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TELOMERES AND TELOMERASE AND THEIR FUNCTIONAL APPLICATIONS IN MYELOPROLIFERATIVE NEOPLASMS AND ACUTE MYELOID LEUKEMIA

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TELOMERES AND TELOMERASE AND THEIR FUNCTIONAL APPLICATIONS IN MYELOPROLIFERATIVE NEOPLASMS AND ACUTE MYELOID LEUKEMIA

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Myeloproliferative neoplasms (MPNs) are a group of diseases characterized by hyperproliferation in the myeloid lineages of the bone marrow, leading to increased levels of circulating mature blood cells from one or more lineages. MPNs consist of polycythemia vera, essential thrombocythemia and primary myelofibrosis. Several recurrent mutations are seen in MPNs, most of them resulting in an activation of the JAK/STAT signaling pathway. Telomeres are non-coding repetitive sequences of DNA located at the end of chromosomes. The telomeres are shortened with every cell division and when they become critically short, the cells enter a permanent growth arrest state called replicative senescence. When the telomeres are very short, the cells become genetically unstable and are more susceptible to genetic aberrations. There is accumulating evidence for a role for telomere dysregulation in the pathogenesis of MPNs and AML, and the overall aim of the studies included in this thesis was to better define this role.

To clarify a potential dysregulation of telomeres and telomere associated proteins in MPNs, we studied the telomere length (TL), TERT expression and expression of telomere associated proteins in 81 patients with MPNs and 43 healthy controls. We found that patients with MPNs have shorter telomeres in their granulocytes compared to that of healthy controls, but also compared to the patients' own lymphoid cells. There was no difference in TERT expression between patients and controls. The expression of two positive regulators of TL was lower, and the expression of two negative regulators of TL was higher in patients with MPNs. The dysregulation of telomere binding proteins may contribute to the telomere shortening seen in MPNs.

Genetic variants in the *TERT* locus are implicated in susceptibility to cancer and other diseases. Recent reports also revealed an association between the SNP *TERT* rs2736100_CC genotype and the risk of developing MPNs in Caucasian populations. We genotyped patients and healthy controls from Sweden and China and found that the *TERT* rs2736100_C allele is associated with an increased risk of MPN development in both populations. The association of the C-allele with an increased risk of MPNs was only seen in male MPN patients, who generally have a worse outcome than female MPN patients. Moreover, the Chinese healthy population had significantly lower frequency of the *TERT* rs2736100_C allele compared to their Swedish counterpart, which may contribute to the lower MPN incidence seen in China compared to that in Europe. Patients with the *TERT* rs2736100_CC had the highest TERT expression, which may make them more susceptible to develop MPNs.

The evolution of an acute promyelocytic leukemia (APL)-clone in a patient with a very late relapse was studied to distinguish between a relapse of the first APL, a secondary APL or a second *de novo* APL, and thereby guide future treatment decisions. Based on identical breakpoints of the PML-RARα gene, but differences in genetic aberrations and mutations in the FLT3-gene, we conclude that the patient most likely suffered a true relapse of her initial APL. We hypothesize that the PML-RARα-bearing pre-leukemic clone survived the initial chemotherapy and did not develop into an APL until seventeen years later, when the clone acquired another FLT3 mutation and other genetic aberrations.

JAK2 inhibitors have proven effective in reducing symptoms and splenomegaly in patients with myelofibrosis, but they do not eliminate the disease initiating clones. A telomerase inhibitor is in clinical trials for MF with promising results, but with severe myelosuppression as a side-effect. We studied the effect of the JAK2 inhibitor LY2784544 in combination with the telomerase inhibitor GRN163L in a $JAK2^{V617F}$ –bearing erythroleukemia cell line. The combination had a larger effect on viability and number of cells than either of the drugs alone. Treatment with LY2784544 alone increased the fraction of HEL cells expressing the stem cell marker CD34, an effect that was partially mediated by an up-regulation of the transcription factor KLF4. Importantly, accumulation of CD34 positive cells was not seen after combined LY2784544/GRN163 treatment. This suggests that combining JAK2- and telomerase inhibition may have a therapeutic benefit, and that KLF4 may be a potential therapeutic target in MPNs. Furthermore, JAK2 inhibition reduced the telomerase activity, indicating a direct effect of JAK/STAT signaling on telomere regulation in MPNs.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. **Dahlström J**, Zhang X, Ghaderi M, Hultcrantz M, Björkholm M, Xu D. *Dysregulation of shelterin factors coupled with telomere shortening in Philadelphia chromosome negative myeloproliferative neoplasms.* Haematologica. 2015; 100, e402-e405; doi: 10.3324/haematol.2015.125765

II. **Dahlström J**, Liu T, Yuan X, Saft L, Ghaderi M, Wei Y B, Lavebratt C, Li P, Zheng C, Björkholm M, Xu D. *TERT rs2736100 genotypes are associated with differential risk of myeloproliferative neoplasms in Swedish and Chinese male patient populations.* Annals of Hematology. 2016; 95, 1825–1832; doi: 10.1007/s00277-016-2787-7

III. Zhang X, Zhang Q, **Dahlström J**, Tran A-N, Yang B, Gu Z, Ghaderi M, Porwit A, Jia J, Derolf Å, Xu D, Björkholm M. *Genomic analysis of the clonal origin and evolution of acute promyelocytic leukemia in a unique patient with a very late (17 years) relapse.* Leukemia. 2014; 28, 1751–1754; doi:10.1038/leu.2014.113.

IV. **Dahlström J**, Björkholm M, Xu D. *JAK2 inhibition in JAK2^{V617F}-bearing leukemia cells enriches CD34 positive leukemic stem cells, an effect abolished by the telomerase inhibitor GRN163L.* In manuscript. 2016.

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LIST OF ABBREVIATIONS

ALT	Alternative lengthening of telomeres
AML	Acute myeloid leukemia
AP2	Activating protein 2
APC	Allophycocyanin
APL	Acute promyelocytic leukemia
ATO	Arsenic trioxide
ATRA	All-*trans*-retinoic acid
BM	Bone marrow
CR	Complete remission
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
ET	Essential thrombocythemia
ER	Estrogen receptor
FISH	Fluorescence in situ hybridization
FITC	Fluorescein isothiocyanate
FLT3	Fms Related Tyrosine Kinase 3
HIF-1	Hypoxia inducible factor-1
HSC	Hematopoietic stem cells
HU	Hydroxyurea
IDH1/2	Isocitrate dehydrogenase 1/2
ITD	Internal tandem duplication
JAK2	Janus kinase 2
KLF4	Krüppel-like factor 4
LSC	Leukemic stem cell
MDS	Myelodysplastic syndromes
MF	Myelofibrosis
MPN(s)	Myeloproliferative neoplasm(s)

P53	Tumor protein P53
PCR	Polymerase chain reaction
PMF	Primary myelofibrosis
POT1	Protection of telomeres 1
PV	Polycythemia vera
RAP1	Repressor/Activator Protein 1
RNA	Ribonucleic acid
RUNX1	Runt-related transcription factor 1
SNP	Single nucleotide polymorphism
SOCS3	Suppressor of cytokine signaling
TER	Telomerase RNA component
TERT	Telomerase reverse transcriptase
TIN2	TRF1 Interacting Nuclear factor 2
TL	Telomere length
TPP1	TINT1, PTOP, PIP1 — POT1-TIN2 organizing protein
TRAP	Telomere repeat amplification protocol
TRF1	Telomeric repeat binding factor 1
TRF2	Telomeric repeat binding factor 2
VTE	Venous thromboembolism
WB	Western Blot
WGS	Whole genome sequencing
WHO	World Health Organization
WT1	Wilms tumor 1

1 INTRODUCTION

1.1 TELOMERES AND TELOMERE MAINTENANCE

1.1.1 Telomeres

Telomeres are nucleoprotein structures located at the chromosome ends and consist of up to 20 kb tandemly repeated TTAGGG sequences and associated proteins[1]. The telomeres are arranged in loop structures that are stabilized by associated proteins. The six key proteins binding to the telomere are TRF1, TRF2, TIN2, POT1, TPP1 and RAP1, which together form structures, the shelterin complex (figure 1), around the nucleotide sequence[2]. The telomere and its associated proteins form protective caps on human chromosome ends and prevents them from being recognized as double strand breaks[3]. They are thus essential for maintaining genomic stability and integrity.

