinery, relevant to maintain a stable long-life hemopoiesis, is still matter of investigation. HSC self-renewal potential is finely regulated, with several molecular pathways involved.

Several studies suggest that HSC, despite their proliferative and self-renewal ability, have a limited proliferative potential, and that the biology beyond this point is still to be elucidated [1]. As observed in other cell populations, the limited proliferative potential observed in HSC seems to be related to Telomere attrition [2]. Telomeres are the terminal sequences of chromosomes, and are constituted by repeated TTAGG sequences. Their function is to protect chromosomes from degradation, fusions and translocations. In most of somatic cells, telomere attrition is observed during cell divisions, until reaching a critical length; once reached a critical threshold, the cell underwent senescence, a phenomenon characterized by irreversible arrest of proliferation [3]. Telomerase enzyme activation determines telomere length maintenance; several studies showed telomerase involvement in regulation of self-renewal machinery. Some studies also showed that tumoral and germ cells have unlimited proliferative potential, probably because of increased

telomerase enzyme activity. According to “Telomeres/Telomerase aging hypothesis”, telomere attrition is the molecular step that causes somatic cells senescence [4]. Several studies showed the relation between telomere length, cell aging and hematopoietic tissue proliferative potential. Both mature and undifferentiated cells underwent progressive telomere attrition [5, 6]. Telomerase activity may be observed on primitive progenitors; this activity increases during committed progenitors proliferation and is negatively regulated during differentiation [7]. Moreover, distress was observed in highly proliferative tissues, as bone marrow, in telomerase knockout mice [8]. It has been shown that HSC may completely reconstitute hematopoiesis in host undergoing myeloablation, and that transplant outcome is influenced by features of infused population. Several authors showed that HSC of patients undergoing HSC transplantation have shorter telomeres than related donor cell populations [9], and that attrition size is inversely proportional with the number of transplanted cells [10]. This aspect suggests that proliferative stress during infused HSC engraftment causes accelerated telomere attrition, limiting HSC proliferative potential. This finding is supported by observation in patients affected by some genetic diseases, like Down Syndrome or Hutchinson-Gilford Progeria, in which hematopoietic failure is related to altered telomere regulation. [11, 12]. At now, it seems clear that biology of telomeres is strictly involved on HSC proliferation regulation. Recently, some advances investigate molecular pathways involved on expansion and differentiation of several cell populations, and telomere regulation on HSC is a matter of active research, because of many possible clinical applications. Some studies showed as a group of hematological malignancies, known as Chronic Myeloproliferative disorders, is caused by mutation in multi-potent hematopoietic progenitors [13]. These disorders have historically been described as indolent diseases, presenting usually for several years as chronic conditions, followed by a rapid evolution into aggressive malignancies as acute myeloid leukemia. However recently, the introduction of new drugs radically changed the scenario of two of these syndromes, Myelofibrosis [14] and Chronic Myeloid Leukemia [15]. These new drugs changed the prognosis and management of patients suffering these conditions, in terms of outcome

and quality of life [14, 15]. The great efficacy of these drugs increased and probably will keep on increasing patients eligible to these treatments, with a great impact in terms of public health. Considering the relevant role of telomeres on HSC proliferative potential regulation [3], I focused my research activity on the study of telomere biology, to identify new biomarkers that allow a better selection of patients to address to these new drugs, pursuing a treatment personalization strategy, with the aim to select the best therapy for every single patient.

Telomeres

Historical perspective

The existence of particular structures at the end of chromosomes is not a recent finding. In fact was the 1938 when a young scientist, Hermann J. Muller, presented the results of his work about at Edinburgh Animal Genetics Institute. He observed that the ends of irradiated Drosophila Melanogaster chromosomes, different from the other genome, didn't present alterations as inversions, deletions or translocations, probably because of a protective cap that he called "telomere" [16].

His observations were confirmed two years later by Barbara McClintock, with his work on Zea Mays genetics. She showed that chromosome break determined adhesion and fusion of their ends, with consequently generation of dicentric chromosomes; further, she found that these broken ends could be healed, substantially thanks to the acquisition of new telomeres, suggesting that these structures play a fundamental role in the integrity of chromosome [17].

These relevant findings were accepted with great skepticism, and only 30 years later genetic research acquired appropriate importance. James Watson, the same that described the double helicoidal structure of DNA, in 1972 recognized the so called "terminal replication problem", that is the cells incapacity to completely replicate linear ends of DNA. He proposed that, because DNA polymerase cannot completely replicate its ends, consequently the telomeres and therefore the chromosomes were shortened. He postulated also the existence of a chromosomal shortening protective machinery [18].

In the same years, Alexsei Matveevich Olovnikov, a Russian scientist, hypothesized the relation between the "terminal replication problem" and the cellular senescence [19], the phenomenon described in 1961 by Leonard Hayflick [20]. Cellular senescence was defined as the condition of

cells exceeding their replicative lifespan, characterized by typical biochemical and morphological alterations. Olovnikov postulated that terminal replication problem was responsible for progressive telomere shortening, and that telomere length acted as a biological clock to determine the number of division that a cell could experience throughout its life [21].

Olovnikov and Watson postulated that cells have strategies to prevent telomere attrition during normal DNA replication. Some years later, in 1985, Elizabeth Blackburn and Carol Greider described that strategy, identifying the enzymatic activity of a protein that could add repeated sequences: they called it Telomerase [22].

I wanted to start my dissertation summarizing these pioneering studies, that are so actual now, at almost 80 years from the first observation of a telomere. The key to understand biology of aging, cancer and probably the cure of some malignancies has to be found on telomeres, and we are going across this way. But, all started from these amazing works.

Telomere Biology

Eukaryotic cell's DNA is scattered in multiple linear chromosomes: the normal genetic patrimony is constituted by 46 chromosomes for each somatic cell. The presence of multiple and independent chromosomes represented a great selective advantage during human evolution. Chromosome linearity permits the increase of recombination frequencies, and consequently the casual chromosome melting during sexual reproduction. So, DNA dispersion in several linear chromosomes permits to increase genetic heterogeneity in a population [23].

It was showed that linear chromosome structure is exposed to degradation and fusion of DNA endings; such machinery is finely tuned by complex protection systems. In fact, 3' and 5' DNA endings are extremely susceptible to nuclease degradation and frequently involved in genetic recombination, because of linking with free DNA ends or integration with centromeric DNA [24, 25]. The "terminal replication problem" is due to the particular pathway of DNA synthesis, as described by Watson in 1972 [18]. Briefly, DNA polymerase could synthesize completely and in a continuous way only the original strand in direction 5' to 3', consequently, the synthesis of the lagging strand is discontinuous and subordinate to little RNA primers, the so-called "Okazaki Fragments" [26]. In most cases, the primers of the Okazaki fragments can be easily replaced with DNA and the fragments connected to form an unbroken strand. However, when the replication fork reaches the end of the chromosome, there is a short stretch of DNA that is not covered by an Okazaki fragment. That is because the polymerization reaction that they catalyze involves attaching the phosphate group of an incoming nucleotide to the hydroxyl group of an existing nucleotide (one that's already part of the strand). Without this hydroxyl group to use as a "hook," a DNA polymerase has nothing to attach nucleotides to and cannot catalyze its reaction to make new DNA [26].

The 5’ end of the lagging strand is shorter than the 3’ end of the other strand, and causes the loss of 50-100 base pairs every replicative cycle [23]. Without other interventions, this DNA loss would determine cellular death. Therefore, during evolution, eukaryotic cells developed specific structures, called telomeres, to resolve this problem [26].

Telomeres are heterochromatin specialized structures, acting as protective caps for chromosome endings [27]. In man and other vertebrates are constituted by short guanine rich repeated sequences [28] associated with proteins: the repeated telomeric sequence is TTAGGG-3’. Every chromosome termination is constituted by 1000-2000 repeats, for a total approximately of 6-12 kb [29, 30]. The number of repeat of the base sequence is variable according to species, but telomeric functions are highly conserved and can be summarized in the following three points:

- Maintenance of chromosome stability, in terms of protection by exonucleases degradation, ligase-induced chromosome fusion, translocation and recombination [31]
- Cellular nucleus spatial organization and chromosome anchoring during DNA replication [31]
- Influencing transcription of genes near telomere endings [28]

Telomeric DNA has a double strand conformation, and is linked to proteins constituting a peptide complex that is involved in maintenance of telomere stability and regulation of telomere length. Beyond the telomere double strand region, 3’ end of guanine-rich strand includes several hundred base pairs organized as a single strand called “G- overhang”. This overhang folds up back creating with telomeric double strand DNA a structure defined as T-loop. The closed configuration of the T-loop provides a protective cap that defines the natural end of the chromosome and masks the telomere from the DNA damage response (DDR) machinery [32]. The repetitive and GC rich nature of telomeric DNA allows forming higher order DNA secondary structures, such as G-quadruplexes, which have been proposed to represent obstacles for the replication machinery.

TTAGGG repeats have also been identified as poor substrates for in vitro nucleosome complex assembly [26].

In human cells, telomere associated proteins are essential to telomere stability maintenance and length regulation. The human telomere protein complex is composed of six individual proteins, TRF1 (Telomeric Repeat binding Factor 1), TRF2, RAP1 (Repressor/Activator Protein 1), TIN2 (TRF1 Interacting protein 1), TPP1 (TINT1/PIP1/PTOP 1) and POT 1 (Protection of Telomeres 1) and is termed Shelterin complex [26].

This complex has several functions; TRF1 and TRF2 bind double stranded telomeric repeats, while POT1 associates with the single stranded overhang [33]. TPP1 and TIN2 bridge these DNA binding modules and are crucial for chromosome end protection and telomere length regulation. TRF1 and TRF2 are constitutively present at telomeres and the proportion of these proteins loaded on telomeres is important for telomere length regulation. TRF1 has also DNA remodeling activity, and has recently been shown to promote efficient replication of telomeres [34, 35, 36]. TRF2 primary function in chromosome end protection is to promote topological changes in telomeric DNA, T-loop assembly [37] and by suppression of ATM (Ataxia Telangiectasia Mutated) -dependent DDR [38] and non-homologous end joining (NHEJ) [39], the major pathway of DNA repair machinery. TRF2 also plays a role in chromatin assembly [40]. The function of RAP1 is still to be defined. In fact, RAP1 does not bind TTAGGG repeats and its telomeric localization seems to be dependent on interaction with TRF2, but recently the factor has been implicated in the inhibition of NHEJ in vitro and in vivo [41,42,43]. POT1 contributes to telomere protection by binding to the overhang and by suppression of ATR (ATM Rad3 related protein) -dependent DDR pathways [44]. Also, it has been proposed that POT1 binds to the displaced G-strand in the T-Loop and “lock-in” the closed configuration of the structure. The loading of POT1 and TPP1 onto the overhang seems to be also an important determinant of telomere length [45,46].

It is evident that the six proteins of Shelterin complex play an immensely complex role on telomere biology. However, Shelterin complex is not the only player of telomere regulation. A recent study of telomeric proteome identified over than 200 proteins that interacted with and might influence telomere structure [47].

Telomerase is a ribonucleoprotein of the great family of inverse transcriptases. Telomerase RNA stamp is integrated in the enzyme machinery [48]; Enzyme function is to connect to 3' eukaryotic chromosome ending and add repeated single strand sequences TTAGGG [49]. Active Telomerase enzyme needs of two essential components: RNA template (hTR, human Telomerase RNA), and a proteic subunit with reverse-transcriptase activity (hTERT). hTR subunit is constituted by 451 nucleotides; 11 of these nucleotides represent the plate sequence CUAACCCUAAC that codifies for repeated telomeric sequences (TTAGGG)n. The codifying gene is located on the distal region of chromosome 3 long arm [31]. TERT protein, that is codified from homonymous gene located on chromosome 5 (hTERT in man, Human Telomerase Reverse Transcriptase), is a 127 kDa polypeptide of reverse-transcriptase family. Further telomerase-associated proteins as TP1/TEP1 seems to play a key role on in-vivo enzyme activity [50]. Telomerase mechanism of action, as illustrated in figure 1, evidenced as enzyme synthesize repeated DNA sequences alternating elongation to translocation [48].

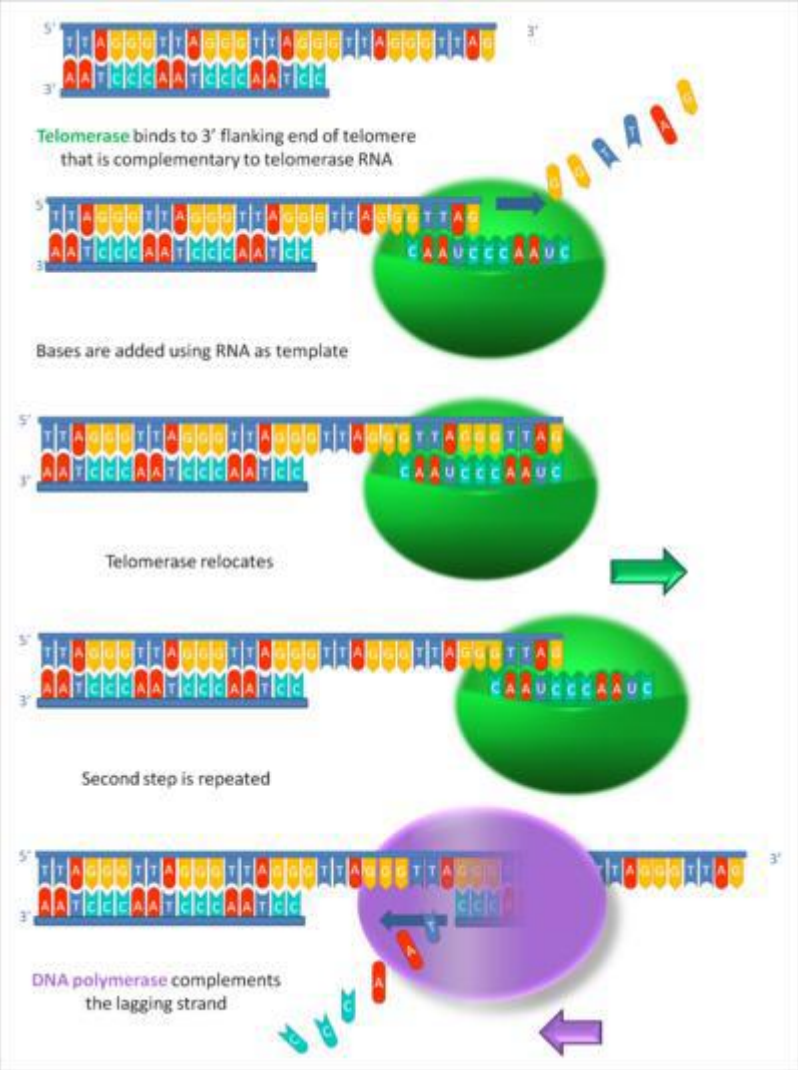

Figure 1. Telomerase mechanism of action on telomere synthesis [51]

Briefly, template region binds to 3' overhang ending of telomeric DNA; next, catalytic subunit stimulates the addition of deoxy-nucleotides in 5'-3' direction and starts synthesis. The elongation is due to RNA subunit translocation on 3' ending of newly synthesized telomere [52]. Telomerase activity has been observed in several human fetal tissues, as muscle, lung and cutis; these observations suggest that the enzyme is active during development and is suppressed on adult tissues [31]. The first normal human cells identified as telomerase positive were stimulated lymphocytes [53]. Also hematopoietic stem cells presented telomerase activity [25, 54].

Other studies showed that, in every tissue, a small subpopulation keeps low levels of telomerase activity, however not sufficient to protect from telomere attrition [55]. In fact, while hTR is expressed virtually by all embryo and somatic cells, hTERT is finely regulated and not identifiable in most of somatic cells. These findings seem to suggest that hTERT expression is the limiting step of telomerase activity [56, 57]. Most human cells, except hematopoietic, germinal and tumoral cells, not have telomerase activity and presented progressive telomere shortening, with consequent senescence. Telomerase activity is finely regulated during development, to prevent unlimited proliferation, with possible neoplastic evolution [58].

Over 40 years ago, it was postulated that most normal cells have a programmed number of cell divisions, not being able to indefinitely divide [20]. This theory was investigated by Harley, with the telomeric aging hypothesis: the theory was that a defined loss of telomeric sequences activated a molecular signal that started cellular senescence. Supporting this theory, telomere shortening in human fibroblasts is prevented by up-regulation of telomerase activity, with consequent immortalization of these cells [59].

Several studies find data that supports this hypothesis [60], but at now there are several points that need to be investigated [27]. There is a considerable variability between single chromosomes ending length, with the short arm of 17 chromosomes usually containing the lowest number of repeated telomeric sequences [61].

Some authors postulated that the recognition of the shortest telomere is more important than mean telomeric length for vitality and chromosomal stability [62]. So, the proliferative potential could be limited by the chromosome with the shortest telomeres, while some data suggest that short telomeres will accumulate before senescence, and that this is not triggered by the first telomere to reach a critical threshold [63]. Further, it has been shown that telomere attrition is related to guanine rich 3’ telomeric overhang. Cells with longer overhang lose more telomeric repeat at every cell division than those with short overhang [64]. Finally, some data suggest that shorter telomeres on

senescent cells may be not able to connect with TRF2, losing the ability to adopt T-loop conformation [65]. Once a critical threshold of telomeric sequence is reached, a genetic damage signal is produced, that causes cellular cycle arrest. The exact mechanism of this pathway is unknown, however several studies on yeast seem to suggest that telomeric DNA loss triggered the same pathways induced by chromosome break (cell cycle arrest, DNA reparation) [66]. Telomeres have a dynamic structure, and seems that 3' extremity triggers pathways of damage signaling involving mutant gene of Ataxia Telangiectasia and TP53 [28,67,68,69].

Telomeres and hematopoietic stem cells

When we use the term hematopoiesis, we speak about the process of formation, developing and differentiation of mature blood elements. This process derives from HSC. These cells have the ability of self-renewal, maintaining simultaneously the ability to differentiate in all the mature blood elements. HSC can also reconstitute the entire hematopoietic system in lethally irradiated host. HSC are rare, and can be detected at a frequency of 1/10000-100000 bone marrow cells. HSC are normally quiescent, and only a fraction enters regularly on cell cycle to proliferate and generate differentiated cells [29].

Unfortunately, there is not a combination of cell surface markers to identify HSC. CD34 surface marker, a sialomucinic adhesion molecule, is expressed on 1-5% of mononucleated cells, included HSC and progenitor cells, endothelial cells and some fibroblasts. Several studies on animal models showed that CD34+ bone marrow cells could repopulate lethally irradiated hosts. Bone marrow, Cord blood and peripheral blood derived CD34+ cells can engraft and repopulate NOD/SCID mice (Non Obese Diabetic/Severe Combined Immunodeficiency Disease) [29]. When compared CD34+ with CD34 –cells, this population seems to have a minor contribution to human cells engraftment on NOD/SCID mice; consequently, it was formulated the dogma that human HSC are CD34+. Consequently, CD34 expression is actually used to quantify on clinical practice the number of transplantable cells, as CD34+ cell count can predict transplant outcome [29].

Self-renewal and differentiation of CD34+ HSC are strictly regulated. [55]. Twenty years ago, it was demonstrated that HSC telomere length decreases in relation to differentiation grade and age [11, 71, 72].

Telomere length evaluation reveals a relevant difference between adult and fetal/neonatal HSC. In fact, in cord blood and fetal derived HSC, telomeres are about 4kb longer than those of adult HSC. These findings support the hypothesis that HSC, although with self-renewal potential, not have an

unlimited replicative potential [71].Telomere loss is rapid during the first year of life and then decrease since 50-60 years of age; next, it increases again [30, 73]. Telomere attrition is great before birth (approximately 240 base pairs for week) and it is related to high proliferation index of HSC in that situation. This attrition is higher in lymphocytes and granulocytes, suggesting that age related telomeric loss compromise immunological functions more than HSC activity. Granulocyte telomere length could be used as a surrogate for HSC telomere length. In normal circumstances, age related telomere length decrease is so slow; making unlikely that HSC will enter in senescence or apoptosis consequently to telomere attrition. Even if occasionally a single HSC will enter in senescence because of excessive telomere attrition, the great number of HSC ensure that hematopoiesis will not be compromised [74]. Many proliferating HSC presents detectable telomerase activity. After an initial increase, telomerase activity is down regulated while HSC and progenitor cells proliferate and differentiate towards mature cells, in which enzyme activity is not detectable. HSC ex-vivo exposure to cytokines raises temporarily hTERT activity that however is down regulated during subsequently differentiation phases. Similarly, in tumoral cells with high self-renewal ability, telomerase is constitutively expressed, suggesting that telomerase activity could be associated to self-renewal potential. This hypothesis means that hTERT over-expression could increase HSC self-renewal potential, with a possible action during engraftment. This could have some clinical application [6, 31, 54, and 75]. An active telomerase doesn't implicate a stable and static telomere length. Telomerase activity is up-regulated when HSC are stimulated to generate progenitor cells or when lymphocytes underwent clonal expansion [76]. However, up-regulation is not sufficient to prevent telomere attrition, but could contribute to cell survival in absence of proliferation [77]. While traditional description of hTERT activity showed that the role of this enzyme is to maintain telomere length during cell division, recent studies suggest that the activity could be more complex and involve other pathways that play a role on cell survival [78].

Chronic Myeloid Leukemia

Chronic Myeloid Leukemia (CML) is a myeloproliferative neoplasm with an incidence of one to two cases per 100,000 adults. It accounts for approximately 15% of newly diagnosed cases of leukemia in adults [79]. The median age at diagnosis is 60–65 years, according to most relevant epidemiological registries. The incidence of CML is slightly higher in males than in females. Since 2000, the year of introduction of imatinib, the annual mortality in CML has decreased from 10–20% down to 1–2% [79]. Therefore, the prevalence of CML in the United States, estimated at about 25-300,000 in 2000, has increased to an estimated 800–100000 in 2015, and will reach a plateau of about 1800000 cases in 2030 [80].

Chronic myeloid leukemia (CML) is a myeloproliferative disorder of hematopoietic stem cell (HSC) origin caused by the t(9;22) chromosomal translocation. The fusion of the breakpoint cluster region (BCR) on chromosome 22 with the Abelson murine leukemia viral oncogene homolog 1 (ABL) tyrosine kinase of chromosome 9 generates the so-called “Philadelphia (Ph) chromosome”, which constitutively express the BCR-ABL fusion gene. The BCR-ABL protein is a constitutively active tyrosine kinase that can influence several relevant signaling pathways involved in cell survival, proliferation, adhesion, and differentiation such as RAS, RAF, JUN kinase, MYC and STAT [81, 82, 83, 84, 85, 86, 87].This machinery is involved in leukemogenesis, creating a cytokine-independent cell cycle with aberrant apoptotic signals in response to cytokine withdrawal. Clinically, it shows itself as an expansion of myeloid cells and an accumulation of differentiating granulocytic precursors and differentiated effector cells, with increased peripheral granulocytosis, anemia, thrombocytosis and splenomegaly [80].

Diagnosis

The diagnosis of classical CML is simple and consists of identification, in the setting of a persistent unexplained leukocytosis (occasionally associated to thrombocytosis and/or anemia), of the Philadelphia (Ph) chromosome abnormality, the t(9;22)(q34;q11), by routine cytogenetics, or the Ph-related molecular BCR-ABL1 abnormalities by fluorescence in situ hybridization (FISH) or by molecular studies [88, 89]. FISH analysis relies on the co-localization of large genomic probes specific to the BCR and ABL genes. Simultaneous marrow and blood samples analyzed by FISH show high concordance. Reverse transcriptase-polymerase chain reaction (RT-PCR) amplifies the region around the splice junction between BCR and ABL1. It is highly sensitive in detecting minimal residual disease. PCR testing can either be qualitative (QPCR), providing information about the presence of the BCR-ABL1 transcript, or quantitative, assessing the amount of BCR-ABL1 transcripts. Qualitative PCR is useful for diagnosing CML; quantitative PCR is actually the gold-standard for monitoring residual disease. However, false-positive and false-negative results can happen with PCR. False-negative results may be from poor-quality RNA or failure of the reaction; false-positive results can be due to contamination. A 0.5–1 log difference in some samples can occur depending on testing procedures, sample handling, and laboratory experience [88, 89]. The Ph chromosome is usually present in 100% of metaphases, often as the sole abnormality. Ten to 15% of patients have additional chromosomal changes involving trisomy 8, isochromosome 17, additional loss of material from 22q or double Ph, or others. This phenomenon seems to be linked to clonal evolution. 90% of patients have a typical t(9;22); 5% have variant translocations which can be simple (involving chromosome 22 and a chromosome other than chromosome 9), or complex (involving one or more chromosomes in addition to chromosomes 9 and 22) [88]. The prognosis of patients with Ph-variants is similar to Ph-positive CML. About 2–5% of patients present with a morphologic picture of CML without the Ph-positivity by cytogenetic studies, with FISH and PCR showing absence of t(9:22) and BCR-ABL1 transcript. Such patients have similar

response and outcome on TKI therapy as patients with Ph-positive CML [88]. Bone marrow aspiration is mandatory for all patients with suspected CML. Bone marrow morphology and cytogenetic analysis are essential to confirm the diagnosis, but also to obtain information for disease staging (blast and basophil percentages). Baseline reverse transcriptase-polymerase chain reaction is imperative to identify the specific type of rearrangement; this data are relevant to assess the response to TKI therapy. About 2-5% of patients have b2a3 or b3a3 variants of p210 BCR-ABL1 or p230 transcripts that may yield a false negative PCR by routine probes and, if not tested at diagnosis, may resemble a false complete molecular response [88].

Patients may be diagnosed with CML in chronic phase, that means fewer of 10% of blast in peripheral blood and bone marrow, or in accelerated or blast phase. CML patients with accelerated phase have a blast count of 10-19% in peripheral blood and bone marrow, or 20% or more basophils, persistent thrombocytosis ($>1000x10^9/l$) or thrombocytopenia ($<100x10^9/L$) and additional chromosomal abnormalities in Ph+ cells at diagnosis or during therapy. CML patients in blast phase have a blast count $\geq$ 20% in peripheral blood or bone marrow [79].

Treatment

Since 18 years ago, the pharmacological therapy for CML was limited to nonspecific agents such as Busulfan, Hydroxyurea, and Interferon-alfa (INF-a) [11]. INF-a led to disease regression and improved survival in a minority of patients, but was hindered by a multitude of toxicities. Allogeneic stem cell transplantation (allo-SCT) is curative in CML, but carries a high risk of morbidity and mortality. Further, allo-SCT is an option only for patients with adequate performance status and organ function, and who have an appropriate stem cell donor. The CML therapeutic landscape was changed dramatically by the development of small molecule tyrosine kinase inhibitors (TKIs) that interfered with the interaction between the BCR-ABL1 oncoprotein and adenosine triphosphate (ATP), blocking the cellular proliferation of the malignant clone. This

“targeted” approach altered the natural history of CML, improving the 10-year survival rate from approximately 20% to 80–90% [79, 80,90].

It is recommended that in practice, outside of clinical trials, the first-line treatment of CP CML can be any of the 3 TKIs that have been approved for this indication and are available nearly worldwide, namely imatinib (400 mg once daily), Dasatinib (100 mg once daily) and Nilotinib (300 mg twice daily). These TKIs can also be used in second or subsequent lines, at the standard or at a higher dose (400 mg twice daily for imatinib, 400 mg twice daily for Nilotinib, and 70 mg twice daily or 140 mg once daily for Dasatinib).Other TKI as Bosutinib (500 mg once daily) has been approved by the FDA and EMA for patients resistant or intolerant to prior therapy [79]. Ponatinib (45 mg once daily) has also been approved by the FDA for patients resistant or intolerant to prior TKI therapy [79]. Other drugs available for patients in whom prior TKI therapy fails are Radotinib, which is available in Korea [91], and Omacetaxine, which is a non-TKI drug approved by the US FDA [92, 93].

Busulfan actually is not recommended, while Hydroxyurea can be used for a short time before initiating a TKI, until the diagnosis of CML has been confirmed. rIFNα alone is recommended only in the rare circumstances in which a TKI cannot be used, as pregnancy. The combinations of TKIs and rIFNα are potentially useful but are still under investigation [94]. Cytotoxic chemotherapy is never recommended in CP but may be useful to control BP and to prepare BP patients for allo-SCT [94].

The choice of the TKI must take into account tolerability and safety, as well as patient characteristics, particularly age and comorbidities, which may be predictive of particular toxicities with the different TKIs. In all cases of unsatisfactory response, research and investigational studies and should be encouraged to improve treatment results.