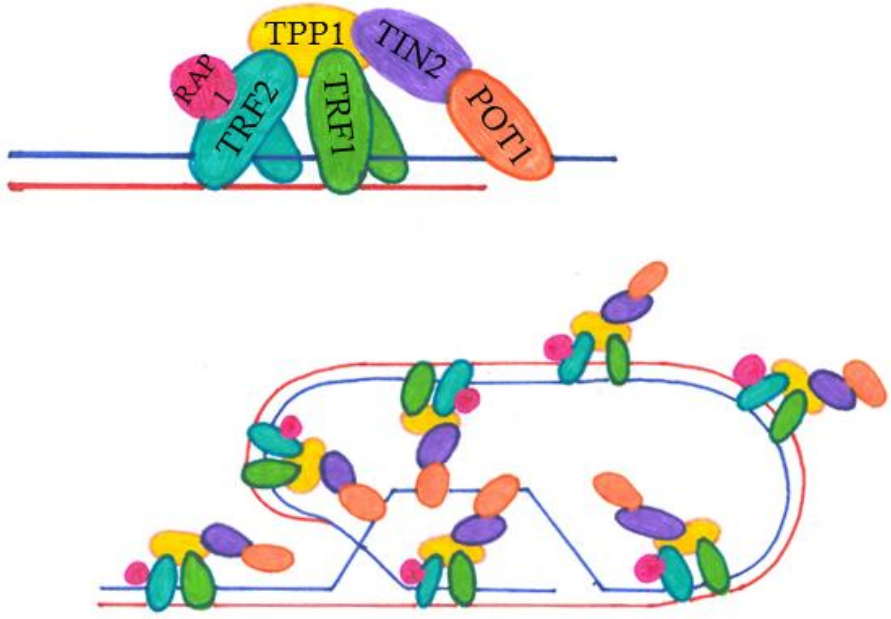

Figure 1. *Telomere structure. Upper picture show how the telomere binding proteins interact with the telomeric DNA in an open state. Lower picture shows a closed telomeric structure that is hold together by the telomere binding proteins.*

DNA polymerase can only replicate in the 5' → 3' direction in the replication fork, which renders it unable to elongate the end of the lagging strand[4] (figure 2). This 'end replication problem' results in progressive telomere shortening, with approximately 50 - 100 bases for every DNA replication[5,6]. The rate of telomere shortening is also affected by environmental

factors[7]. When a telomere reaches a critical length, it activates a DNA damage response and triggers the cell to enter a permanent growth arrest stage called replicative senescence. Senescence mediated by telomere shortening is suggested as an anti-tumor mechanism, but also contributes to the aging of mitotic tissues[8]. The telomere length (TL) in normal human cells varies between 5-15 kb[9]. Many factors influence the TL and telomere maintenance is a delicate dynamic process of lengthening and erosion.

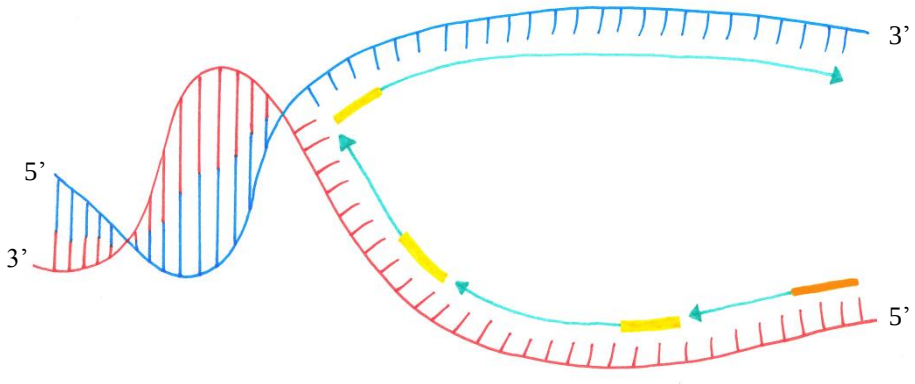

Figure 2. *Illustration of the end replication problem. Replication of the lagging strand occurs stepwise. The primers (yellow) required for initiation of replication by polymerase are degraded and the gap filled in. The space where the outermost primer binds (orange) cannot be filled in and the telomere is shortened.*

1.1.2 Telomere binding proteins

The shelterin proteins are crucial for telomere protection and maintenance. They govern and stabilize the 3D structure of the telomeric sequence[2]. TRF1 and TRF2 bind directly to the double stranded telomeric sequence whereas POT1 binds directly to the single stranded overhang of telomeres[10]. The other shelterin proteins interact indirectly with telomeric DNA by binding to TRF1, TRF2 or POT1 (figure 1). Shelterin proteins affect telomerase function and protect telomeres from DNA damage responses by inhibiting ATM and ATR dependent pathways[11]. Shelterin proteins are thought to mainly affect telomerase function by regulating its access to the telomeres. One of these shelterins, POT1, is a negative regulator of TL and the depletion of POT1 from the single strand overhang results in excessive telomere lengthening[12]. In contrast to POT1, TPP1 is essential for recruiting telomerase to the telomeres, and certain mutations in TPP1 result in excessive telomeric loss[13].

1.1.3 Telomerase

Telomerase is an enzyme that can synthesize the telomeric sequence and hence lengthen telomeres. Telomerase consists of two core components; the rate-limiting catalytic subunit TERT and a RNA template for telomeric DNA called TER[14]. Telomerase is very tightly regulated and only active in cells in need of extensive proliferation potential, such as activated lymphocytes, stem cells and embryonic tissues[15]. The main regulator of telomerase activity is the expression of TERT[15]. Ectopic expression of TERT is enough to induce telomerase activity, suggesting that TERT is the rate limiting component of telomerase[16].

The positive relationship between cellular life-span and telomere length and telomerase expression has been well established[17]. Stem/progenitor cells with great proliferation potential have a higher expression of telomerase to be able to compensate for the telomere loss during cell divisions[18]. Impaired telomere maintenance or telomerase deficiency may lead to defective hematopoietic cell proliferation and bone marrow failure, while aberrant activation of telomerase is essential for immortalization and transformation of human cells including hematopoietic cells[19].

1.1.4 Regulation of TERT expression

The *TERT* gene is localized at chromosome 5p15.33 and is mainly regulated at a transcriptional level[15,20]. The *TERT* promotor is extensively studied and binding sites for an abundance of both activating and suppressing transcription factors have been identified. There are many activating factors, such as c-Myc, HIF-1, AP2 and ER, and suppressing factors include mostly tumor suppressors, such as p53, WT1, and Menin[21]. *TERT* transcription is highly influenced by the general transcription factor SP1 that binds to the TATA binding motif[21]. Interestingly, no TATA box has been found in the *TERT* promoter, yet mutations affecting SP1 binding sites can attenuate or even eliminate *TERT* promoter activity[22].

TERT is also regulated post-transcriptionally by alternative splicing. Seven mRNA variants have been identified, but only the full length transcription variant is translated into a functional protein[23,24]. Phosphorylation of TERT can either activate or suppress TERT, depending on which site is phosphorylated[25]. Telomerase can be inactive in cells despite the presence of full length TERT mRNA, suggesting that posttranscriptional regulation of TERT potentially plays an important role[26,27]. The *TERT* promoter harbors one CpG island and

epigenetic regulation of TERT is evident in differentiation and in cancers[28]. There are conflicting data regarding the association between methylation status of the TERT promoter and TERT expression. Some authors report that TERT expression is activated upon promoter demethylation[29], whereas others describe an activation of TERT upon hypermethylation of the promoter[30,31]. It is also proposed that the *TERT* promoter is highly regulated by histone modifications, including repression by hypoacetylation of core histones[32-34]. Apart from TERT regulation, histone modifications and methylation of the subtelomeric region have also been shown to play a role in regulating TL[35].

1.1.5 Alternative lengthening of telomeres

Some tumors do not express telomerase but still have an unlimited replicative potential and the ability to maintain their telomeres[36,37]. In these cells, telomeres are lengthened by recombination, often called alternative lengthening of telomeres (ALT) (figure 3)[38]. This mechanism for telomere lengthening is mostly found in sarcomas and high grade astrocytomas[36,39]. Telomere lengthening with ALT is more prone to functional errors than lengthening by telomerase[40]. ALT typically causes a great variation in TL within an individual cell, ranging from atypically long to undetectably short[41]. It is debated whether telomerase activity and ALT are mutually exclusive, but there is evidence that they can coexist and that ALT can be induced if telomerase activity is repressed[42].

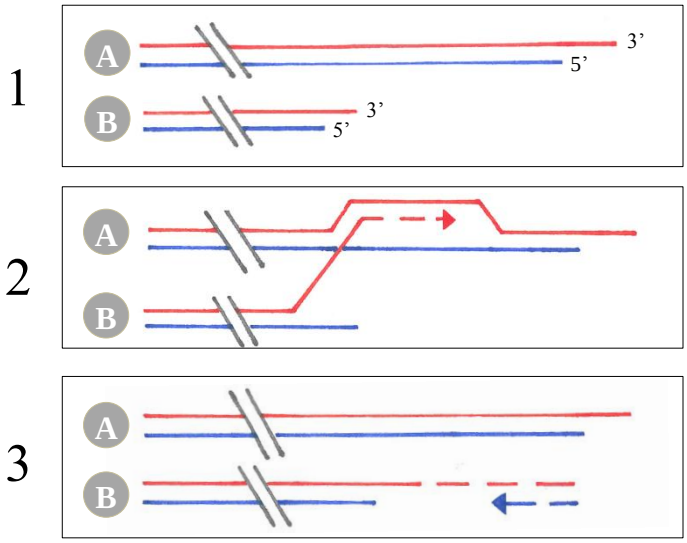

Figure 3. *Illustration of alternative lengthening of telomeres. (2) The 3' end of telomere B places itself alongside telomere A and is elongated, using telomere A as a template. (3) This elongation of the B telomere's 3' end enables the synthetization of its complementary strand.*

1.1.6 Telomeres and telomerase in in human diseases

Telomere dysfunction have been shown to play a role in many different diseases, from depression[43] to rheumatoid arthritis[44], but telomere biology has mostly been highlighted in malignant tumors. In order for a malignant cell to obtain indefinite dividing capacity, it must acquire the ability to elongate its telomeres. In the vast majority of tumors this is accomplished by activating telomerase, which has been shown to be detectable in 90% of all tumor types[45-47]. The other 10% are thought to elongate their telomeres by ALT or other similar mechanisms[40]. Telomerase activation is thus necessary for the development of several cancer types[48-50], but activation of telomerase alone is not sufficient for malignant transformation[51,52]. Even though telomerase is aberrantly expressed in most tumor cells, these often have shorter telomeres[53]. This could be secondary to tumor cells' high proliferation resulting in accelerated telomere erosion, which cannot be fully compensated by telomerase activity. Alternatively, cancer may originate from cells having short telomeres have bypassed replicative senescence and are genetically instable (figure 4).

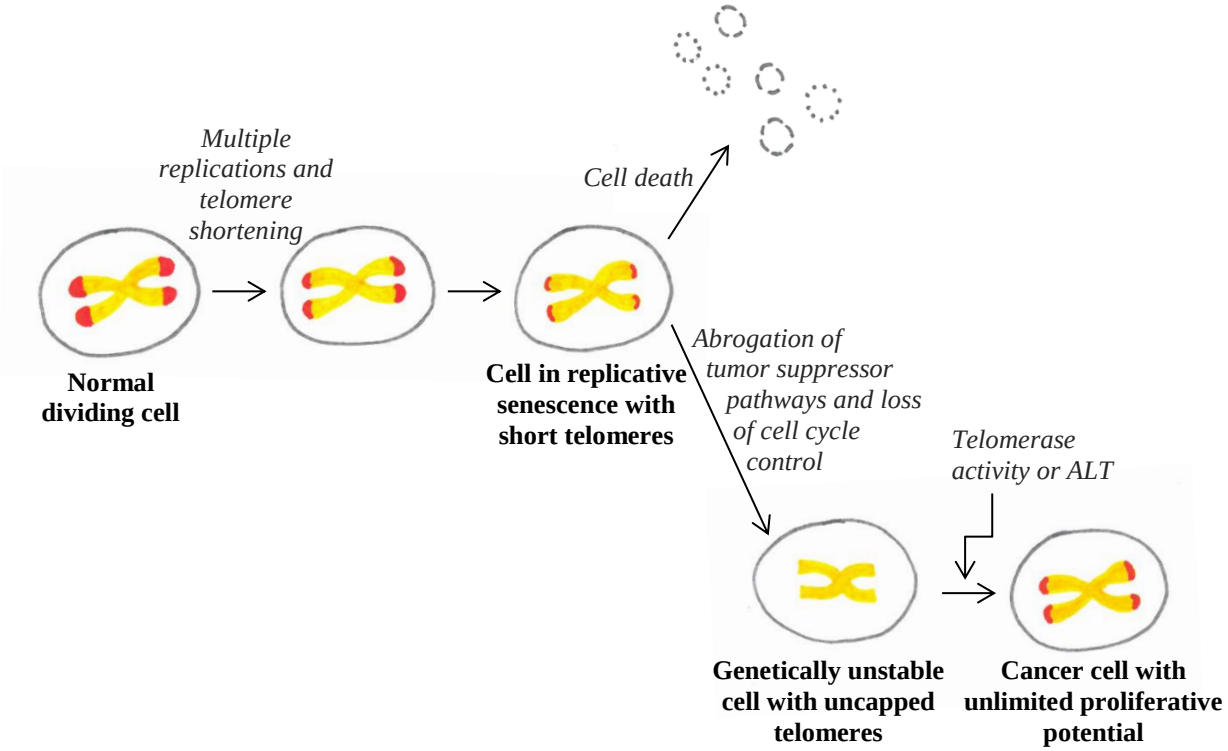

Figure 4. *Illustration of telomere shortening and how malignant transformation can occur when cells manage to bypass replicative senescence.* Italic *text describes events whereas* ***bold*** *text describes the state of the cell.*

Growing amount of data supports the notion that telomerase has an active role in tumorigenesis independent of its telomere lengthening function[54,55]. This role is so far undefined, but a possible contributing mechanism could be that TERT make cells more resistant to apoptosis[56,57]. Despite strong evidence for telomerase being a key player in tumorigenesis, its activation has also been proposed to mitigate genomic instability in tumor cells caused by shorter and dysfunctional telomeres[53]. Several mutations in the telomere associated genes have been linked to tumors and other diseases. For example, germline mutations in the *TERT* promoter and inactivating mutations in POT1 have recently been associated with familial melanoma[58-60], and mutations in TPP1 have recently been shown to cause bone marrow failure including aplastic anemia[61].

1.2 MYELOPROLIFERATIVE NEOPLASMS

Myeloproliferative neoplasms (MPNs) are a group of clonal disorders within the myeloid lineages of the bone marrow (BM). MPNs are characterized by hyperproliferation, resulting in excessive numbers of terminally differentiated cells from one or more of the myeloid lineages. MPNs consist of polycythemia vera, essential thrombocythemia, and primary myelofibrosis. The incidence of MPNs varies between different parts of the world, with a higher incidence in Europe (5.8/100 000)[62,63] compared to East Asia (2/100 000)[64]. The acquired mutation $JAK2^{V617F}$ and mutations in *JAK2* exon 12, *Calreticulin (CALR)* and *MPL* are found in the majority of patients with MPNs (see chapter 1.2.4).

1.2.1 Polycythemia vera

Polycythemia vera (PV) is characterized by excessive proliferation in the erythroid lineage leading to a high number of mature erythrocytes in the peripheral blood. In Sweden PV has an incidence of 1.9-2.6/ 100 000 persons/year[65,66]. The median age at diagnosis is 70 years and the condition affects men and women equally. Symptoms include fatigue, pruritus, head ache, sleeping difficulties and blushing[67]. Patients have a hypercellular BM dominated by erythropoiesis (figure 5). In many patients there is also hyperproliferation in other myeloid lineages, leading to high platelet and white blood cell counts[67]. PV is diagnosed according to the World Health Organization (WHO) 2008 classification system (table 1)[68,69]. Mutations in *JAK2* are seen in the majority of patients with PV (see chapter 1.2.4).

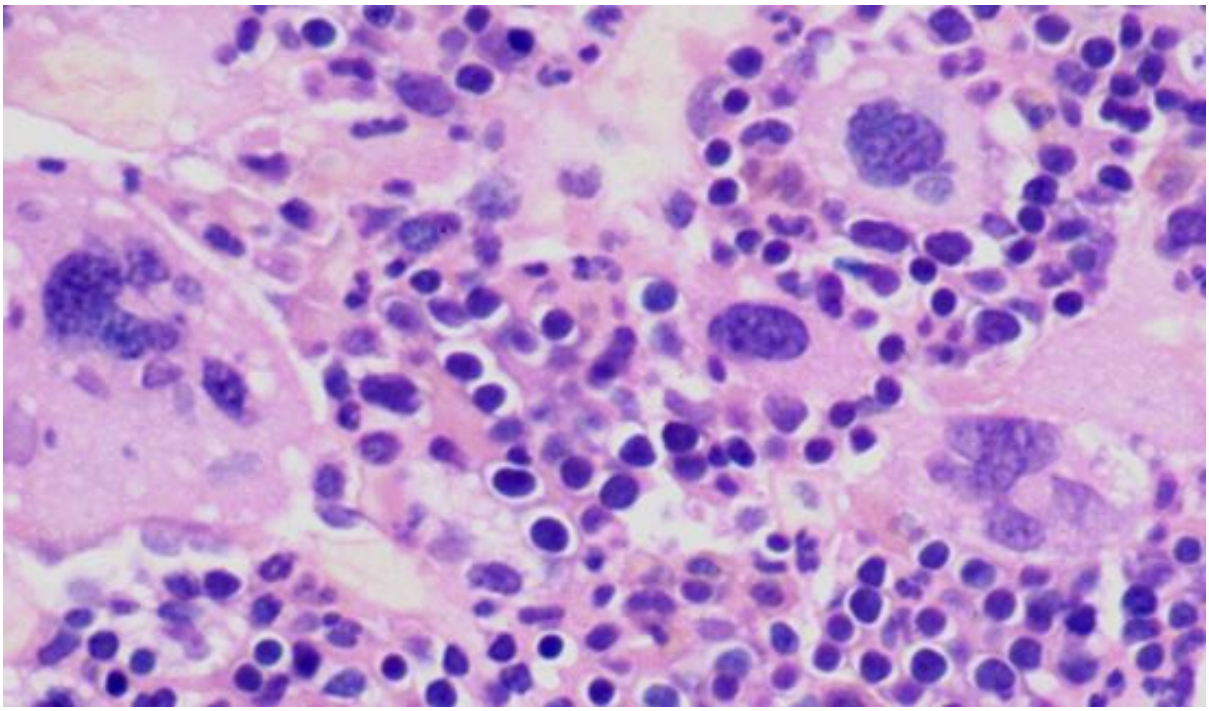

Figure 5. *Bone marrow from a patient with polycythemia vera showing a dominating erythropoiesis and large megakaryocytes.*

Table 1. The 2008 WHO classification system for diagnosis of PV[69].

The presence of both major criteria and one minor criterion or the presence of the first major criterion together with 2 minor criteria is required for diagnosis.

Major criteria

1. Hemoglobin >185g/L in men, >165g/L in women, or hematocrit >0.52 in men and >0.48 in women, or other evidence of increased red cell volume*
2. Presence of JAK2 ^{V617F} or other functionally similar mutation such as the JAK2 exon 12 mutation

Minor criteria

1. Bone marrow biopsy showing hyper cellularity for age with tri-lineage growth with prominent erythroid, granulocytic, and megakaryocytic proliferation
2. Subnormal level of erythropoietin in serum
3. Endogenous erythroid colony formation in vitro

1.2.2 Essential thrombocythemia

Essential thrombocythemia (ET) is characterized by megakaryocyte proliferation resulting in an abnormally high platelet count. The symptoms are typically mild and ET is often discovered at a routine medical examination. ET is also diagnosed according to WHO criteria and requires that the patient has a high platelet count (>450 × 10^9/L), proliferating megakaryocytes seen in the BM (figure 6), and an absence of characteristic mutations causing secondary thrombocythemia (table 2). Patients with ET have an elevated risk for venous thromboembolism (VTE), but paradoxically also for hemorrhages at very high platelet counts (>1,500 × 10^9/L)[70]. The risk of progression to myelofibrosis (MF) and transformation to Myelodysplastic Syndromes (MDS) and Acute Myeloid Leukemia (AML) is low (see chapter 1.2.6). A majority of patients with ET carries mutations in *JAK2*, the thrombopoetin receptor *MPL* or *C ALR* (see chapter 1.2.4).