Imatinib

Imatinib mesylate was the first TKI to be approved by the Food and Drug Administration (FDA) for the treatment of patients with CML in chronic phase. Its way of action is the competitive inhibition of the ATP-binding site of the BCR-ABL1 oncoprotein, which prevents the phosphorylation of proteins involved in cell signal transduction. It inhibits the BCR-ABL1 kinase, but also blocks the platelet-derived growth factor receptor (PGDFR) and the C-KIT tyrosine kinase [95]. The International Randomized Study of Interferon and STI571 (IRIS) study is considered a miliar stone of clinical trial for TKIs and CML [96]. The investigators randomized 1,106 patients in CML-CP to receive imatinib 400 mg/day or INFα plus low-dose Cytarabine. After a median follow-up of 19 months, outcomes for patients receiving imatinib were significantly better than in those treated with INFα plus Cytarabine; in fact, imatinib treated patients showed higher rates of CCyR (74% vs. 9%, $P < 0.001$), and freedom-from-progression to AP or BP at 12 months (99% vs. 93%, $P < 0.001$) [96]. Furthermore, there was a high crossover rate from IFNα to imatinib due to intolerance. The responses to imatinib were durable: in an 8-year follow-up of the IRIS study [90], the estimated event-free survival rate was 81%, and the overall survival (OS) rate was 93% when only CML-related deaths were considered. However, while imatinib is a very efficacious drug, only 55% of patients enrolled in the IRIS study remained on therapy at the 8-year follow-up time. This underscored the need for additional treatment options for patients who had failed or were intolerant to imatinib. This need led to the development of second generation TKIs that could effectively treat patients unable to continue on imatinib therapy.

High-dose imatinib and imatinib-based combination

Other strategies for frontline therapy with Imatinib include using higher doses or combining with an additional agent, such as interferon. In the Tyrosine Kinase Inhibitor Optimization and Selectivity (TOPS) study, patients were randomized to receive imatinib 400 mg once daily or twice daily (800

mg) [97]. The primary endpoint was Major molecular response (MMR) rate at 12 month, with cytogenetic response and time to such responses collected as secondary outcomes. Patients in the high-dose group achieved faster CCyR and MMR, but rates were not significantly different at 12 months. Interferon has recently re-emerged as an interesting therapeutic option in CML, specially with the advent of pegylated formulations requiring less frequent administration and with improved tolerability. In a phase III randomized study, patients were assigned to one of four treatment arms (imatinib 400 mg once daily, imatinib 600 mg once daily, imatinib 400 mg once daily plus peg-interferon alfa-2a, or imatinib 400 mg once daily plus subcutaneous Cytarabine) [98]. Patients were initially assigned to receive peg-interferon alfa-2a at a dose of 90 mcg once weekly. Because of a high rate of discontinuation due to toxicity, the dose was later modified to 45 mcg once weekly. At 12 months, rates of CCyR were similar among the four groups. The imatinib plus peg-interferon alfa-2a treated group obtained higher rates of MMR and deeper molecular responses, but follow-up was not sufficient to define the impact on long-term outcomes.

Dasatinib

Dasatinib is a second generation TKI, 350 times more potent than imatinib in vitro [99–100-101]. It also inhibits the Src family of kinases, which may be important in modifying several critical cell signaling pathways [102]. Initially was evaluated in patients in the second line\salvage setting, but later compared in frontline CML to imatinib to evaluate the hypothesis that frontline therapy with a more potent TKIs might improve outcomes. The DASISION trial was a phase III randomized study comparing imatinib 400 mg once daily to Dasatinib 100 mg once daily in 519 newly diagnosed patients with CML [103]. The primary endpoint was confirmed CCyR (cCCyR) at 12 months. Patients assigned to Dasatinib achieved cCCyR at 12 months more often than those on imatinib (77% vs. 66%, P = 0.007). A five-year follow-up showed that Dasatinib induced more rapid and deeper responses at early time points compared to imatinib [104]; achievig this response predicted

for better progression-free survival and OS. Transformations to CML-AP or CML-BP were fewer in patients treated with Dasatinib versus imatinib (4.6% vs. 7.3%). In another multicenter trial, patients with newly diagnosed CML-CP patients were randomized to either Dasatinib 100 mg once daily or imatinib 400 mg once daily [105]. Similar to the results of the DASISION study, Dasatinib treated patients achieved higher rate of CCyR (84% vs. 69%, P 5 0.04). However, patients in the Dasatinib arm presented more toxicity and Grades 3 or 4 adverse events (58% with Dasatinib and 35% imatinib), mostly hematologic toxicity [105]. A third Phase 3 randomized study, SPIRIT 2, compared imatinib 400 mg daily with Dasatinib 100 mg daily [106]. The interim results showed the 12-month MMR rates with Dasatinib versus imatinib to be 58% versus 43% (P<0.001). The 12-month CCyR rates were 51% and 40% (P <0.002), respectively. Progression rate to CML-AP was 0.7% with imatinib and 0.5% with Dasatinib. The progression rate to CML-BP was 1.7% versus 1%. However, Pleural effusions occurred more frequently on Dasatinib (19% vs. <1%). Other side effects of Dasatinib include myelosuppression (20%), and rarely pulmonary hypertension (1–2%).

Nilotinib

Nilotinib is a structural analog of Imatinib. It was showed that its affinity for the ATP-binding site on BCR-ABL1 is 30-50 times greater in vitro [107]. Like Dasatinib, Nilotinib initially demonstrated the ability to induce hematologic and cytogenetic responses in patients who had failed Imatinib first line therapy. Nilotinib was also compared to Imatinib in a large, international, randomized study (ENEST-nd). In ENEST-nd, two schedules of Nilotinib (300 mg or 400 mg twice daily) were compared to Imatinib 400 mg once daily [108]. The primary endpoint was the rate of MMR at 12 months. Patients treated with both doses of Nilotinib achieved statistically significantly higher rates of MMR compared to Imatinib (44% and 43% vs. 22%, P < 0.001). The cumulative incidence of CCyR by 24 months was 87% with Nilotinib 300 mg twice daily, 85% with Nilotinib 400 mg twice daily, and 77% with Imatinib 400 mg daily (P < 0.001) [108]. With a follow-up of 5 years, the two

arms of Nilotinib demonstrated better early results compared with Imatinib [109]. The cumulative incidences of MMR by 60 months were 77%, 77%, and 60%, respectively (P <0.0001%). The incidences of BCR-ABL1 transcripts [IS] < 0.0032% (equivalent to a 4.5 log reduction of disease) by 72 months were 56%, 55%, and 33%, respectively (P <0.0001%). The incidences of transformation to AP or BP were 3.9%, 2.1%, and 7.4%, respectively (P 5 0.06 and 0.003, respectively). The estimated 5-year EFS rates were 95%, 97%, and 93%, respectively. The estimated 5-year survival rates were 94%, 96%, and 92%, respectively. Nilotinib was superior to Imatinib across all Sokal score categories in inducing higher rates of CCyR and MMR; instead, the advantage in reducing the rates of transformation was more pronounced in patients with intermediate- and high-Sokal risk CML. In fact, among patients with low Sokal risk, those who were treated with Nilotinib 300 mg orally twice daily and 400 mg twice daily presented a rate of transformation of 1%, compared with the 0% of those with Imatinib. Instead, the rates of transformation were respectively 2%, 1%, and 10% among patients with intermediate-Sokal risk and 9%, 5%, and 11% among patients with high-Sokal risk. A second randomized trial with the same design that enrolled 267 patients; the MMR rate was 52% at 12 months with Nilotinib compared with 28% with Imatinib [110]. However, the rates of both CCyR (84% vs. 87%) and progression-free survival (95% each) were similar at 24 months. Overall, six patients in each arm progressed to AP/BP. In both arms, the estimated 2-year survival rate was 98%. While Nilotinib therapy was well tolerated, there was an increased risk of cardiovascular adverse events during therapy. The 6-year cumulative cardiovascular event rates were 9.9%, 15.9% among patients treated with Nilotinib 300 mg twice daily, Nilotinib 400 mg twice daily, while was 2,5% for Imatinib treated patients [109]. Other notable side effects were headache and skin rashes, self-limited elevation of indirect bilirubin (10%), elevations of blood sugar (10–20%), and rarely pancreatitis (1–2%).

Multiple TKIs are available for patients with newly diagnosed patient with CML in CP; every drug has a distinct toxicity profile, that is mandatory to consider when deciding on a therapy. Most TKIs are well tolerated with adequate monitoring and supportive care. For patients at risk of developing pleural effusions or pre-existing lung injuries, TKI other than Dasatinib should be selected. This should be considered for patients with a history of lung disease, cardiac disease (as those with cardiac failure), or uncontrolled hypertension. Pulmonary arterial hypertension (PAH) is also a rare but important complication of Dasatinib [111], and patients with preexisting PAH may be considered for alternative TKIs in the frontline setting. Dasatinib also inhibits platelets function [112], and therefore patients who need to be treated with concomitant anticoagulants may be at an increased risk of hemorrhagic complications [113]. Nilotinib has been associated with hyperglycemia; the drug should be avoided or prescribed with caution in patients with diabetes or history of pancreatitis. Nilotinib was shown to potentially prolong the QT interval; potassium and magnesium should be repleted to appropriate serum levels before starting Nilotinib or determining an individual patient's QT interval. Nilotinib has also been associated with vasospastic and vaso-occlusive vascular events, such as ischemic heart disease, ischemic cerebrovascular events, and peripheral artery occlusive disease (PAOD) [114,115,116,117]. In the 6-year follow-up on the ENEST-nd trial [109], approximately 10% of patients experienced vascular events. Nilotinib use should be limited in patients with risk factors such as diabetes mellitus or coronary or cerebrovascular artery disease. Imatinib causes side-effects as weight gain, fatigue, peripheral and periorbital edema, bone and muscle aches, nausea and others. However, most are mild to moderate. Less than 5–10% of the patients will experience elevations in creatinine with long-term therapy. Patient's age plays an important role in treatment decision. Patients younger than 50 years are expected to live 30,1 more years. Therefore, inducing a durable CMR may potentially lead to therapy discontinuation. Second generation TKIs induce a significantly higher rate of CMR compared with Imatinib. The issue of durable CMR and potential therapy discontinuation plays a

less important role in elderly patients where the expected survival is shorter and discontinuing therapy is less relevant.

Second-line treatment with Nilotinib, Dasatinib, or Bosutinib can yield high rates of response in patients who have inadequate response to Imatinib, including high rates of MMR. The dose escalation of Imatinib can improve response rates in patients with inadequate response to standard-dose Imatinib [118], but likely switching to second-line TKI is more effective [119]. In the TIDEL-II study, patients who had suboptimal response to Imatinib and were switched to Nilotinib had a higher rate of CMR at 12 months than patients who had dose escalation of Imatinib prior to being switched to Nilotinib [120]. In a retrospective pooled analysis of three clinical studies of second-line Dasatinib for patients resistant to or intolerant of Imatinib, patients who were switched to Dasatinib after the loss of MCyR (early intervention group) had higher rates of CHR, CCyR, and MMR, as well as 24-month EFS, TFS, and OS, than patients who were switched after the loss of both MCyR and CHR (late intervention group) [121]. Although this analysis included studies with different study designs and various dosing schedules of Dasatinib, the essential finding that earlier switch to a second generation was associated with better outcomes. Ponatinib is a third generation TKI, and the first TKI in class to exhibit activity against CML with T315I mutation [122]. It was showed that is 500 times more potent than Imatinib at inhibiting BCR-ABL1. In the PACE trial, 449 patients with heavily pretreated CML or Ph-positive acute lymphoblastic leukemia (ALL) were treated [123]. Patients enrolled for this trial had resistance or intolerance to Dasatinib or Nilotinib, or were affected by CML with T315I mutation. Ponatinib dose was 45 mg once daily, and patients were stratified according disease phase and presence or absence of a T315I mutation. Of the 267 patients who received Ponatinib in CML-CP, 56% achieved a MCyR by 12 months, which included 45/64 (70%) patients with a T315I mutation. Patients who received fewer TKI responded more favorably. With a median follow-up of 3.5 years, 59% of patients achieved MCyR at any time; 83% of those remained in MCyR at 3 years [124]. Furthermore, 39% of patients achieved a MMR or

better. The 3-years PFS and OS rates were 60% and 81%, respectively [124]. Arterial occlusive events occurred in 28% of patients (23% serious). The most common adverse events, occurring in 40% of CPCML patients were abdominal pain (46%), rash (46%), thrombocytopenia (45%), headache (43%), constipation (41%), and dry skin (41%) [124]. Other relevant toxicities include severe skin rash (4-7%), pancreatitis (7%), and severe hypertension (20%). In 2014, Ponatinib was associated with a high risk of thrombotic events (13% per year), vascular occlusions, heart failure, and hepatotoxicity [125]. Vascular occlusion adverse events were more frequent with increasing age and in patients with prior history of ischemia, hypertension, diabetes, or hyperlipidemia [124]. Factors associated with increased risk of vascular occlusion events include older age, higher dose, history of myocardial infarction or prior vascular events, and longer duration of CML [124]. In community practice, it seems to be safe to use Ponatinib 30 mg daily (and lower the dose to 15 mg daily for toxicities) rather than use the FDA approved dose of 45 mg daily.

Monitoring Treatment Response

Because patients with CML on TKI therapy are expected to live for a long time, surrogate markers of outcome are important. In general, achieving a deeper response faster has been associated with improved outcome, although the result of molecular testing is dependent on the laboratory and their techniques. New assays on peripheral blood have made possible to avoid the need for traditional bone marrow examinations for treatment monitoring. The response to TKI is the most important prognostic factor. The responses are defined as "optimal" or "failure". Optimal response is associated with the best long-term outcome: patients with optimal response have a greater probability to achieve a duration of life comparable with that of the general population. Failure means that the patient should receive a different treatment to limit the risk of progression and death. Between optimal and failure, there is an intermediate zone, which was previously referred to as "suboptimal" and that now is designated as "warning." A warning implies that the characteristics of the disease and the response to treatment require more frequent monitoring to permit earlier changes in therapy in case of signs of treatment failure [94].

At baseline, all patients should undergo a bone marrow examination to establish the diagnosis, assess percentage of blasts and basophils, and perform cytogenetic analysis to confirm the presence of the Philadelphia chromosome and to exclude clonal evolution. Bone marrow examination at 3, 6 and 12 months after starting therapy probably may be not necessary anymore [94].

FISH and PCR to assess cytogenetic response may be also executed on peripheral blood. If a patient is responding optimally, and the FISH study is negative at 6 or 12 months and or BCR-ABL1 transcripts [IS] < 10% at 3 months, this could discriminate into high and low risk categories for long-term outcomes (i.e., progression, survival) [104,108,126,127].

In the definition of response, a point still matter of active investigation is the value of early molecular response, particularly after 3 months of treatment. A BCR-ABL1 transcripts level >10%

was reported to be significant for prognosis in several studies [126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137]. However, a single measurement of BCR-ABL transcripts level is not sufficient to define as failure necessitating a change of treatment, whereas at least 2 consecutive assays (at 3 and 6 months) with increasing BCR-ABL transcript are mandatory for the decision to change the treatment. Failures must be distinguished as either primary (failure to achieve a given response at a given time) or secondary (loss of response). The criteria for identifying optimal response, warning and failure are resumed on table 1.

Table 1. Response definition for any TKI in first line

Time-point	OptimalResponse	Warning	Failure
3 months	BCR-ABL ≤10% on PCR analysis, and/or Ph+ cells ≤ 35% on cytogenetic analysis (pCyR)	BCR-ABL >10% Ph+ cells 36-95%	No CHR Ph+ 95%
6 months	BCR-ABL <1% Ph+ 0% (CCyR)	BCR-ABL 1-10% Ph+ 1-35%	BCR-ABL >10% Ph+ >35%
12 months	BCR-ABL ≤0.1% (MMR)	BCR-ABL 0.1-1%	BCR-ABL>1% Ph+ >0
Then, and at any time	MMR or better	CCA/Ph- (-7, or 7q)	Loss of CHR Loss of CCyR Loss of MMR, confirmed Mutations CCA/Ph+

The definitions of the response to second-line treatment are based on the same concepts. These definitions have relevant implications, because they define a threshold beyond which to consider allo-SCT as a clinical option. Allo-SCT remains an important treatment option for the management of post TKIs failure and in advanced CML phases. Allo-SCT still remains the only available treatment that can potentially cure CML patients. However, the associated procedural-related morbidity and mortality remains a major deterrent. German CML Study Group conducted a trial who reported on 84 patients (median age, 37 years) receiving myeloablative allo-SCT between 2003 and 2008, as either first-line therapy (n = 19) or after Imatinib failure (n = 37 in CP, n = 28 in AP) and with related (36%) or unrelated (64%) donors [138]. OS at 3 years was 88% in patients transplanted after first line therapy, 94% in those transplanted in chronic phase after Imatinib failure and 59% in those transplanted in accelerated phase. Transplant-related mortality was 8% and chronic graft-versus-host disease occurred in 46% of patients. Another retrospective study of the

Center for International Blood and Marrow Transplant (CIBMTR) reported on 306 patients >40 years of age who received reduced-intensity conditioning or non myeloablative procedures between 2001 and 2007 [139]. Half of the patients were in CP at the time of transplant and 74% had received Imatinib before their graft. Patients were stratified in the 3 age groups: 40-49, 50-59 and >60 years. OS, leukemia-free survival, transplant-related mortality, and relapse incidence were 54%, 52%, and 41%; 35%, 32%, and 16%; 18%, 20%, and 13%; and 36%, 43%, and 66%, respectively. Chronic graft-versus-host disease was reported in ∼50% of patients. Pavlu et al [140] updated the Hammersmith results for patients transplanted between 2000 and 2010, with a 6-year OS of 89%, 60%, and 30% for patients transplanted with EBMT risk scores of 0 to 2, 3, and >3, respectively. Outcome for patients transplanted in blast crisis was very poor, with an OS of <10%.

Discontinuation Treatment

The Stop Imatinib (STIM) trial investigated the risk of relapse in patients on Imatinib with ongoing complete molecular response (CMR) for greater than two years in which treatment was stopped [141,142]. In the most recent update, 100 patients with a median follow-up of 50 months were monitored closely for evidence of molecular relapse. Overall, 61% experienced a molecular relapse, with 95% of the relapses occurring within 7 months of stopping Imatinib. Once Imatinib therapy was restarted, almost all patients were able to achieve CMR. Low-risk Sokal score and duration of Imatinib therapy greater than 60 months were predictive factors for sustained CMR after therapy cessation. These results were confirmed in other large studies of CML. The TWISTER study followed 40 patients who stopped Imatinib after being without detectable minimal residual disease for greater than two years [143]. Follow-up was executed for a minimum of 15 months (median 43 months) from Imatinib interruption; 22 of 40 patients became molecularly positive; nearly 70% of the molecular relapses occurred within the first 6 months of treatment cessation and patients who resumed TKIs were able to recapture deep molecular responses. Interestingly, analysis with highly sensitive patient-specific PCR was able to detect the original CML clone in several patients who remained off Imatinib for several years. This finding indicates that probably it may not be necessary to completely eradicate the disease to allow patients to enjoy what we could define a “functional cure”. The previous studies included heterogeneous groups of patients, particularly ones exposed to interferon prior to the Imatinib era. The French group conducted a follow-up study to STIM enrolling patients who have only been exposed to Imatinib as CML therapy (STIM2) [144]. The inclusion criteria were similar to those used in STIM1. There were 124 patients identified who stopped Imatinib therapy. With a median follow-up of 12 months, 48 patients had molecular relapse; 94% of relapses occurred within 6 months of TKI withdrawal. All patients remained sensitive to Imatinib or a second generation TKI upon re-challenge. In this study and in others, patients with low level positivity for BCR-ABL1 transcripts seems to be able to remain off therapy

with close monitoring. French investigators address recently this point, finding that resuming patients on TKI therapy if their transcript level became greater than 0.1% (i.e., loss of MMR) seems safe. [145]. The EUROSKI study aimed to define factors associated with durable deep molecular response (MR) after stopping TKI. An interim analysis on 200 patients with 6-month follow-up of molecular events was reported [146]. Were eligible only adult CML patients in CML-CP on TKI treatment in confirmed deep MR (MR4, BCR-ABL1 transcripts [IS] 4 log reduction on TKI therapy for >12 months confirmed by three consecutive PCR tests) and under TKI treatment for at least 3 years. The duration of TKI treatment was 8 years (range, 3–12.6 years), and median duration of MR4 before TKI cessation was 5.4 years (range, 1–11.7 years). Overall, 123 of the 200 patients remained without relapse in the first 6 months. The loss of MMR was observed in 47/114 patients (47%) treated for <8 years, compared to 27/86 patients (27%) treated for >8 years (P = 0.003). So, the duration of MR4 >5 years vs. <5 years was showed to be a predictive factor for a lower relapse rate (P = 0.03).

TKIs discontinuation studies in patients with durable CMR demonstrate that stopping TKI therapy is feasible, and some patients may be cured. While the results thus far are encouraging, it is still recommended that TKI therapy be stopped only under the auspices of a clinical trial.

Myelofibrosis

Myelofibrosis (MF) is a hematologic malignancy that belongs to the category of myeloproliferative neoplasms (MPNs) and may presents as a primary disorder (primary myelofibrosis [PMF]) or evolve from indolent myeloproliferative disorders as polycythaemia vera (PV) or essential thrombocythaemia (ET): these secondary forms are defined post-PV or post-ET MF [147]. The most important features of this disorder is the clonal proliferation of a pluripotent haematopoietic stem cell [148], with the release from an abnormal stem cell population of several cytokines and growth factors into the bone marrow microenvironment; this machinery leads to an increase in bone marrow fibrosis but alsoto stromal changes, involvement of extramedullary organs such as the spleen and liver, and subsequent clinical manifestations [149]. The incidence of Myelofibrosis is about 0.58 new cases per 100 000 person-years; however, the prevalence is higher, about 6 per 100000 person-years because of chronic and disabling course.[150]The median age at diagnosis is 67 years, with similar distribution between the sexes. Median survival ranges from approximately 3.5 to 5.5 years, [151,152]. The principal cause of death in patients with MF is transformation to acute myeloid leukaemia (20%), but several patients die because of other disease-related events, such as progression without transformation, infections, and thrombo-haemorrhagic complications [151].

Clinical Manifestations

The most common clinical manifestation of MF is splenomegaly; it is estimated that more than 80% of patients with PMF present splenomegaly [151]. In a large retrospective analysis, one third of patients with PMF had marked splenomegaly, defined as palpable spleen length >10cm (153). Splenomegaly may cause simptoms of variable severity, ranging from early satiety and abdominal discomfort to severe abdominal pain, and potentially important complications as portal hypertension and splenic infarcts (154; 155). Other organs, particularly the liver, may be affected by extramedul-

lary hematopoiesis, and the occurrence of hepatomegaly is a major concern of palliative splenectomy [155,156]. Other classical disease manifestations are anemia, observed approximately in 50% of patients with PMF [153], and debilitating constitutional symptoms secondary to chronic cytokine-driven hypercatabolism (e.g., weight loss, fatigue) and inflammation (e.g., fever, pruritus, night sweats; [157,158]. Although the nature and severity of MF-related symptoms may vary substantially [157], overall symptom burden is a major contributor to the often observed poor QOL among patients with MF [158].

Diagnosis

The diagnosis of MF is currently based on the World Health Organization 2016 criteria, which include the *JAK2*V617F mutation that is detected in 50% to 60% of all cases [147]. Recently, several authors have described mutations in genes other than *JAK2*. *MPL* mutations, observed at a frequency of 5%-10% [159,160] and somatically acquired mutations in the *CALR* gene, with a reported frequency of 15%-20% [161,162]. However, about 10% of patients with MF do not present any known mutation and are considered to have 'triple-negative' MF [163]. Several other somatic mutations involving epigenetic processes (*EZH2*, *TET2*, *ASXL1*, and *DNMT3A*), spliceosome machinery (*SRSF2*, *SF3B1*, and *U2AF1*), and disease evolution have been identified in PMF patients [164,165,166]. Interstingly,mutations in *ASXL1*, *SRSF2*, and *EZH2* seem to predict short survival in a large cohort of patients. Tefferi et al [167] also reported that the *CALR*−/*ASXL1*+ profile is the most detrimental mutation profile in PMF. However, the genetic trigger of MF still remains unknown. The criteria for PMF diagnosis are resumed in table 2; the diagnosis requires all three major criteria and at least one of minor criteria.

Table 2. PMF diagnosis criteria

Major Criteria	Megacariocyte atypia and proliferation with fibrosis (reticulin and/or collagen)
	Absence of WHO criteria for other myeloid neoplasms
	Presence of JAK2, CALR or MPL mutations or othere clonal marker, in absence of reactive fibrosis
Minor Criteria	Leucoerythroblastosis
	Increased serum LDH
	Leucocytosis (<11000x10^9L)
	Palpable splenomegaly
	Anemia

Prognosis and Scoring Systems

MF prognosis could currently be predicted using 3 different prognostic scoring systems, which mainly refer to variables as age, constitutional symptoms, anaemia, white blood cell counts, and percentage of peripheral blood blasts. The scoring systems are the International Prognostic Scoring System (IPSS), which is applicable at diagnosis [151], and the Dynamic International Prognostic Scoring System (DIPSS)[168] and the DIPSS-plus, which can be applied at any time during follow up. The last scoring system incorporates 3 additional independent risk factors: red blood cell (RBC) transfusion requirement, platelet counts of $<100 \times 10^9$/L, and an unfavourable karyotype [169]. The scoring systems are resumed on Table 3.

Table 3. Prognostic scoring systems used in MF

Variables	IPSS	DIPSS	DIPSS-plus
Age >65 y	1	1	1
Constitutional symptoms	1	1	1
Hb<10g/dl	1	2	1
WBC count>25x10^9/L	1	1	1
Peripheral blood blast ≥1%1	1	1	1
PLT count<100x10^9/L	---	---	1
RBC transfusion need	---	---	1
Unfavourable karyotipe (+8, -7/7q, i(17q), -5/5q-, 12p-, inv(3), 11q23	---	---	1

Treatment

MF has historically been a disease orphan of curative treatments: allogeneic hematopoietic stem cell transplantation was the only treatment with a clearly demonstrated impact on disease progression; however, as predictable, treatment-related mortality is high and only a minority of patients are eligible for a such intensive and aggressive therapy [170]. Classically used treatments (Corticosteroid, Hydroxyurea, Alkylating agents, Epo, Metotrexate….) were palliative and able to achieve only limited benefits in terms of QoL and symptom control.

Recently, the discovery of *JAK2* mutations, that is proven to be involved on dysregulation of the *JAK-STAT* signaling pathway, consequently being a major contributor to the pathogenesis of MPNs, has dramatically changed the therapeutic scenario of MF [171,172]. In fact, the discovery of mutations leading to constitutive activation of the *JAK-STAT* signaling pathway raises hope that MF may be cured by selective *JAK1*/*2* inhibitors, as happens in the case of chronic myeloid leukemia treated with *BCR-ABL1* tyrosine kinase inhibitors. This hope has led to the development of small-molecule *JAK1*/*2* inhibitors, the first of which (Ruxolitinib) has recently been approved for the treatment of MF in the United States and Europe.

Ruxolitinib (Jakavi; Novartis, Basel, Switzerland) was the first *JAK1/2* inhibitor to become commercially available for the treatment of MF [173]. In preclinical *JAK2*V617F-positive MPN mouse models, Ruxolitinib induced a relevant downregulation of *JAK*-dependent proinflammatory cytokines, with evidence of drug-related antiproliferative and pro-apoptotic activities. Drug-exposed mice showed reduced splenomegaly.

Two phase III randomized studies addressed clinical applications of Ruxolitinib: COMFORT-I (Ruxolitinib vs placebo) and COMFORT-II (Ruxolitinib vs best available therapy[BAT]) [174,175]. The primary endpoint of both studies was a >35% reduction in spleen volume after 24 (COMFORT-I) or 48 weeks of treatment (COMFORT-II). The primary end-point was reached by, respectively, 41.7% and 28.5% of the patients treated with Ruxolitinib, as against, respectively, 0.7% and 0% of the patients receiving placebo or BAT ($P < 0.0001$) [174,175]. Overall, more than 90% of the patients enrolled in both studies experienced some reduction in spleen volume at some time during the follow-up, and the reduction remained stable in most of the patients after a median follow-up of 3 (COMFORT-I) and 5 years (COMFORT-II) [176,177].

Unlike the drugs previously used to treat MF, it is efficacious also in relieving constitutional symptoms. Several studies showed that Ruxolitinib is highly efficacious in reducing abdominal discomfort, appetite loss, itching, fatigue, and night sweats; and improving the QoL of most treated patients. Interestingly, the drug's activity seems to be independent of *JAK2* mutational status and not specific for the neoplastic clone, as the response rate is similar in patients with and without the *JAK2*V617F mutation.