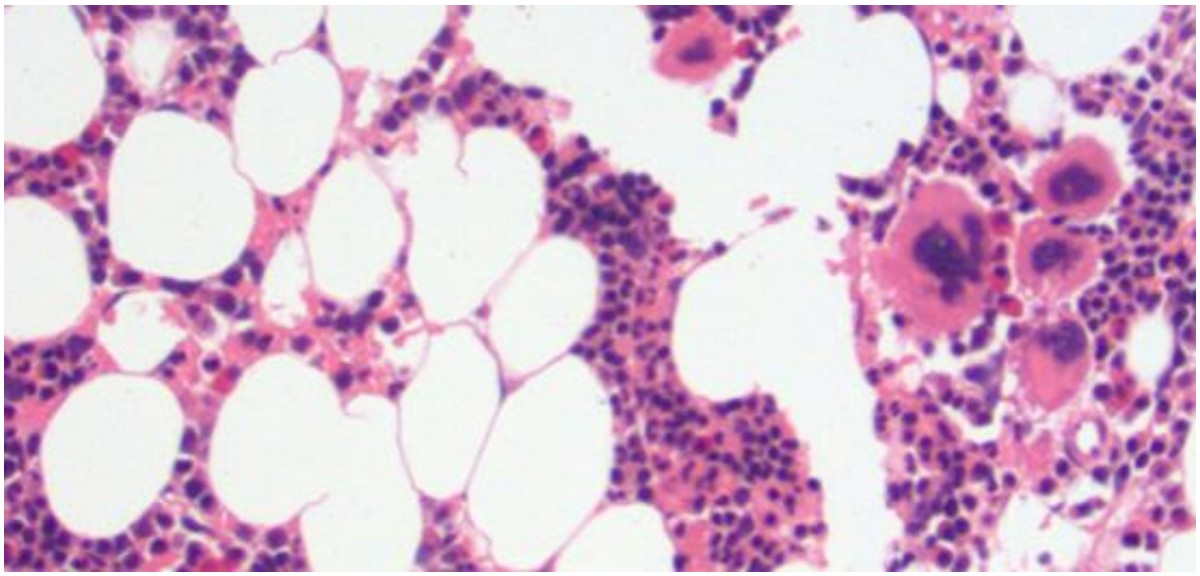

Figure 6. *Large mature megakaryocytes in the bone marrow from a patient with essential thrombocythemia.*

Table 2. The 2008 WHO classification system for diagnosis of ET[69].

All four major criteria have to be met for the diagnosis of ET.

Major criteria

1. Platelet count >450 × 10^9/L
2. Proliferating megakaryocytes with large and mature morphology. No or insignificant granulocyte or erythroid proliferation.
3. Not meeting the WHO criteria for any other myeloid neoplasm
4. Presence of $JAK2^{V617F}$ or other clonal marker. If no marker is identified, all causes of reactive thrombocytosis have to be excluded.

1.2.3 Primary myelofibrosis

Primary myelofibrosis (PMF) is a myeloproliferative neoplasm that is characterized by myeloproliferation, atypical megakaryocytes and BM fibrosis (figure 7)[71]. Diagnostic criteria are presented in table 3. Anemia and splenomegaly secondary to fibrosis of the BM are common. Symptoms include fatigue, night sweats and weight loss and the condition affects about 0.6/100 000 persons per year in Sweden[65]. As the name suggests, PMF does not develop from a preexisting hematological disease. However, the pathology is similar to secondary myelofibrosis derived from a previous ET or PV. PMF patients have an increased risk of VTE, but not as high as that of the patients with ET or PV. PMF has the worst prognosis of all MPNs and the median survival time after a PMF diagnosis is only 6 years[72].

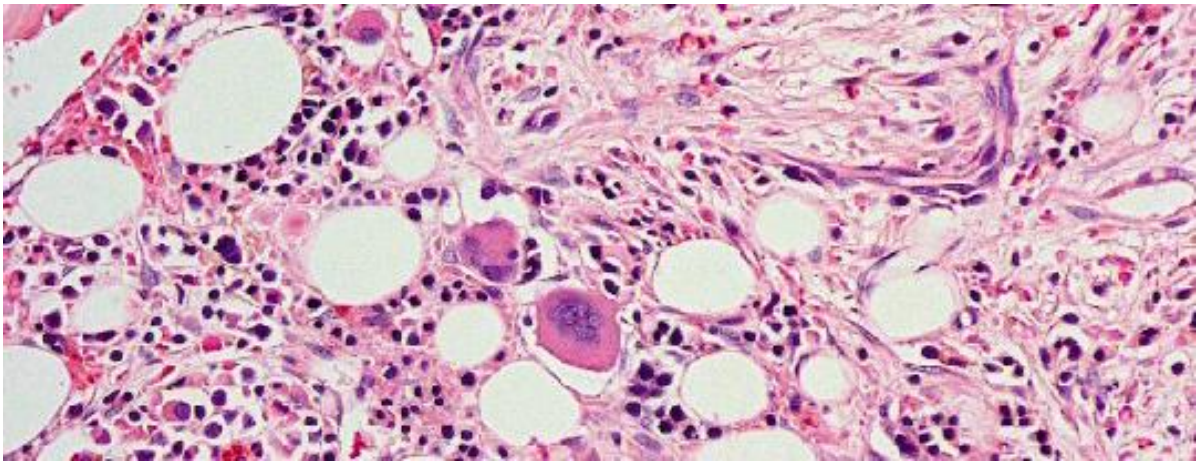

Figure 7. *Fibrosis in the bone marrow from a patient with primary myelofibrosis.*

Table 3. The 2008 WHO classification system for diagnosis of PMF[69].

All three major criteria and two minor criteria have to be met for the diagnosis of PMF.

Major criteria

1. Proliferating atypical megakaryocytes combined with either collagen and/or reticulin fibrosis. In the absence of fibrosis, the changes in megakaryocytes must be accompanied by a hypercellular bone marrow, granulocytic proliferation and often suppressed erythropoiesis (pre-fibrotic PMF).
2. Not meeting the WHO criteria for any other myeloid neoplasm
3. Presence of $JAK2^{V617F}$ or other clonal marker. If no marker is identified, causes of reactive myelofibrosis must be excluded.

Minor criteria

1. Leukoerythroblasts in the peripheral blood
2. Elevated lactate dehydrogenase
3. Splenomegaly
4. Anemia

1.2.4 Molecular and cytogenetic background of myeloproliferative neoplasms

In 2005 a gain of function mutation of *Janus Kinase 2 (JAK2)* in MPNs was described by several research groups simultanously[73-75]. The mutation leads to phenylalanine being substituted by valine at position 617 ($JAK2^{V617F}$), making the kinase constitutively active. Normally when the ligand binds to the transmembrane receptor, the receptor undergoes conformational changes and the JAK2 phosphorylates the cytoplasmic region of the receptor. Upon activation, JAK2 also phosphorylates members of the STAT family of transcription factors which mediates the downstream intracellular signal[76]. However, when JAK2 is constitutively active a proliferation signal will be sent to the nucleus of the cells, even in the absence of the proper ligand-signal. The $JAK2^{V61F}$ is present in 95% of PV-patients and 60% and 50% of ET- and PMF patients, respectively[77]. The presence on $JAK2^{V617F}$ has been linked to an increased risk of secondary myelofibrosis in ET and PV patients[73,78] and it is one of the risk factors for thrombosis in ET patients[79]. The reports regarding the clinical role of $JAK2^{V617F}$ in PMF are somewhat contradictive, where some authors report a reduced overall survival when the mutation is present[80], and others report that a low allele burden has been associated with shorter survival[81].

$JAK2^{V617F}$ is the most common mutation in MPNs, but other recurrent mutations also exist. Mutations in exon 12 of the *JAK2* gene is seen in 16% of PV patients without the $JAK2^{V617F}$, and is more often seen in younger patients with a more isolated effect on the erythroid lineage[82-84]. About 5% of ET and PMF patients harbor activating mutations in the thrombopoietin receptor *MPL* (W515L or W515K), resulting in cytokine-independent growth mediated thorough the JAK/STAT signaling cascade[85].

Mutations in the *CALR* gene in 67-88% of $JAK2^{V617F}$ and *MPL* mutation-negative ET and PMF patients were discovered in 2013 and these mutations also result in a hyperactivation of the JAK/STAT pathway[86,87]. Authors have reported that ET patients with *CALR* mutations have a prognostic advantage over those with the $JAK2^{V617F}$ mutation[88]. Other more rare mutations have also been associated with MPNs (e.g. *SOCS1-3*, *TET2*, *EZH2*, *ASXL1*, and *RUNX1*) and most of them affect JAK/STAT signaling[89,90]. *EZH2* and *ASXL1* have been associated with an increased risk of disease progression, but the potential prognostic relevance of most of those mutations in MPNs is not yet known[91,92].

Cytogenetic abnormalities can be found in 11%, 7% and 33% of patients with PV, ET and PMF, respectively[93-95]. Most frequent abnormalities are shared with other hematological malignancies, but +9 and del(13q) mainly occur in MPNs[96].