Actually, Ruxolitinib is the only *JAK* inhibitor approved in the United States for the treatment of splenomegaly in subjects with intermediate/ high-risk MF and in Europe for the treatment of splenomegaly and/or constitutional symptoms in patients with intermediate-2/high-risk MF [178].

Further studies have investigated the efficacy of Ruxolitinib in patients at intermediate-1 risk. The phase II ROBUST study evaluated the safety and efficacy in patients with MF, including those at

intermediate-1 risk. The treatment was successful in 50% of the population as a whole and 57% of the intermediate-1–risk patients. Reduction in spleen length and symptoms was observed in all the risk groups, and improvements in the Myelofibrosis Symptom Assessment Form Total Symptom Score were seen in 80% of intermediate-1, 72.7% of intermediate-2, and 72.2% of high-risk patients [179]. Similarly, the phase IIIb JUMP trial found that the drug's safety and efficacy profile inintermediate-1-risk patients was consistent with that previously reported in intermediate-2-risk and high-risk patients [180].

These studies showed that Ruxolitinib has some relevant side effects and toxicities: in the COMFORT studies, thrombocytopenia was the dose-limiting toxicity, and anemia was the most common hematologic AE. The toxicity of Ruxolitinib treatment is mainly hematologic due to the drug's interference with an essential pathway for hematopoiesis. Another important issue during Ruxolitinib treatment was the incidence of infective complications. Several studies have shown that Ruxolitinib affects many cytokines and interferes with the immune process necessary for the pathogenesis of MPNs, but it also interfere with the function of various immune cells and may therefore facilitate opportunistic and non opportunistic pathogens [181,182]. It was shown that Ruxolitinib impairs natural killer cell differentiation and function and inhibits dendritic cell activation and migration; also, antigen specific T-cell responses are inhibited in a dose-dependent manner in vitro and in vivo.

Despite these warnings about increased infective risk [183, 184, 185], a recent update of the JUMP study described surprisingly a low incidence of infections. The all-grade infections observed in ⩾1% of patients included nasopharyngitis (6.3%), urinary tract infection (6%), pneumonia (5.3%), bronchitis (4.2%), herpes zoster (3.6%), influenza (3%), upper respiratory tract infection (2.9%), cystitis (2.5%), gastroenteritis (1.8%), respiratory tract infection (1.8%), and oral herpes (1.6%). Other infections included tuberculosis in 3 patients (0.3%) and *Legionella* pneumonia in 1 patient (0.1%). No hepatitis B reactivation was reported, and only 6 patients (0.5%) discontinued treatment

because of grade ⩾3 pneumonia [180]. Recently, our center described a case of hepatitis B reactivation in a patient treated with Ruxolitinib [183]. In the COMFORT-II study, patients treated with Ruxolitinib experienced higher rates of viral and bacterial infection than those receiving conventional therapy, but most of the infections were grade 1 or 2 and did not lead to any dose reductions or the discontinuation of trial medication. Furthermore, the rates of infection tended to decrease with longer exposure to the drug. However, as patients with MF are already predisposed to infections [186] and the long-term risks of Ruxolitinib treatment are still unknown, treated patients should be carefully monitored, and prophylaxis for herpes zoster or other infections should be considered on a case-by-case basis, depending on local risk. The impact of Ruxolitinib on overall survival is debated, with some authors reporting improved overall survival, and others showing no difference between BAT and Ruxolitinib treated patients.

Given its promising results, a further indication for Ruxolitinib treatment may be as a therapeutic bridge to allo-HSCT. Recently, an increasing number of reports appeared in the literature, describing the morphologic changes in the bone marrow occurring in Ruxolitinib treated patients, mostly focusing on modifications in bone marrow fibrosis degree [187, 188, 189, 190, 191]. Allogeneic hematopoietic stem cell transplantation is still the only intervention that has been shown to be a potential cure for MF or a means of prolonging the survival of these patients. Data from the most recent studies suggest that the expected 3-year progression-free survival rate in transplanted patients is in the range of 40% to 50% [192]. The introduction of reduced intensity conditioning regimens has recently made allo-HSCT applicable to a larger proportion of patients, [170] but decisions concerning the indication to allo-HSCT and require a considerable professional experience. The relevant problems are patient selection, donor selection, pre- and post-transplant management, conditioning regimen, prevention and management of post-transplant relapses and center experience. International prognostic scoring systems (ie, IPSS, DIPSS, and DIPSS-plus) [151,168,169] are the most comprehensive means of risk stratification currently available to guide

therapeutic decision making. The influence of driver mutations and the acquisition of additional mutations during the natural course of the disease may further refine this process, but this is still a matter of active investigation. All patients with MF aged <70 years with IPSS, DIPSS, or DIPSS-plus intermediate-2–risk or high-risk disease and a reasonable performance status, and without any significant competing comorbid conditions, should be considered potential candidates for allo-HSCT. Patients aged <65 years with intermediate-1-risk disease should only be considered candidates if they present with refractory, transfusion-dependent anemia or >2% of peripheral blood blasts, or adverse cytogenetics (as defined by the DIPSS-plus classification). Patients with low risk disease should not undergo allo-HSCT [193]. It is important to note that peripheral blood is considered the most appropriate source of hematopoietic stem cells in the case of HLA-matched sibling and unrelated donors. Splenectomy before allo-HSCT may favor disease eradication; some authors have also shown faster engraftment in splenectomized patients, but the pre-transplant use of splenectomy remains controversial as no study has yet prospectively evaluated the effect of protocol based splenectomy before transplantation. In the case of older patients and/or those with comorbidities, a less intense conditioning regimen seems to be more appropriate. Patients with advanced disease and a good performance status should undergo a more intensified regimen, but prospective studies are lacking [194]. Finally, in patients relapsing after stem cell transplantation with constitutional symptoms or splenomegaly, *JAK1*/*2* inhibitor treatment is recommended but remains experimental.

Aim of the work

Recent advances on CML and MF treatment, with the introduction respectively of TKI and JAK-STAT inhibitors, radically change the management of patients affected by these malignancies. Particularly, 8 years survival for patients with CML treated with Imatinib reaches 93%, while recent studies showed for patients treated with second generation TKIs as Dasatinib and Nilotinib similar overall survival with better progression free survival [90, 104, 109]. It was estimated that actually the prevalence of CML patients in USA is around 800000-100000; because of improved survival and very low mortality (e.g. 1%/year) in 2030 will be 1800000 patients affected by CML undergoing long-life treatment [80]. The monthly cost of each drug is substantial (Imatinib 3147,80 E, Nilotinib 4629,36 E, Dasatinib 6626,92 E) [194] and it's evident as a similar number of patients that underwent treatment would overcome any sanitary system. Recent studies showed that a proportion of CML patients (40-50%) remains in complete molecular remission after prolonged time without treatment with TKI. At now, there are no data to discriminate the patients who could safely interrupt the treatment, and so this strategy can't be applicable outside clinical trials, because of patients hesitation to interrupt efficacious drugs and physicians fear of relapse. To identify patients that could safely interrupt TKI therapy is a stringent need, to adequately manage sanitary resources.

About MF, while the impact of JAK-STAT inhibitors in terms of quality of live has been considerable, some studies questioned Ruxolitinib effect on overall survival [152, 177, 178, 179, 180]. Several studies showed that Ruxolitinib administration determines in most patients a great reduction of spleen volume and improvement of systemic symptoms, that are two of the factors greatly responsible of decreased quality of life in MF patients. At now, many international guidelines suggest Ruxolitinib treatment in MF patients with DIPSS score intermediate 2 or higher

[178]. However, patients treated with this drugs didn’t seem to beneficiate from therapy in terms of overall survival.

Therefore, as telomeres are greatly involved on stem cell proliferation regulation, I tried to investigate the relation between telomere length and these patients outcome, with the aim to discover new therapeutic targets and outcome predictive factors, to discriminate patients that could safely stop therapy, saving resources that could be used to treat patients who can’t interrupt treatment.

Part 1: Telomere length shortening is associated with treatment free remission in chronic myeloid leukemia patients

Background

Recent works have shown new insight on the potential of using telomere length as biomarker for diagnosis, disease progression, and prognosis of many types of hematological disorders. It's widely accepted that telomeres are specialized repeat structures of TTAGGG and nucleoprotein complexes localized at the ends of human chromosomes, playing a crucial role in cellular homeostasis by maintaining genome stability and integrity and protecting the cell from progressive DNA shortening, that happens during each round of DNA replication. Telomerase, the enzyme that polymerizes the successive TTAGGG sequence motifs, is highly expressed in cancer cells and confers to the cell a potential endless proliferative capacity or "immortalization" [2,3]. Yet, despite high telomerase activity, global telomere shortening can still occur in cancer [72]. Telomere biology has been more extensively studied in chronic myeloid leukemia (CML) than in any other blood cancer.

Myeloid cells from patients with CML show accelerated telomere shortening, likely because of the increased turnover of the BCR-ABL positive hematopoietic compartment. Interestingly, this shortening was found to be more accelerated in CML patients with a high-risk Hasford score at diagnosis than in low-risk patients, and telomere length correlated with the remaining time of chronic phase before disease progression [195].

The isolation and characterization of the leukemic stem cell (LSC) population has facilitated steady progress in understanding how such cells may attain an immortal phenotype. One prerequisite is the ability to maintain telomere length via expression of the widely conserved enzyme telomerase. It has been reported that CD34+ cells of CML patients present significant higher levels of telomerase

activity when compared to normal cell populations. According with several observations in solid tumors, the enhanced telomerase activity in CML is likely to be related to increased ratio of leukemic stem cells actively dividing in CML. Telomerase activity is increased in chronic phase CML, and further up-regulated during disease progression to accelerated phase and blast crisis [196]. Telomerase inhibition could represent a novel therapeutic approach in the treatment of myeloproliferative disorders, in association with TKIs. Successful therapy with TKI (Imatinib) was found to be associated with an increase in mean telomere length [197]: however, recent investigations reported persistent telomere reduction also in CML patients in sustained complete cytogenetic remission, virtually independent of which treatment was used to induce remission [198]. As underlined before, treatment discontinuation is a field of active investigation. Identifying biomarkers that could predict treatment free remission would be a major advance on chronic myeloid leukemia therapy. As yet, no studies have considered the possible association between telomere length and treatment-free remission (TFR) after discontinuation of therapy with tyrosine kinase inhibitors (TKIs).

Objective

My objective was to investigate the relation between telomere length and outcome in patients with CML. As prolonged survival and good quality of life after TKI introduction are not questionable, I tried to identify new factors that may help to discriminate patients who can safely stop treatment and can be defined “cured” from those who will relapse and in which the so-called “stop-therapy” may be a high risk procedure.

Patients and study design

My study focused on a population of chronic-phase CML patients who discontinued TKI treatment after achieving complete molecular remission (CMR) for at least 18 months. This group was selected within a population of over 100 patients followed on General Hematology Office of Hematology Department of R. Binaghi Hospital.

To select this group, I looked for patients who received Imatinib therapy for more than 24 months, as long term TKI exposure seems to be an important criteria to discriminate patients who can beneficiate from treatment suspension. I choose to include two patients with previous molecular relapse, successfully treated with Nilotinib second line therapy, who experienced a long term molecular remission. Finally, thirty-two patients were selected to be prospectively enrolled on the study. The median follow-up after discontinuation was 30 months (range 18–60). During follow up, I performed every three months clinical evaluation. In particular, I analyzed blood counts to investigate white cells increase, and physical exam to detect hepatomegaly and splenomegaly. I asked patients about systemic signs such fever, night sweat, chill, itch. Patients underwent blood sample to evaluate BCR/ABL transcript on peripheral blood. I defined complete molecular response (CMR) as undetectable breakpoint cluster region-Abelson (BCR/ABL1) by real-time quantitative polymerase chain reaction (qRT-PCR) with a sensitivity of the assay corresponding to molecular response MR4 and MR4.5. When a patient presented a loss of CMR, that means a detectable BCR/ABL1 transcript, I call back patients and perform a new sample within one months. If loss of CMR was confirmed, with increasing BCR/ABL transcript on peripheral blood, I considered it as impending relapse, and so I resume TKI treatment. If BCR/ABL transcript values seems stable, I used a wait and watch strategy, performing new evaluation on subsequent month, to investigate the trend. Instead, if BCR/ABL transcript was again undetectable, I considered the patient still in CMR, so I prosecuted follow up as scheduled.

Relative Telomere length was evaluated with a peripheral blood sample during follow up (median 26 months, range 18-30). In those patients who presented relapse early, telomere length was evaluated during relapse.

Sample characteristics

DNA extracted from cells previously was sorted from PB or BM of CML patients and delivered to our operating unit in our dry ice in an amount equal to 150 ng per patient.

PCR analysis of BCR-ABL transcript

QRT-PCR analyses were performed in the central laboratory at R. Binaghi Hospital. QRT-PCR of patient specimens, standard dilutions, and negative controls were analyzed in triplicate using a LightCycler instrument (Roche Diagnostics, Indianapolis, IN). BCR-ABL transcripts were amplified in 20 µl reactions containing 0.1 µl of cDNA; 10 mM Tris-HCl, pH 8.3; 50 mM of KCl; 4 mM MgCl2; 0.2 mM of each dNTP; 5 µg BSA; 1.25 U AmpliTaq Gold DNA polymerase (PE Biosystems, Foster City, CA); 300 nM of each primer; and 100 nM of probe. Similarly, ABL transcripts were amplified in order to compensate for differences in RNA integrity and cDNA synthesis efficiency. The final concentrations of primer and probe were 400 nM and 100 nM, respectively. The BCR-ABL p210 transcripts were amplified using previously published primer and probe sequences. The p190 and ABL transcripts were detected using the following primer and probe sequences: (p190 5') GCAGATCTGGCCCAACGAT, (p190 3') TCAGACCCTGA-GGCTCAAAGTC, and (p190 probe) 6FAM-CATGGAGACGCAGAAGCCCTTCAGC-TAMRA; (ABL 5') AAAATGACCCCAACCTTTTCG, (ABL 3') CCA TTCCCCATTGTGATTATAGC, and (ABL probe) 6FAMTCTAAGCATAACTAAAGGTGAAAAGCTCCGGGT CTT-TAMRA. Standard TaqMan™ PCR parameters (ABI PRISM 7700 SDS) were applied to all BCR-ABL and

ABL amplifications. The absolute quantities of BCR-ABL and ABL transcripts in patient specimens were determined by reference to standard curves. All standard curves were generated from 5-fold serial dilutions of CML cell line cDNA (ranging from 80 pg to 250 ng) containing the appropriate BCR-ABL transcript. Real-time RTPCR results were reported as a ratio or normalized quotient (NQ) of BCR-ABL/ABL. NQ values ≤ 0.0001 are below the level of detection with our assay and would be considered a 'molecular remission'. The sensitivity of each assay was between 10-6 and 10-7 for the p190 transcript and between 10-5 and 10-6 for both p210 transcripts.