1.2.5 Treatment of myeloproliferative neoplasms

PV is often treated with phlebotomy and low-dose aspirin. High-risk patients with ET are often also prescribed low-dose aspirin, but this should be avoided in patients with very high platelet count (>1,500 × 10^9/L) due to the risk of paradoxical bleeding. High-risk PV and ET patients are often offered cytoreductive treatment with pegylated interferon-α (younger patients) or hydroxyurea (HU) (older patients)[97]. The treatment of PMF is focused on reducing symptoms. Patients with anemia often receive erythropoietin stimulation and patients with high blood cell counts get cytoreductive treatment[97]. Splenomegaly is often reduced by cytoreductive treatment, but can also be treated with JAK2 inhibitors or more rarely splenectomy. Allogeneic stem cell transplantation is considered in young patients with an intermediate or high-risk PMF.

An abundance of JAK2 inhibitors are currently in clinical trials for MPNs. The most well studied and established inhibitor is ruxolitinib (Jakavi®), which is now approved for treatment of PMF and refractory PV. Most JAK2 inhibitors also affect other targets than JAK2, but very little is known about how their effect on other targets influences their efficiency in treating MPNs. Interestingly, patients with and without mutations in JAK2 benefit equally from JAK2 inhibition, suggesting a more general effect on the JAK/STAT pathway[89]. Even though JAK2 inhibitors are effective in reducing symptoms for many patients, they are not curative. Some JAK2 inhibitors are shown to reduce the $JAK2^{V617F}$ allele burden in a fraction of patients, but it does not seem to eliminate the disease clone[98,99].

The telomerase inhibitor GRN163L (Imetelstat®) has been used in a clinical trial to treat MF. In one study, 21% of patients treated with GRN163L either had a complete remission (CR) (defined as normalization of hepatosplenomegaly, blood counts and leukocyte differential together with reversal of BM fibrosis) or a partial remission (defined with the same criteria as for complete remission apart from reversal of the BM fibrosis)[100]. It should be noted that some patients developed severe myelosuppression.

1.2.6 Transformation to myelofibrosis, myelodysplastic syndromes and acute myeloid leukemia

Patients with PV and ET have a well-recognized risk of developing secondary MF. The risk of developing MF is reported to be 4.9 – 6% per 10 years for PV and 0.8 – 4.9% per 10 years for ET[101].

MPNs can also transform into MDS, which is a group of disorders with BM failure resulting in cytopenias and/or malfunction of the circulating mature cells, or into AML. The risk of transformation to MDS/AML is 5 – 10% per 10 years for PV and 2 – 5% per 10 years for ET[102]. Patients with PMF have the highest risk of transformation, 8 - 20% during a 10 year observation[102]. The molecular events driving this transformation are unclear. An AML secondary to a MPN is associated with a more complex karyotype, which is thought to contribute to the worse prognosis seen in these patients[103,104]. Treatment of MPN with radioactive phosphorous (P^{32}) and alkylators has been linked with an increased risk of transformation into AML[102,105,106]. Whether HU has a leukemogenic effect is still debated[102,107], but 25% of MPN patients transforming to AML/MDS had no previous cytoreductive treatment[102].

Several recurrent mutations in MPNs, such as *RUNX1*, *TP53*, *SRSF2*, *IDH1/2*, *IKZF1*, *NF1*, *NRAS*, and *DNMT3A*, are linked to a higher risk for transformation[104,108-110]. Other risk factors for leukemic transformation are abnormal karyotypes, leukocytosis and reticulin fibrosis[111]. The most common mutation in MPNs, $JAK2^{V617F}$, has not been linked to an increased risk for transformation into AML[112]. However an association between $JAK2^{V617F}$ allele burden in PV and ET and the transformation to MF has been reported[113]. Interestingly, sometimes a $JAK2^{V617F}$ positive MPN transforms into a $JAK2^{V617F}$ negative AML, suggesting that the clone giving rise to the secondary AML is a more primitive one than the $JAK2^{V617F}$ clone driving the MPN[114].

1.2.7 Telomeres in myeloproliferative neoplasms

Telomere length in MPNs has been assessed by several research groups and shown to be shorter in cells from patients with MPNs compared to those of healthy individuals[115-117]. Authors have also reported that short telomeres in BM cells could predict progression of MPN[115]. The mechanism causing this telomere shortening and how telomere shortening

potentially contributes to the pathology and progression of MPNs is not understood. The telomerase activity is reported to be higher in the BM of MPN patients compared to that of healthy individuals[115,118]. Recently, a single nucleotide polymorphism (SNP) variant in the TERT promoter has been linked to the risk of MPN development[119,120]. Any association between telomerase activity and/or TL and prognosis in MPNs has so far not been established.

1.3 ACUTE MYELOID LEUKEMIA

AML is a common term for diseases characterized by clonal expansion of precursor cells of the myeloid lineage and a block in their differentiation (figure 8)[121]. The accumulation of immature cells and block in myeloid differentiation in the BM results in impaired normal hematopoiesis which causes anemia, thrombocytopenia and granulocytopenia.

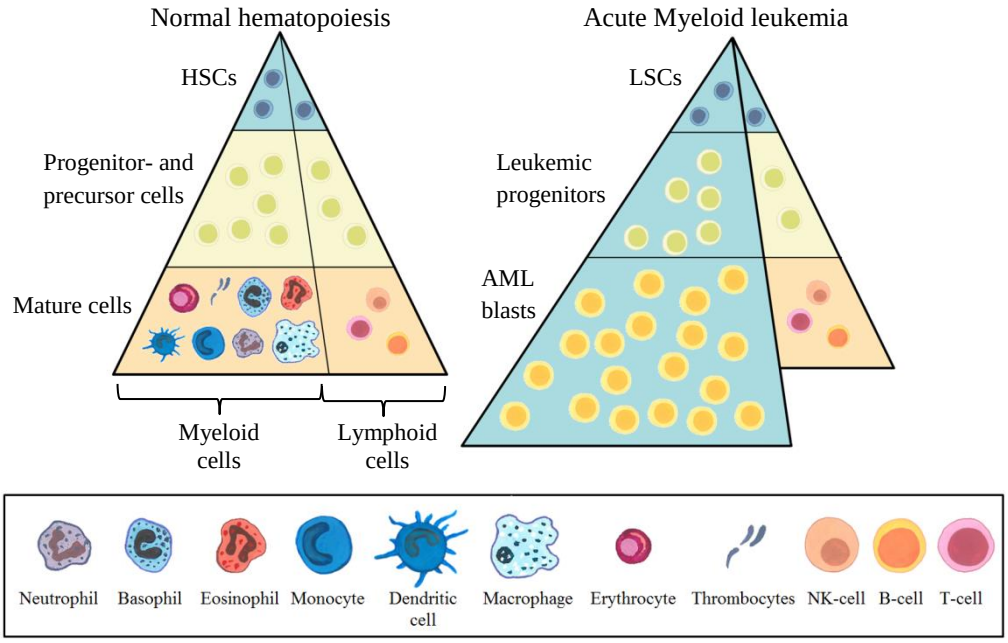

Figure 8. *Normal and leukemic hematopoiesis. Hematopoietic stem cells (HSCs) give rise to progenitor and precursor cells that are committed to specific hematopoietic lineages. Those cells later differentiate to become mature blood cells. Leukemic stem cells (LSCs) generate leukemic progenitors and the more differentiated, but still immature, leukemic blasts.*

Diagnostic criteria for AML are the presence of ≥20% myeloblasts in the BM or even less if characteristic genetic aberrations are detected[68]. Historically, AML has been classified using the FAB-classification system described in table 4[122]. Today the WHO 2008 classification is used, which is based on the presence of genetic aberrations and is shown in table 5[68]. A

revision of this classification is underway. The incidence of AML increases with age to reach a maximum of 150/100 000 in individuals 80-85 year of age in Sweden[123]. The median age at diagnosis is approximately 70 years and AML is slightly more common among men than among women[121].

Table 4. French-American-British classification of acute myeloid leukemias

M0: Acute myeloblastic leukemia, minimally differentiated
M1: Acute myeloblastic leukemia, without maturation
M2: Acute myeloblastic leukemia, with granulocytic maturation
M3: Acute promyelocytic leukemia
M4: Acute myelomonocytic leukemia
M5: Acute monoblastic leukemia
M6: Acute erythroid leukemias
M7: Acute megakaryoblastic leukemia

Table 5. WHO 2008 classification of acute myeloid leukemia

- Acute myeloid leukemia with recurrent genetic abnormalities
- AML with myelodysplasia-related changes
- Therapy-related myeloid neoplasms
- Myeloid Sarcoma
- Acute myeloid leukemia, not otherwise specified
- Acute leukemia of ambiguous lineage

The development of AML is a multi-step process in which several mutations and chromosomal abnormalities are aquired[124]. It has been proposed that both the acquisition of mutations that activate signaling pathways and give hematopoietic cells a proliferative advantage and mutations in transcription factors impairing hematopoietic differentiation are crucial for AML development[125]. Apart from transcription factors and genes involved in signaling pathways, tumor suppressors, splicing factors, and genes involved in DNA methylation are also frequently mutated in AML[121]. There are many recurring mutations such as *FLT3*, *C-KIT*, *N-RAS*, *RUNX1*, *WT-1* and *ASXL1*, to name but a few[121,126]. The variety of mutations seen in AML results in a very heterogeneous group of diseases. About one fourth of AMLs are secondary to other hematological diseases, such as MPNs or MDS, or previous treatment with chemo- or radiotherapy.