PCR analysis of telomere length

The method of investigation for the determination of telomere length that currently allows greater flexibility, compared to the number of samples and to the possibility of automation, makes use of Quantitative PCR (q-PCR). The analysis in qPCR was performed according to the technique described by Cawthon in 2002 [199]. This method provides relative quantification of average telomere length in a genomic DNA sample, by measuring the number of telomere repeats (T) normalized to the single copy reference gene (S), and expressed as a T/S ratio. The procedure provides for the separate amplifications T and for the reference gene in single copy (the 36B4, a gene coding for the ribosomal phosphoprotein PO). As standard calibration was used the DNA of the cell line Raji (diploid cell line from a patient with Burkitt's lymphoma). As control samples inserted in each plate to check the repeatability of amplification, were used the DNA of the cell lines 1301 (human T-cell leukemia characterized by very long telomeres) and HL60 (human promyelocytic leukemia whose telomeres are particularly short). The standard curve was used to normalize for differences in DNA concentration between the telomere and single copy gene product. The amplification reaction, based on the use of the Sybr-Green, was performed in a volume of 25 μ l, in 96-well plates. To the mixture of amplification will be used SYBR Select Master Mix for CFX. In the reaction mixture was used 5 ng of DNA in a volume of 10 μ l for both the DNA of

the patients that for those of calibration and control. Each sample was analyzed in triplicate. The efficiency of the reaction was determined by means of a standard curve with scalar concentrations of DNA (20-10-5-2,5-1,25 ng) of the calibration sample. Each point of the curve was performed in quadruplicate. For the amplification mixture of telomeres were used the primers tel1b(For) 5′- CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT-3′ (270 nM) and tel2b(Rev) 5′-GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT -3′(900 nM). For gene amplification primers 36B4 were used 36B4u (For) 5′-CAG CAA GTG GGA AGG TGT AAT CC-3′ (300 nM) and 36B4d (Rev) 5′-CCC ATT CTA TCA TCA ACG GGT ACA A-3′ (500 nM).

The amplification was carried out in a thermocycler Bio-Rad C1000 equipped with the module CFX96. For amplification of telomeres was used the following profile: 2 min. at 95 ° C followed by 30 cycles at 95 ° C for 15 sec and then 54 ° C for 2 min. For amplification of the gene 36B4 profile provides 95 ° C for 2 min. followed by 30 cycles at 95 ° C for 15 sec. then 58 ° C for 1 min.

At the end of the amplifications was performed a cycle of melting to identify possible artifacts.

Data analysis

The data obtained from amplification were analyzed by the method of Livak-Schmittgen, also known as a method of 2-ΔΔCT. This method assumes that the amplification efficiency of the target gene (T) and gene reference (S) is close to 100% with a tolerance of ± 5%. If the amplification efficiency, monitored by means of the standard curve, within the parameters provided, it calculates the difference (ΔCt) between the threshold cycle of amplification of telomeres (CtTel) and that of the single-copy gene (Ct36B4). When the efficiency of the reaction is equal to 100%, the ratio T / S is 2-ΔΔCt. Normalizing for the ΔCt of the calibrator (ΔΔCtCal), T/S = 2-ΔΔCT. So the T / S ratio of the samples measures the variation of the relationship between the target gene and the one present in single copy compared to the calibration sample.

Age-corrected RTL (acRTL) represented the difference in telomere length between patients and age- and sex-matched controls.

Statistical analysis

The Kaplan-Meier method was used to calculate the cumulative probability of achieving TFR. The log-rank test compared the groups of patients according to RTL. Comparison between the 2 groups (patients achieving or not TFR) were analyzed using the Mann-Whitney U test or contingency tables, were appropriate. Pearson's correlation was used to investigate a possible linear association between age and telomere length. Moreover, RTL, age at diagnosis >45 years, gender, white blood cell (WBC) and platelet (PLT) counts, Sokal risk (low versus intermediate-high), previous interferon treatment, rapidity in obtaining CMR and overall treatment duration >60 months were included in univariate and multivariate analysis. Variables with a p-value lower than 0.2 in univariate analysis were included in multivariate analysis using a multi-step forward binary logistic regression model, where TFR was considered a dependent variable. P-values $\leq$ 0.05 were considered to be statistically significant.

Results

The characteristics of 32 chronic phase CML patients are shown in Table 4.

Table 4. Characteristics of patients enrolled in the study

	Age at diagnosis	Sex	Sokal score	Follow-up (months)	TKI therapy before stop (months)	Time to MR 4.5 (months)
1	42	M	Low	132	99	14
2	73	M	Intermediate	154	113	18
3	75	M	Intermediate	142	112	19
4	31	M	Low	111	67	7
5	63	M	Intermediate	177	106	53
6	64	M	Intermediate	203	100	84
7	66	M	Intermediate	127	96	19
8	66	F	Intermediate	128	98	6
9	47	M	Low	140	110	10
10	51	M	Low	192	141	93
11	58	M	Low	104	74	3
12	26	M	Low	120	94	6
13	54	F	Low	150	114	16
14	39	F	Low	51	45	11
15	74	M	High	94	35	7
16	71	F	Intermediate	52	32	10
17	37	M	High	160	143	85
18	68	F	High	102	66	48
19	34	M	Low	136	98	24
20	64	M	Intermediate	90	61	30
21	25	F	Intermediate	140	91	84
22	45	M	Intermediate	144	102	76
23	65	M	Intermediate	135	104	48
24	54	F	Intermediate	172	72	6
25	72	M	High	90	53	26
26	63	M	Low	164	127	72
27	56	M	Low	144	108	88
28	76	M	High	108	78	56
29	64	F	Intermediate	56	24	10
30	32	M	Low	120	36	24
31	37	M	Low	132	80	62
32	64	M	Low	102	24	17

Thirteen patients (41 %) showed loss of CMR during surveillance. All relapsed patients regained CMR after restarting treatment with TKIs. The 36-month cumulative probability of TFR was 59.4 %.). The majority of relapses occurred within 9 months of therapy interruption (mean 8.7 months, range 2–20).

Management of the patients

Patient 1: Treated with Imatinib. MR 4.5 obtained in 14 months. Therapy interruption after 99 months of TKI treatment. Still in CR after 132 months of follow-up

Patient 2: Treated with Imatinib. MR 4.5 obtained in 18 months. Therapy interruption after 113 months of TKI treatment. Still in CR after 154 months of follow up

Patient 3: Treated with Imatinib. MR 4.5 obtained in 19 months. Therapy interruption after 112 months of TKI treatment. Still in CR after 142 months of follow up

Patient 4: Treated with Imatinib. MR 4.5 obtained in 7 months. Therapy interruption after 67 months of TKI treatment. After 18 months from the interruption of treatment, detectable BCR-ABL transcript. I decided to perform a new sample after 30 days, that showed a little bit raising values of the transcript. A next assay performed at 20 months of follow up showed a significant increase of BCR-ABL transcript on peripheral blood. I prescribed therapy with 2nd generation TKI Nilotinib. The patient regained rapidly molecular remission and is still on treatment at 111 months of follow up.

Patient 5: Treated with Imatinib. MR 4.5 obtained in 54 months. Therapy interruption after 106 months of TKI treatment. Still in CR after 177 months of follow up

Patient 6: Treated with Imatinib. MR 4.5 obtained in 84 months. Therapy interruption after 100 months of TKI treatment. Still in CR after 203 months of follow up.

Patient 7: Treated with Imatinib. MR 4.5 obtained in 19 months. Therapy interruption after 96 months of TKI treatment. Still in CR after 127 months of follow up.

Patient 8: Treated with Imatinib. MR 4.5 obtained in 6 months. Therapy interruption after 98 months of TKI treatment. After 2 months from the interruption of treatment, detectable BCR-ABL transcript. I decided to perform a new sample after 30 days, finding a rapidly increasing BCR-ABL transcript. Patient asked to wait for another assay before reintroducing therapy; new control performed at 4 months from the interruption showed increasing values of BCR-ABL transcript on peripheral blood. I decided to reintroduce Imatinib because of comorbidities (diabetes, pleural effusion). Patient 8 regained CR and is still on treatment after 128 months of follow up.

Patient 9: Treated with Imatinib. MR 4.5 obtained in 10 months. Therapy interruption after 110 months of TKI treatment. After 4 months from the interruption of treatment, detectable BCR-ABL transcript. The next sample performed at 30 days showed rapidly increasing values of the transcript on peripheral blood. I decided to start therapy with 2nd generation TKI Dasatinib, because of favorable schedule (mono-administration). The patient regained rapidly CR and is still on treatment at 140 months of follow up.

Patient 10: Treated with Imatinib. MR 4.5 obtained in 93 months. Therapy interruption after 141 months of TKI treatment. After 6 months from the interruption of treatment, detectable BCR-ABL transcript. I prescribed a new sample at 30 days, but the patient missed the control to our center. The next evaluation performed at 8 months from the interruption of treatment confirmed loss of MR, and I start treatment with 2nd generation TKI Nilotinib.

Patient 11: Treated with Imatinib. MR 4.5 obtained in 3 months. Therapy interruption after 74 months of TKI treatment. Still in CR after 104 months of follow up.

Patient 12: Treated with Imatinib. MR 4.5 obtained in 6 months. Therapy interruption after 94 months of TKI treatment. Loss of MR at 5 months from treatment interruption. Because of rapidly increasing BCR-ABL transcript at 30 days evaluation, Imatinib was reintroduced. The patient regained CR, and is still under treatment at 120 months of follow up.

Patient 13: Treated with Imatinib. MR 4.5 obtained in 16 months. Therapy interruption after 114 months of TKI treatment. BCR-ABL transcript detectable at 7 months from treatment interruption.

Assays performed at 8 and 9 months from interruption showed raising values of the transcript. Nilotinib treatment reintroduced with new CMR. Patient still under treatment and in remission at 150 months of follow up.

Patient 14: Treated with Nilotinib because of previous molecular relapse during Imatinib treatment. Therapy interruption after 45 months of Nilotinib therapy. Loss of CMR at 1 month from treatment interruption, confirmed with the following assay performed at 2 months. I immediately resume Nilotinib treatment. The patient experienced a new rapid remission. Still under treatment at 51 months of follow up

Patient 15: Treated with Imatinib. MR 4.5 obtained in 7 months. Therapy interruption after 35 months of TKI treatment. Still in CR after 94 months of follow up.

Patient 16: Treated with Nilotinib, because of previous molecular relapse during Imatinib treatment. Therapy interruption after 32 months of Nilotinib therapy. Still in CR after 52 months of follow up.

Patient 17: Treated with Imatinib. MR 4.5 obtained in 85 months. Therapy interruption after 143 months of TKI treatment. Loss of CMR at 9 months from treatment interruption. Because of rapidly increasing values of transcript, treatment with Dasatinib was started. Patient regained a new CR, and is still under treatment at 160 months of follow up.

Patient 18: Treated with Imatinib. MR 4.5 obtained in 48 months. Therapy interruption after 66 months of TKI treatment. Loss of CMR at 17 months from treatment interruption, confirmed at the 18-month evaluation. I reintroduced Imatinib because of age of patient and fear for vascular side effects. The patient rapidly regains CR, and is still under treatment at 102 months of follow up.

Patient 19: Treated with Imatinib. MR 4.5 obtained in 24 months. Treatment interruption after 98 months of TKI treatment. Detectable BCR-ABL transcript with loss of CMR at 10 months from treatment interruption. Evaluation of peripheral blood at 11 months from interruption showed undetectable BCR-ABL transcript, as 12-months assay. Unfortunately, the evaluation performed at 13 months from treatment interruption showed again detectable transcript; this finding was confirmed by a new evaluation performed at 14 months from treatment interruption, so I decided to

start treatment with 2nd generation TKI Nilotinib. After 6 months of therapy the patient regained CR. Still in CR and under treatment at 136 months of follow up.

Patient 20: Treated with Imatinib. MR 4.5 obtained in 30 months. Treatment interruption after 61 months of TKI therapy. Loss of CMR at 10 months from treatment interruption, confirmed by 11-month evaluation. Imatinib was reintroduced, with a new CR. The patient is still under treatment and in CR with a follow up of 90 months.

Patient 21: Treated with Imatinib. MR 4.5 obtained in 84 months. Treatment interruption after 91 months of TKI therapy. Loss of CMR detected at 2 months from treatment interruption, confirmed by 3-month evaluation. Nilotinib treatment was started, with new CR. The patient is still under treatment and in CR 140 months of follow up.

Patient 22: Treated with Imatinib. MR 4.5 obtained in 76 months. Treatment interruption after 102 months of TKI therapy. Still in CR at 144 months of follow up.

Patient 23: Treated with Imatinib. MR 4.5 obtained in 48 months. Treatment interruption after 104 months of TKI therapy. Still in CR at 135 months of follow up.

Patient 24: Treated with Imatinib. MR 4.5 obtained in 6 months. Treatment interruption after 72 months of TKI therapy. Still in CR at 172 months of follow up.

Patient 25: Treated with Imatinib. MR 4.5 obtained in 26 months. Treatment interruption after 53 months of TKI therapy. Still in CR at 90 months of follow up.

Patient 26: Treated with Imatinib. MR 4.5 obtained in 72 months. Treatment interruption after 127 months of TKI therapy. Still in CR at 164 months of follow up.

Patient 27: Treated with Imatinib. MR 4.5 obtained in 88 months. Treatment interruption after 108 months of TKI therapy. Still in CR at 144 months of follow up.

Patient 28: Treated with Imatinib. MR 4.5 obtained in 56 months. Treatment interruption after 78 months of TKI therapy. BCR-ABL transcript detectable 12 months after TKI interruption. Transcript determination was stable at 13-month, 14-month and 15-month evaluation, then BCR-ABL was again undetectable. Still in CR at 108 months of follow up.

Patient 29: Treated with Imatinib. MR 4.5 obtained in 10 months. Treatment interruption after 24 months of TKI therapy. Still in CR at 56 months of follow up.

Patient 30: Treated with Imatinib. MR 4.5 obtained in 24 months. Treatment interruption after 36 months of TKI therapy. Still in CR at 120 months of follow up.

Patient 31: Treated with Imatinib. MR 4.5 obtained in 62 months. Treatment interruption after 80 months of TKI therapy. Loss of CMR at 10 months after therapy interruption. 11-month evaluation showed increasing values of BCR-ABL transcript, so I decided to start Nilotinib treatment. Patient regained CR and is still under treatment at 132 months of follow-up.

Patient 32: Treated with Imatinib. MR 4.5 obtained in 17 months. Treatment interruption after 24 months of TKI therapy. Still in CR after 102 months of follow up.

The differences between patients with sustained TFR and relapsing are summarized in table 5.

Table 5. Univariate analysis of difference between patients relapsing and patient with sustained treatment free remission

	Patients in TFR N=19	Patients relapsing N=13	p-value
Age at diagnosis (mean, range)	74 (47–88)	56 (37–77)	0.004
Leucocytes at diagnosis X 103/uL (mean, range)	50.47 (8.15–221)	69 (19.8–263)	Ns
Platelets at diagnosis ×103/uL (mean, range)	472 (178–918)	357 (171–695)	Ns
Months to CMR (median, range)	28 (3–88)	30 (6–93)	Ns
Months of TKIs (median, range)	86 (24–127)	84 (45–143)	Ns
Months of TKIs >60 (no., %)	13(68.4)	12 (92.3)	Ns
Male gender (no., %)	16 (84.2)	8 (61.5)	Ns
Sokal risk (no., %)			Ns
Low	6(31.6)	8 (61.5)	Ns
Intermediate	10 (52.6)	3 (23.1)	Ns
High	3 (15.8)	2 (15.4)	Ns
First-line TKI treatment (no., %)	11 (57.9)	11 (84.6)	Ns
Previous IFN treatment (no., %)	8 (42.1)	2 (15.4)	Ns

Imatinib first-line TKI treatment (no., %)	19 (100)	13 (100)	Ns
Nilotinib second-line TKI treatment (no., %)	1 (5.3)	1 (7.7)	Ns
Age-corrected relative telomere length (mean ± SD)	0.01 ± 0.14	0.20 ± 0.21	0.01

Relative Telomere Length

Overall, median RTL was slightly shorter in patients than in controls (0.97 vs 1.05). The median value of acRTL in the CML cohort was 0.09 (range −0.26, +0.86). Although the male gender was more frequent in TFR patients, we did not find any significant difference in telomere length between male and female. acRTL was not related to Sokal Risk score, nor to timing to obtain MR 4.5. TKI treatment duration was not related to acRTL. The Mann-Whitney Utest showed shorter acRTL in TFR patients compared to patients with molecular relapse (mean ± SD = 0.01 ± 0.14 vs 0.20 ± 0.21;p= 0.01) .Patients were stratified according to the median value of acRTL≤0.09. TFR was significantly higher in CML patients with acRTL≤0.09 in comparison to those with longer telomeres (78.9 vs 30.8 %, p= 0.002) (Fig. 2).

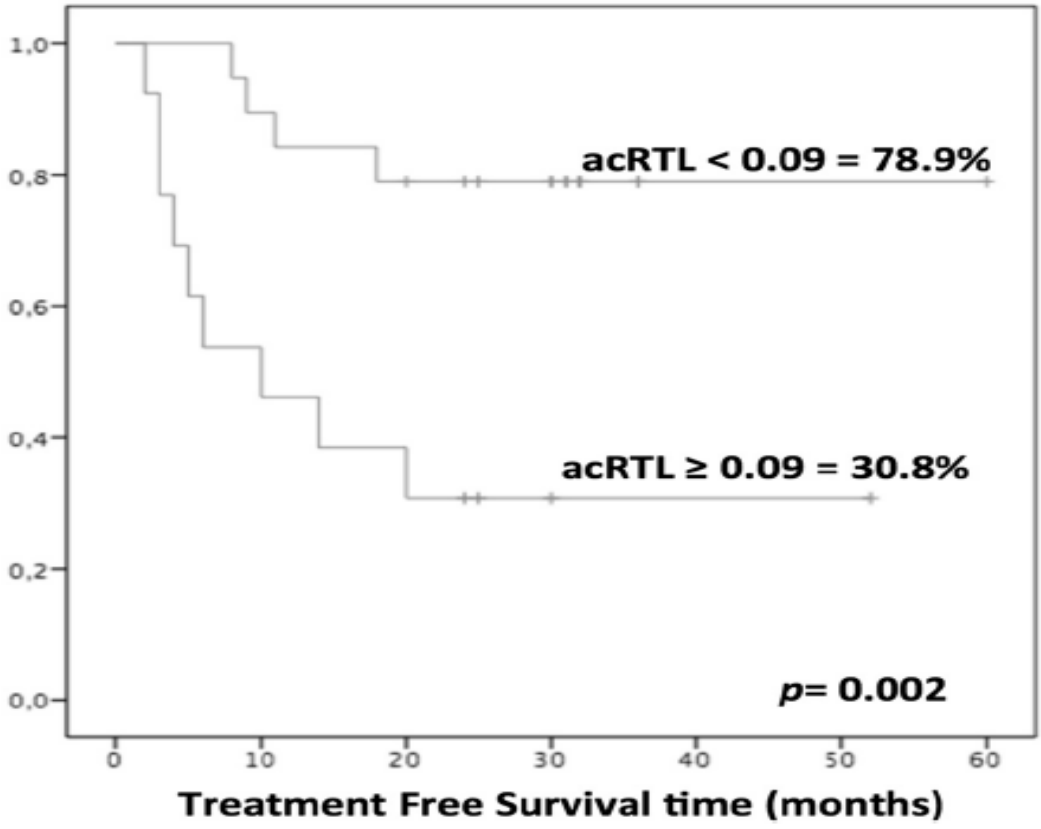

Fig 2. Treatment free remission according to age corrected relative telomere length (acRTL) ≥0.09 in 32 CML patients

Discussion

Previous studies suggest a dual-step model for telomere length changes in CML. In the earlier phases, increased turnover of Philadelphia positive (Ph+) progenitors seems to result in telomere shortening, leading to genetic instability. Later, CML cells likely escape senescence and apoptosis through upregulation of telomerase and restored telomere length. It is likely that this machinery promotes the occurrence of genetically unstable CML sub-clones with a selective growth advantage [197]. Discontinuation of TKIs is the next hurdle to be overcome in the management of CML patients. Several factors have been identified as potentially capable of predicting durable TFR and hopefully definitive recovery [145,200]. A significant correlation between younger age and molecular relapse was reported [201]. In our study, CML patients had a slightly shorter telomere length than healthy controls and we found a statistically significant correlation between aging and telomere shortening. However, the most interesting finding was that patients with sustained TFR showed significantly shorter acRTL compared to molecular relapses. A possible explanation is that quiescent CML stem cells harboring longer telomeres are prone to escape senescence mechanisms and maintain a proliferative potential even after discontinuation of imatinib treatment; however, this hypothesis should be supported by CML stem cell telomere assessment in patients with molecular response.

Some limitations need to be noted in this study. First, the cohort of patients was relatively small and a longitudinal telomere assessment from diagnosis is lacking. Furthermore, myeloid compartment sorting was not executed to determine telomere length; however, previous reports showed that no significant differences in CML telomere lengths are observed when comparing peripheral mononuclear blood cells, fractionated peripheral neutrophils, and non-fractionated bone marrow mononuclear cells [198]. In conclusion, the present study is the first to suggest that patients with longer telomeres would seem to be more susceptible to relapse after TKI treatment. This findings is relevant because for the first time a genetic biomarker predictive of TFR has been evidenced in

CML patients. Furthermore, because patients with longer telomeres seem to be prone to molecular relapse after TKI treatment interruption, this data focus on telomerase as new potential therapeutic target. This hypothesis deserves to be investigate in further studies.

Part 2: Ruxolitinib therapy and telomere length in myelofibrosis

Background

Some authors recently observed reduced telomere length in patients with Myelofibrosis, apparently without relation with previous treatments [202, 203, 204]; this finding suggest a possible prognostic relevance for telomere length as a biomarker in this patient population. Actually, prognosis in MF patients can be predicted using scores as IPSS, DIPSS, DIPSS plus [149, 151, 152, 168, 169]. DIPSS and DIPSS plus introduced variables as blood transfusion requirement and cytogenetics, and first allow evaluation during time, as these scores could be used during patient follow up. This point is relevant, as myelofibrosis seems to be a dynamic disease, that typically starts with a chronic phase or even a pre-fibrotic phase, and further evolves in advanced disease.

Actually, no studies have investigated telomere dynamics in patient with myelofibrosis.

As stated before, the introduction of Ruxolitinib has dramatically changed the clinical management of patients affected by MF. Ruxolitinib is a JAK 1/2 inhibitor approved for the treatment of intermediate-2 and high risk MF, primary or post-polycythemia vera (PV) and essential thrombocythemia (ET). Several studies showed that Ruxolitinib treatment significantly improves quality of life and systemic symptoms. Interestingly, the presence of JAK2 V617F mutation seems to be not essential for the response to treatment; in fact, patients with unmutated JAK2 seem to have similar improvement in quality of life and systemic symptoms than patients with JAK2 mutation. The reason for this phenomenon has still to be investigated [175, 176, 177].

Patients and study design

My study focused on a population of 11 patients affected by Myelofibrosis. Eight patients had primary MF, and three patients were affected by a secondary MF, evolving from Essential Thrombocythemia. This group was selected from a population of over 40 patients followed General Hematology Office of Hematology Department of R. Binaghi Hospital.

To select this group, I searched for patient eligible for Ruxolitinib treatment, with a life expectancy of almost 12 months that could be prospectively evaluated for telomere dynamics. I included also patient who were already in treatment with Ruxolitinib but for whom was available a DNA sample before treatment start. I opted to investigate only patients eligible for Ruxolitinib treatment, with the aim of studying a homogenous population. In fact, MF is a complex disease, and patient affected from this malignancy may have important differences in terms of risk of transformation, previous treatment, comorbidities. Because of the relatively small numbers of the cohort of MF in our center, I tried to minimize confounding factors.

The treatment schedule was with 15 mg or 20 mg of oral Ruxolitinib twice daily (BID), according to baseline platelet counts (100 000/μl to 200 000/μl or200 000/μl, respectively).

I escalated the drug dose to 25 mg BID in patients with an inadequate response and reduced when platelet counts dropped to100 000/μl. I interrupted the treatment when platelet levels fell below 50 000/μl.

Telomere length was assessed with a peripheral blood sample before treatment with Ruxolitinib, and at a median of 1000 days after treatment. Peripheral blood samples were also collected from 11 age-and sex-matched controls from a larger database of 100 healthy subjects.

PCR analysis of telomere length

The method of investigation for the determination of telomere length that currently allows greater flexibility, compared to the number of samples and to the possibility of automation, makes use of Quantitative PCR (q-PCR). The analysis in qPCR was performed according to the technique described by Cawthon in 2002 [199]. This method provides relative quantification of average telomere length in a genomic DNA sample, by measuring the number of telomere repeats (T) normalized to the single copy reference gene (S), and expressed as a T/S ratio. The procedure provides for the separate amplifications T and for the reference gene in single copy (the 36B4, a gene coding for the ribosomal phosphoprotein PO). As standard calibration was used the DNA of the cell line Raji (diploid cell line from a patient with Burkitt's lymphoma). As control samples inserted in each plate to check the repeatability of amplification, were used the DNA of the cell lines 1301 (human T-cell leukemia characterized by very long telomeres) and HL60 (human promyelocytic leukemia whose telomeres are particularly short). The standard curve was used to normalize for differences in DNA concentration between the telomere and single copy gene product. The amplification reaction, based on the use of the Sybr-Green, was performed in a volume of 25 µ l, in 96-well plates. To the mixture of amplification will be used SYBR Select Master Mix for CFX. In the reaction mixture was used 5 ng of DNA in a volume of 10 µ l for both the DNA of the patients that for those of calibration and control. Each sample was analyzed in triplicate. The efficiency of the reaction was determined by means of a standard curve with scalar concentrations of DNA (20-10-5-2,5-1,25 ng) of the calibration sample. Each point of the curve was performed in quadruplicate. For the amplification mixture of telomeres were used the primers tel1b(For) 5′- CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT-3′ (270 nM) and tel2b(Rev) 5′- GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT -3′(900 nM). For gene amplification primers 36B4 were used 36B4u (For) 5′-CAG CAA GTG GGA AGG TGT AAT CC-3′ (300 nM) and 36B4d (Rev) 5′-CCC ATT CTA TCA TCA ACG GGT ACA A-3′ (500 nM).

The amplification was carried out in a thermocycler Bio-Rad C1000 equipped with the module CFX96. For amplification of telomeres was used the following profile: 2 min. at 95 ° C followed by 30 cycles at 95 ° C for 15 sec and then 54 ° C for 2 min. For amplification of the gene 36B4 profile provides 95 ° C for 2 min. followed by 30 cycles at 95 ° C for 15 sec. then 58 ° C for 1 min.

At the end of the amplifications was performed a cycle of melting to identify possible artifacts.

Data analysis

The data obtained from amplification were analyzed by the method of Livak-Schmittgen, also known as a method of 2-ΔΔCT. (5) This method assumes that the amplification efficiency of the target gene (T) and gene reference (S) is close to 100% with a tolerance of ± 5%. If the amplification efficiency, monitored by means of the standard curve, within the parameters provided, it calculates the difference (ΔCt) between the threshold cycle of amplification of telomeres (CtTel) and that of the single-copy gene (Ct36B4). When the efficiency of the reaction is equal to 100%, the ratio T / S is 2-ΔΔCt. Normalizing for the ΔCt of the calibrator (ΔΔCtCal), T/S = 2-ΔΔCT. So the T / S ratio of the samples measures the variation of the relationship between the target gene and the one present in single copy compared to the calibration sample.

Age-corrected RTL (acRTL) represented the difference in telomere length between patients and age- and sex-matched controls.

Statistical Analysis

Related samples Wilcoxon signed-rank test was used to compare mean acRTL between patients and healthy controls. The Mann-Whitney U Test was used to compare patients with High and Int-2 IPSS in relation to telomere length. Univariate and multivariate analyses included the following parameters: primary MF, presence of the JAK2 V617F mutation, high IPSS score, a decrease in splenomegaly of >50%, >50% bone marrow (BM) cellularity before and after treatment, duration of treatment>1000 days and total drug dose of>22 g. Variables with a P-value lower than 0.2 in univariate analysis were included in multivariate analysis using a multi-step forward binary logistic regression model, where RLT>15% from baseline was considered a dependent variable.