A special case is the old M3 (FAB) class of leukemia, acute promyelocytic leukemia (APL), where 97% of the patients have a characteristic translocation resulting in the fusion protein PML-RARα which blocks the differentiation and drives proliferation at the promyelocytic stage[127,128].

2 AIMS

The overall aim of this PhD project was to better define the role of telomeres and telomerase in the pathogenesis of MPNs and AML. The specific aims for each paper are here described after their Roman numerals.

I. The aim of this study was to assess the potential association between the expression of shelterin proteins and TL in MPN patients, in order to better understand the mechanism causing telomere shortening in MPN patients. Further, we aimed to define the influence of MPN subtype, $JAK2^{V617F}$ mutation status, disease duration and therapy on telomere regulation in MPNs.

II. Here our aim was to elucidate the potential relationship between TERT rs2736100 genotypes and the risk of MPNs in two different ethnical populations with different incidences of MPNs. We also sought to determine how the different rs2736100 genotypes influence TL and TERT expression in MPN patients.

III. In this paper we aimed to define the clonal evolution in a patient with APL who after 17 years of clinical remission presented with the same disease. The goal was to elucidate whether this patient suffered a true APL relapse, a secondary APL or a second *de novo* APL, and thereby guide treatment decisions.

IV. This study was designed to outline the effect of the JAK2 inhibitor LY2784544 in the $JAK2^{V617F}$-bearing cell line HEL, in order to better understand why MPN patients have a limited response to JAK2 inhibitors. Secondly, we wanted to reveal how the telomerase inhibitor GRN163L achieves its therapeutic effect in MPNs. We also sought to determine whether combined inhibition of JAK2 and telomerase can have a synergistic therapeutic effect in MPNs.

3 METHODS

3.1 PATIENT SAMPLES (PAPERS I-III)

Peripheral blood from patients diagnosed with MPN was taken at the outpatient clinic at the Karolinska University Hospital in Solna (papers I & II) and at Qilu Hospital, Shandong University, Jinan, China (paper II). The APL patient studied in paper III was sampled and treated at the Karolinska University Hospital in Solna. Informed consent was registered from all patients and the studies were approved by regional ethics committees in Stockholm and in Shandong. Erythrocytes were removed, using Hetasep, from the samples collected in Sweden, whereafter mono- and polynuclear cells were separated with Ficoll-Hypaque density gradient centrifugation. Whole blood from the Swedish patients was also prepared for flow-FISH (see 3.4).

3.2 CELL LINES AND CULTURE CONDITIONS (PAPERS I & IV)

All cell lines used in this thesis were purchased from DSMZ. The HEL cell line was established in 1980 from the peripheral blood of a 30 year old man diagnosed with acute erythroleukemia[129]. The HEL cell line is homozygous for the $JAK2^{V617F}$ mutation and displays cytogenetic abnormalities with loss of long-arm material from both chromosome 5 and 7[130,131]. HEL cells were cultured in RPMI1640 supplemented with 10% FBS, 2mM L-glutamine and 100U/ml penicillin-streptomycin and kept at a density of 0.2-1.2 $\times$ 10^6/ml. When treating cells with drugs (chapter 3.3), medium and drugs were changed every other day.

3.3 DRUGS (PAPERS I & IV)

In this project the JAK2 inhibitor LY2784544 (Gandotinib®) was used. This inhibitor was chosen because of its concentration dependent selectivity for $JAK2^{V617F}$. LY2784544 is reported to have an IC50 of 55nM for inhibiting $JAK2^{V617F}$ driven proliferation compared to an IC50 of 2.26 µM for wild-type JAK2[132]. A phase I trial on 38 patients (31 MF, 6 PV, 1 ET) showed that a daily dose of 120 mg/day was well tolerated. Higher doses led to a substantial increase in serum creatinine. Three of the 10 patients receiving the 120 mg daily dose achieved clinical improvement. Across all dosage levels, 56% of the patients scored a >50%

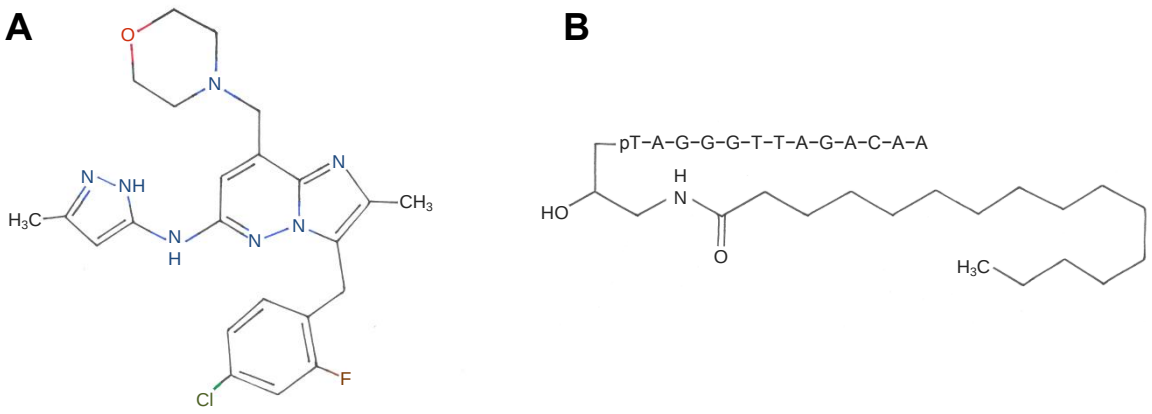

Figure 9. *Chemical structure of: A) LY2784544, B) GRN163L.*

improvement in their total symptom score (assessed by the Myeloproliferative Neoplasm Symptom Assessment Form[133])[134]. Phase II trials are now ongoing.

The telomerase inhibitor GRN163L (Imetelstat®) was used in these papers. GRN163L is an oligonucleotide with the complementary sequence to the RNA component of telomerase and binds to it with high affinity. The component also has a thio-phosphoramidate backbone which makes it more resistant to nucleases and increases its affinity to TER.

3.4 FLOW-FISH OF TELOMERE LENGTH (PAPERS I, II & IV)

The average TL was assessed with flow-FISH according to the protocol by Baerlocher et al[135] with some modifications. Calf thymocytes were kindly donated from Ö-slakt AB and included in all samples as positive controls. In short, fluorescent PNA probes (Panagene, Daejeon, Korea) were hybridized to the telomere sequence and the fluorescent signal was measured on a Gallios flow cytometer (Beckman Coulter, Brea, CA, USA) and analyzed using the Kaluza software (figure 10) (Beckman Coulter, Brea, CA, USA). Fluorescent MESF-FITC beads (Bangs Laboratories, Fishers, IN, USA) were used and the fluorescent signal was quantified using the QuickCal v.2.3 data analysis program (Bangs Laboratories, Fishers, IN, USA). The TL was then calculated using the following formula:

$$Telomere\ length, kB = \frac{MESF\ x\ n_{base}}{n_{chr}\ x\ 1000}$$

where MESF is the fluorescent intensity given by QuickCal v.2.3, n_{base} is the number of bases per PNA probe and n_{chr} is the number of chromosomes in the species of the cells' origin.

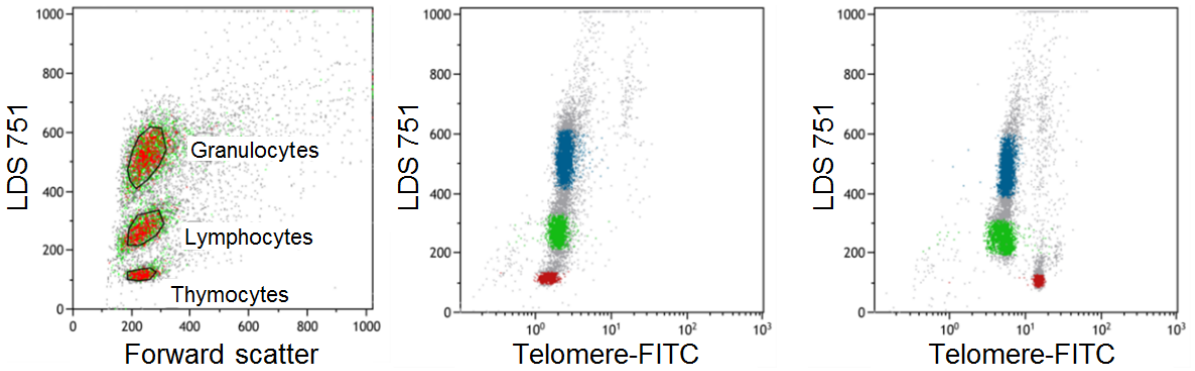

Figure 10. Flow-FISH assessment of telomere length in blood cells from MPN patients.

Left panel: Density plot with forward scatter on the x-axis and cell cycle stain with LDS 751 on the y-axis. Cell populations were gated as shown into three populations: (i) *Calf thymocytes (red in the middle and right panels),* (ii) *lymphocytes (green in the middle and right panels) and* (iii) *granulocytes (blue in the middle and right panels). Middle panel: Whole blood sample hybridized without telomeric probe. Right panel: Whole blood sample hybridized with FITC-labeled telomeric probe.*

3.5 RNA EXTRACTION AND QUANTITATIVE REAL-TIME PCR (PAPERS I, II & IV)

Total RNA was isolated using Trizol (Life Technologies) and the concentration was measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Reverse transcription was performed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). Quantitative real time polymerase chain reaction (qRT-PCR) was performed in triplicate using SYBR Green PCR Master Mix (Life Technologies, Carlsbad, CA, USA) with QuantStudio 7 Flex Teal-Time PCR system (Applied Biosystems, Waltham, MA, USA).