Results

The median age at diagnosis was 72 years (range 53-83). The JAK2V617F mutation was detected in seven patients, while CALR and MPL were found in two and one patient, respectively. One patient was triple negative. All patients had splenomegaly with a median enlargement of 17 cm below the costal margin. Based on the IPSS scores, six patients were assigned to the intermediate-2 risk category and five to the high risk category. Ruxolitinib was administered for a median of 1000 days (range 113–1152).

The characteristics of patients included in the study are resumed in Table 6.

Table 6. Characteristic of patients enrolled in the study

	Age	Mutations	IPSS	Bone marrow cellularity before treatment	Days of Ruxolitinib Treatment	RTL
1	65	JAK2+	Int-2	90%	899	0,85
2	54	JAK2+	Int-2	90%-100%	1470	1,4
3	82	CALR+	High	5%	852	1,13
4	76	JAK2+	Int-2	70%	1360	0,58
5	85	JAK2+	High	90%	1112	0,82
6	72	JAK2+	Int-2	80%	1370	0,99
7	81	JAK2+	High	100%	1005	0,77
8	84	MPL+	High	50%	1355	1,31
9	68	CALR+	High	IPO	1470	1,05
10	57	JAK2+	Int-2	70%	113	1,05
11	69	-	Int-2	90%	820	1,97

Overall, patients received a median of 22 g of Ruxolitinib (range 4.6-44.5). All patients showed improvement in constitutional symptoms and quality of life. Median weight gain was 7 kg (range 4-14 kg). Splenomegaly decreased by 60% (range 20-100%).

Management of the patients

Patient 1: I started Ruxolitinib at 20mg for 2 times/daily. After 30 days of treatment, because of decreasing platelet count (70.000/mm3) I reduced dose to 10 mg twice daily. After 60 days, with platelet count >200000/mm3, I increased Ruxolitinib dose to 20 mg twice daily. Still in treatment, stable disease. Improvement of systemic signs and splenomegaly (100% reduction)

Patient 2: I started Ruxolitinib at 20 mg for 2 times/daily. After 6 months of treatment, because of decreasing platelet count, I reduced the dose to 15 mg twice daily. After seven months at this dose, I

tried to increase the dosage to 20 mg twice daily. However, after 6 months, because of thrombocytopenia (80000/mm3) I have to reduce the dose newly to 15 mg twice daily. Still in treatment, stable disease. Improvement of systemic signs and splenomegaly (60% reduction).

Patient 3: I started Ruxolitinib at 20 mg for 2 times/daily. After 7 months of treatment, because of decreasing platelet count (87000/mm3), I reduced the dose to 10 mg twice daily. Next, after 60 days at this dosage, I increased the dose to 15 mg twice daily. The patient had need for blood transfusion, that persists after Ruxolitinib treatment. Still in treatment, stable disease. Improvement of systemic signs and splenomegaly (50% reduction).

Patient 4: I started Ruxolitinib at 15 mg for 2 times/daily because of a platelet value of 120000/mm3. After 30 days of therapy, I found a platelet value of 85000/mm3, and I decreased Ruxolitinib to 10 mg twice daily. After 30 days of therapy, because of decreasing platelet values, I further reduce the dose to 5 mg twice daily for three months. Then I tried to increase the dose to 10 mg twice daily for 15 days, but a new drop on platelet count forced me to resume 5 mg dosage. In the following months I tried two times to increase the Ruxolitinib dose, but with 10 mg dose the patient presented immediately worsening thrombocytopenia. During treatment moderate improvement of systemic signs and splenomegaly (20% reduction). Loss of response and therapy interruption after 1360 days of treatment.

Patient 5: I started Ruxolitinib at 20 mg for 2 times/daily. Still in treatment, stable disease. Improvement of systemic signs and splenomegaly (70% reduction).

Patient 6: I started Ruxolitinib at 20 mg for 2 times/daily. After 14 months of therapy, because of worsening thrombocytopenia (90000/mm3), I reduced the dose to 10 mg twice daily. After 5 months at this dosage, concomitantly with an infection, worsening thrombocytopenia occurs (50000/mm3); I reduced the dose to 5 mg twice daily. After the infection resolution, I increased the dosage to 15 mg twice daily, that he's actually doing. Still in treatment, stable disease. Improvement of systemic signs and splenomegaly (60 % reduction).

Patient 7: I started Ruxolitinib at 15 mg for 2 times/daily because of a platelet value of 136000/mm3. After 9 months of treatment, I reduced the dose because of thrombocytopenia to 15 mg twice daily. Because of worsening values, after 30 days of therapy, I further reduce to 10 mg twice daily. After 30 days, I increased the dose to 15 mg twice daily, but I could maintain this dosage only for 6 months because of new finding of decreasing platelet count (70000/mm3). A new control after 15 days showed however platelet count 49000/mm3. I decided to don't stop the treatment, reducing only the dose to 5 mg twice daily. I started investigations with a bone marrow sample, that showed a leukemic blast count of 26%, that means evolution to acute myeloid leukemia. The patient was addressed to our acute leukemia referral for further treatment.

Patient 8: I started Ruxolitinib at 20 mg for 2 times/daily. After 10 months, I reduce to 10 mg twice daily because of platelet value of 90000/mm3. At the 30 day control, the blood count showed platelet 135000/mm3, so I increased the dosage to 15 mg twice daily. The platelet count progressively decreased over the following three months to 85000/mm3, so I decided to pass to 10 mg twice daily dosage. Still in treatment at this dosage. Improvement of systemic signs and splenomegaly (70% reduction).

Patient 9: I started Ruxolitinib at 20 mg for 2 times/daily. Because of lack of response, after three months of treatment I decided to increase dosage to 25 mg x twice daily. With the new dosage the patient experienced remission of systemic signs and reduction of splenomegaly; however, platelet count dropped from over 300000 mm3 to 140000/mm3, so I decided to reduce the dose to 20 mg twice daily. The patient maintained symptoms control, however platelet count progressively decreased to 88000/mm3 during the following 8 months, so I reduced further to 15 mg twice daily. After 5 months, ensured by a stable platelet value of 180000/mm3, I could increase the dose to 20 mg twice daily. During treatment, improvement of systemic signs and splenomegaly (90% reduction). Loss of response and treatment interruption after 1460 days of therapy.

Patient 10: I started Ruxolitinib at 10 mg for 2 times/daily because of a platelet count of 90000/mm3. Because of improvement of platelet count, after 3 months of treatment I could increase the dose to 15 mg twice daily and next to 20 mg twice daily after 30 days. After three months at this dosage, the patient presented systemic signs and worsening thrombocytopenia. During treatment improvement of systemic signs and splenomegaly (40% reduction). Loss of response and treatment interruption after 113 days of therapy.

Patient 11: I started Ruxolitinib at 15 mg for 2 times/daily because of a platelet count of 140000/mm3. Still in treatment at this dose. During treatment, improvement of systemic signs and splenomegaly (50% reduction). Clinical changes after Ruxolitinib treatment are resumed on Table 7.

Table 7. Clinical changes after Ruxolitinib treatment

	Spleen reduction (%)	BM cellularity after treatment (%)	Evolution to AML
1	100	70	No
2	60	70	No
3	50	5	No
4	20	50	No
5	70	70	No
6	60	50	No
7	50	40	Yes
8	70	30	No
9	90	IPO	No
10	40	20	No
11	50	70	No

Telomere length

Related samples Wilcoxon signed-rank test performed before treatment with Ruxolitinib showed that the mean RTL was shorter in patients compared with age-and sex-matched healthy controls (1.08 vs1.26, respectively; P= 0.09). I found that IPSS score was significantly related to telomere length, as the Mann-Whitney Utest showed shorter acRTL in MF patients with high IPSS compared to patients with intermediate-2 IPSS (mean ± SD = 1.016 ± 0.22 vs 1.34 ± 0.14; p= 0.03)

After treatment, median RTL increased significantly (1.30 vs 1.08; P= 0.018), showing overlapping values with the healthy controls (Figure 3). Median RTL elongation from baseline was 15%. Only pretreatment BM cellularity of >50% significantly correlated with >15% telomere elongation (P= 0.004).

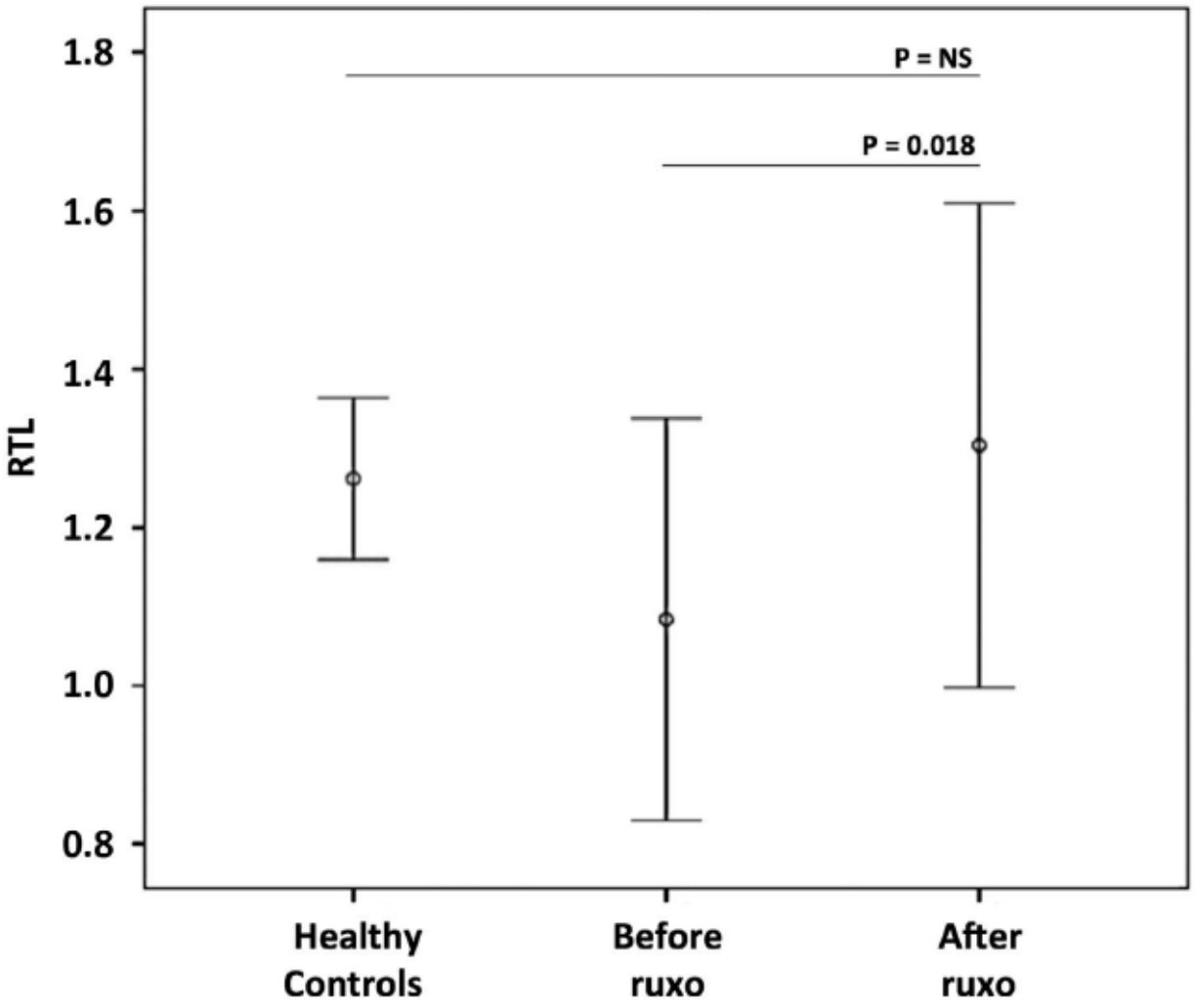

Fig.3 Telomere elongation after Ruxolitinib treatment

Discussion

In this small cohort of patients, I investigate the dynamics of telomeres in myelofibrosis patients treated with Ruxolitinib. The principal finding was that telomere length was restored to normal values after treatment with Ruxolitinib. This observation could stem from a non-specific anti-cytokine action or qualitative changes in clonal hematopoiesis. Indeed, it is possible that Ruxolitinib mediates modulation of the BM microenvironment [205], thereby stimulating stem cell hematopoiesis. Moreover, It has been demonstrated that oxidative stress and in inflammation contributes to a significant decrease in telomerase activity causing telomere shortening. Ruxolitinib suppresses pro-inflammatory cytokines through interference with JAK-signal transducer and activator of transcription (STAT) signaling [206] and thus it's possible that this machinery may reverse a potential mechanism of telomere shortening. Curiously, baseline bone marrow cellularity >50% seems to be related to telomere elongation subsequent to Ruxolitinib administration. This finding could suggest that patient with a preserved cellularity on bone marrow may be different from others in terms of susceptibility to treatments. No differences in terms of telomere elongation were noted between patients with JAK2V617F mutation and patient with others or any mutations. However, the small numbers of the studied population may have masked weaker associations, and analysis of larger cohorts may reveal different impact of Ruxolitinib on telomere elongation between patients with JAK2V617F mutation and those with wild-type gene. This study confirms that patients with Myelofibrosis have shorter telomeres than healthy people, as reported by other authors [203]. A new interesting finding is that baseline acRTL is related to IPSS, with patients with high IPSS having shorter telomeres than those with Intermediate-2 IPSS. This finding, if confirmed in larger cohorts, could indicate a relation between telomere length and high risk features of myelofibrosis; telomere length may be a useful biomarker, with the advantage to not rely on clinical assessment and evaluation of symptoms that may be under or overestimate by patients and clinicians. Despite the uniqueness of this study some limitations need to be noted. First of all, the

cohort of patients was relatively small and I can't determine sorted myeloid compartment telomere length. However, previous reports showed that unsorted peripheral blood telomere length is a good surrogate of myeloid compartment telomere length. In conclusion, these findings for the first time showed that telomeres are involved in Myelofibrosis natural history, and that Ruxolitinib treatment could restore telomere length in exposed patients. This study suggest that some pathways in Myelofibrosis patients interfere with telomere length. This is a speculative hypothesis, but it's likely that telomeres and telomerase may deserve to be investigate as therapeutic targets on myelofibrosis patients.

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