3.6 WESTERN BLOT (PAPERS I & IV)

Whole cell protein was extracted from granulocytes using Trizol according to the manufacturer's instructions (paper I) or from cultured cells using RIPA lysis buffer (paper IV). Protein concentrations were measured using a DC protein assay (Biorad, Hercules, CA, USA). Proteins were separated on SDS-PAGE gels and transferred to PVDF membranes. The membranes were blocked in milk and then probed with antibodies against POT-1, TIN-2, TPP-1, KLF4, SOCS3, MT1x and GSTP1, followed by anti-mouse, rabbit or goat antibodies

conjugated with horse radish peroxidase. The signal was detected with enhanced chemiluminescent (ECL) substrate. β-actin was used as a loading control.

3.7 TELOMERASE ACTIVITY ASSAY (PAPERS I & IV)

Telomerase activity was assessed using the telomerase repeat amplification protocol (TRAP)-ELISA kit (Roche, Basel, Switzerland). This method allows a semi-quantitative measurement of telomerase activity[136,137]. Briefly, the telomerase elongates the 3' end of a biotin-labeled synthetic primer and the elongation product is then amplified with PCR. The amplified products are denatured and hybridized to digoxigenin (DIG)-conjugated detection probes specific to the telomeric sequence. The products are then affixed to a streptavidin-coated microplate by the biotin. Amplified products stuck to the microplate are then bound to antibodies against DIG, conjugated with horseradish peroxidase and the peroxidase substrate[136].

3.8 DNA EXTRACTION AND GENOTYPING OF *TERT* rs2736100 (PAPER II)

DNA was extracted from peripheral blood (Swedish and Chinese patients and Chinese controls) using QIAmp DNA blood kit (Qiagen, Hilden, Germany). DNA was extracted from saliva from Swedish healthy controls using Oragene saliva collection kit (DNA Genotek Inc., Ottawa, Canada)[43]. DNA concentration was measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). TERT rs2736100 genotyping was performed using pre-designed TaqMan SNP genotyping assay kits on a QuantStudio 7 flex system (Applied Biosystems, Waltham, MA, USA). The assay included negative controls and was run with the following protocol: 95°C for 10 min, followed by 40 cycles of 92°C for 15s and 60°C for 1 min. The genotyping success rate was >95%.

3.9 WHOLE GENOME SEQUENCING (WGS) (PAPER III)

Genomic DNA from the APL patient's BM at initial diagnosis and relapse, and peripheral blood from when she was in CR were used. Libraries of qualified genomic DNA were prepared for paired-end analysis by the Illumina HiSeq 2000. After the generation of clusters of template DNA, they were sequenced by the Illumina HiSeq 2000 platform. Each sample

was >30-fold haploid coverage. After a strict QC test, the sequencing data were subjected to bioinformatics analysis. The raw sequencing data were filtered and then aligned using the Burrows-Wheeler Aligner. The 13 human genome build 37 (Hg19) was used as the reference genome for mapping. Then, the generated BWA files were processed by the SOAPsnp, SAMtools, BreakDancer and ANNOVAR to analyze and annotate the variants. In order to find the precise translocation sites of PML-RARα, the intrachromosomal translocation analysis of paired-end sequence data of initial and second APL samples was performed using BreakDancer[138]. First, all Maq-mapped reads within 3000bp of chromosomal locations of PML and RARα were extracted by SAMtools[139]. Then the data were analyzed for structural variants using BreakDancer.

3.10 SOUTHERN BLOT FOR TELOMERE LENGTH ASSAY (PAPER III)

Genomic DNA from BM and peripheral blood was digested overnight at 37°C with *HinfI* and *RsaI* restriction enzymes. The completely digested genomic DNA was separated on a 0.8 % agarose TAE gel and vacuum transferred to a Hybond-nylon membrane using 10 × SSC buffer. The membrane was air-dried and UV cross-linked. Hybridization of DNA fragments and chemiluminescent detection were performed using the TeloTAGGG Telomere Length Assay kit according to the manufacturer's protocol (Roche, Basel, Switzerland). Briefly, the separated DNA fragments were hybridized to a DIG-labeled probe specific for the telomere sequence, followed by incubation with a DIG-specific antibody linked to alkaline phosphate (AP). The signal is then detected with a chemiluminescent substrate for AP. The chemiluminescence signal was detected in the Quantity One® Software (Biorad, Hercules, CA, USA) and the data was analyzed according to Roche's instructions.

3.11 ARRAY-COMPARATIVE GENOMIC HYBRIDIZATION (PAPER III)

Array-comparative genomic hybridization (CGH) of DNA isolated from BM samples was performed using the platform from Oxford Gene Technology (Oxford, UK) with four arrays of 180K oligonucleotide probes (60-mer). This platform gave a complete genome-wide survey with an average resolution of 20-50 Kb. Hybridization was performed according to the manufacturer's recommendation. The arrays were scanned on an Agilent Microarray Scanner and data was analyzed in the CytoSur Interpret Software (OGT, Oxford, UK).

3.12 MUTATIONAL ANALYSIS OF FLT3-ITD AND FLT3-D835 (PAPER III)

DNA at first and second diagnosis of the APL patient was extracted from BM using QIAamp Blood & Cell Culture DNA Kit (QIAGEN, Hilden, Germany). *FLT3-ITD* and *FLT3-D835* mutations were studied qualitatively using the fragment length analysis method. PCR primers were fluorescently labeled with 6-FAM, NED or HEX. Amplified fragments were detected using Applied Biosystems 3130 XL and the length of each dye-labeled fragment was calculated by comparing it to a size standard using the GeneMapper software.

3.13 COLONY FORMATION ASSAY (PAPER IV)

Colony formation assay was performed in 6-well plates containing 500 HEL cells/well, seeded in Methocult H4100 (Stemcell Technologies, Vancouver, Canada) supplemented with RPMI1640 and incubated for 10 days at 37°C with 5% CO_2. The number of colonies consisting of 25-50 cells and >50 cells were counted after 10 days of incubation.

3.14 FLOW CYTOMETRY ANALYSIS OF CD34 FRACTION AND APOPTOSIS (PAPER IV)

Cells were washed in PBS and blocked in mouse serum for 15 min. Cells were then incubated with APC anti-CD34 antibodies (BD Biosciences #555824) for 45 min at 4°C. Apoptosis was measured using an Annexin V-FITC/7-AAD kit following the manufacturer's protocol (Beckman Coulter, Brea, CA, USA). Annexin V is a protein with a high affinity for phosphatidylserine, a protein that is translocated from the inside of the plasma membrane to the outer side in an early stage of apoptosis[140]. 7-AAD is a fluorescent molecule which binds and stains DNA in cells with compromised plasma membrane. It is therefore often used as a viability staining[141]. 7-AAD alone can also be used as an apoptosis marker due to its partial uptake in apoptotic cells, giving a weaker fluorescent signal compared to dead cells[142].

3.15 LENTIVIRAL TRANSFECTION (PAPER IV)

HEL cells were seeded at a density of 0.4×10^6/ml 24h prior to transduction. Lentiviral particles (Origene, Rockville, MD, USA (TL316853V)) were used at a multiplicity of

infection of 20 together with 5µg/ml Polybrene (Santa Cruz Biotechnologies, Dallas, TX, USA). Medium was changed 16h after transduction and selection with puromycin was started 48h after transduction. In some experiments cells were sorted using a BD Influx (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

3.16 WHOLE TRANSCRIPT EXPRESSION ANALYSIS

Microarray analysis was performed using Affymetrix whole-transcript expression analysis and the WT assay gene ST 1.1 (Affymetrix, Santa Clara, CA, USA) in association with the Bioinformatics and Expression Analysis Core Facility (BEA), Karolinska Institutet.

3.17 STATISTICAL ANALYSES

The comparison of TL and mRNA expression of telomere binding proteins was made using 2-tailed Student's t-test (paper I). Age adjustment of telomere length was performed in Graphpad Prism 5 using the ANCOVA-based function "compare slopes and intercepts" (paper I). For correlation analyses Pearson's correlation coefficient was generated using the correlation tool in Excel's Analysis ToolPak add-in software. A t-value was generated using the following formula:

$$t = \frac{r \times \sqrt{n-2}}{\sqrt{1-r^2}}$$

The T-distribution calculation tool in Excel was then used to generate a *P*-value. For all calculations a two tailed test was used (Paper I). Differences in telomere length and mRNA expression of TERT among different genotypes of TERT rs2736100 were determined using Mann-Whitney *U* test (paper II). For comparison of genotype distributions of TERT rs2736100 in MPN patient and controls, Fisher's exact test was used for generation of odds ratio (OR), confidence interval (CI) and *P*-value (paper II). When comparing the distribution of TERT rs2736100 variants in healthy populations in China and Europe, a Chi-square test was used (paper II). Results from TRAP, mRNA expression, colony formation assay and flow-FISH of TL were compared using the two-tailed Student's t-test (paper IV). All analyses were performed in Graphpad Prism 5 if not stated otherwise. *P*-values <0.05 was considered statistically significant.

4 RESULTS AND DISCUSSION

4.1 PAPER I

4.1.1 Telomere shortening in granulocytes from patients with Myeloproliferative neoplasms

The characteristics of the 81 patients with MPNs and the 43 healthy controls are presented in table 6. As MPNs affects the myeloid lineages, TL was determined using flow-FISH in both granulocytes and lymphoid cells in peripheral blood samples from patients and healthy controls. TL in granulocytes was significantly shorter in MPN patients than in healthy controls (mean ± SD: 7.27 kb ± 2.11 vs 8.66 kb ± 1.82, $P < 0.001$; figure 11). As previously reported[143], TL was highly age-dependent in patients ($r = -0.34$, $P < 0.001$). The significant difference in TL between patients and healthy controls remained after correcting for age ($P = 0.046$). In contrast, there was no difference in TL between lymphoid cells in patients and in

Table 6. Summary of clinical features of patients with MPNs and demographics of healthy controls

Characteristics	MPN	HC
N. of individuals	81	43
Sex		
Male n. (%)	36 (44)	16 (37)
Female n. (%)	45 (56)	27 (63)
Age (years)		
Median (range)	68 (25-106)	56 (16-84)
Mean ± SD	67 ±13	52 ± 18
Subtype		
ET	24	
PV	41	
PMF	12	
Unclassified	4	
JAK2 ^{V617F} status		
Positive	65	
Negative	16	
Disease duration, years		
Median (range)	4 (1-36)	
Mean ± SD	6.6 ± 6.9	
Unknown (n. patients)	5	
Treatment n.		
None	8	
Hydroxyurea	50	
Interferons	2	
Other (Aspirin, warfarin, venesectio)	14	
Unknown	7	

MPN: myeloproliferative neoplasm; HC: healthy controls; ET: essential thrombocythemia; PV: polycythemia vera; PMF: primary myelofibrosis

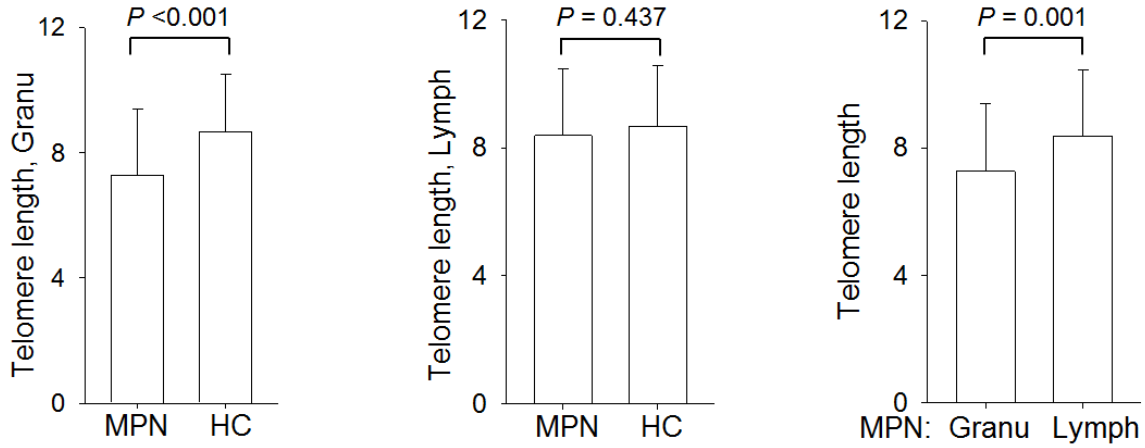

Figure 11. *Telomere length (kb) in granulocytes (Granu) and lymphoid cells (Lymph) derived from patients with MPNs and healthy controls (HC).*

healthy controls (mean ± SD: 8.38 kb ± 2.08 vs 8.67 kb ± 1.89, *P* = 0.437; figure 11). Furthermore, patients' granulocytes exhibited significantly shorter TL compared to their own lymphoid cells (*P* = 0.001; figure 11), whereas no difference was seen between granulocytes and lymphoid cells in heathy controls. These results collectively suggest that accelerated telomere attrition was restricted to myeloid lineage cells in patients with MPNs. Significantly shorter granulocyte telomeres were seen in all subtypes of MPNs compared to controls before age-adjustment. However, after age adjustment only PV patients displayed a significant difference (*P* = 0.025). There was no difference in TL between subtypes. TL was not affected by sex, disease duration, treatment or $JAK2^{V617F}$ status.

4.1.2 Dysregulation of shelterin proteins in patients with Myeloproliferative Neoplasms

After verifying that telomere shortening occurred in granulocytes of MPN patients, we sought to determine whether the expression of shelterin factors was altered in patient granulocytes. mRNA levels of the six shelterin proteins were analyzed using qRT-PCR. There were no differences in TRF1 or TRF2 mRNA expression between MPN patients and healthy controls. Granulocytes from MPN patients had a higher expression of POT1 (mean ± SD: 1.85 ± 0.62 vs 1.15 ± 0.54, *P* < 0.001) and TIN2 (mean ± SD: 1.32 ± 0.35 vs 1.07 ± 0.33, *P* <0.001) compared to that in healthy controls. In contrast, RAP1 and TPP1 was lower in granulocytes from MPN patients compared to controls (mean ± SD: 0.69 ± 0.18 vs 1.01 ± 0.35, *P* <0.001 and 0.83 ± 0.29 vs 1.0 ± 0.39, *P* <0.01 respectively). RAP1 mRNA levels were lower in all three MPN subtypes compared to healthy controls (*P* <0.001, <0.001, and =0.015 for ET, PV

and PMF, respectively), whereas TPP1 expression was significantly lower only in patients with PV compared to healthy individuals (P = 0.004). Moreover, TPP1 mRNA expression was only reduced in patients who had received HU, suggesting that the aberrant expression of TPP1 is secondary to treatment with HU. The mRNA expression of shelterins was not affected by age, sex or disease duration. Telomere length showed a significant negative correlation with POT1 mRNA expression (r = -0.28, P= 0.002), but was not associated with the expression of other shelterin proteins. The higher expression of POT1 and TIN2 and the lower expression of TPP1 were also confirmed at the protein level by Western blot (WB). No difference in RAP1 protein expression could be detected.

4.1.3 POT1 expression is associated with JAK2^{V617F}

POT1 was the only shelterin protein that was expressed differently depending on the MPN patient's JAK2^{V617F} status (P = 0.04). Furthermore, POT1 mRNA tended to correlate with JAK2^{V617F} allele burden. However, the correlation was not statistically significant (P = 0.08), probably due to the rather limited number of patients. To determine whether the elevated POT1 expression was influenced by JAK2^{V617F} status we treated HEL cells with a JAK2 inhibitor (LY2784544). JAK2-inhibition decreased the expression of POT1 mRNA to approximately half of the original level (P = 0.001). A slight decrease in the protein expression of POT1 was seen after JAK2 inhibition.

4.1.4 Discussion

Telomere shortening in MPN has been reported before[115,116]. It is reasonable to believe that excessive proliferation is one of the mechanisms causing telomere shortening, but all factors underlying telomere shortening in MPNs are still not understood. Both POT1 and TIN2 are known negative regulators of TL[12,144], whereas TPP1 and RAP1 have telomere lengthening functions. Our findings of a higher expression of negative regulators and lower expression of positive regulators of TL support that dysregulation of the shelterin proteins contributes to the telomere shortening seen in MPNs. Understanding this mechanism is of great importance since patients with short telomeres are reported to be more prone to develop secondary MF and to transform to MDS and AML[115]. Our finding that TPP1 expression is lower in patients that received HU needs to be verified by mechanistic studies and its possible effect on MPN pathogenesis evaluated. Whether HU increases the risk of disease transformation is, as mentioned in the introduction, a point of contention[107]. A telomerase inhibitor is in clinical

trials for PMF with promising preliminary results[100]. Given the telomere shortening and expression changes seen in patients with MPNs it is worth investigating if these factors can be used to predict a patients' response to telomerase inhibition.

Previous investigators report an elevated telomerase activity in the BM cells derived from patients with MPNs[115,118]. We analyzed the TERT mRNA expression in granulocytes and could not detect any differences between MPN patients and healthy controls. This finding suggests that telomerase is in general not activated in MPN cells.

4.2 PAPER II

4.2.1 Different *TERT* rs2736100 allele distributions in healthy Swedish and Chinese populations

Genotyping of *TERT* rs2736100 was performed in healthy controls in Sweden (n = 756) and China (n = 101). The A-allele was more frequent in the Han Chinese population (57%) than in the Swedish population (47%) (P = 0.006). Healthy Chinese individuals also had more AA genotypes and less CC genotypes compared to the Swedish population (Table 7). To verify our finding, we collected genotyping data from previously published studies from China[145,146] and Europe[147,148]. The genotype distribution in published studies was very similar to our data for both Han Chinese and Caucasians. When pooling the studies we included a total of 2910 and 1359 healthy individuals from China and Europe, respectively, finding that Europeans had a higher frequency of the C allele and a lower frequency of the A allele (48 vs 57% and 52 vs 43% for A and C respectively, P <0.001, Table 7)

Table 7. Published rs2736100 genotype distribution of healthy populations in China and Europe

Author	N	AA (%)	AC (%)	CC (%)	A (%)	C (%)	Area	Reference
China								
Dahlström et al	101	33 (32.7)	50 (49.5)	18 (17.8)	116 (57.4)	86 (42.6)	North"	This study
Yuan et al	289	86 (29.8)	144 (49.8)	59 (20.4)	316 (54.7)	262 (45.3)	North"	[145]
Wei et al	2520	814 (32.3)	1269 (50.4)	437 (17.3)	2897 (57.5)	2143 (42.5)	South#	[146]
Total	2910	933 (32.1)*	1463 (50.3)*	514 (17.6)*	3329 (57.2)*	2491 (42.8)*		
Europe								
Dahlström et al	756	167 (22.1)	377 (49.9)	212 (28.0)	711 (47.0)	801 (53.0)	Sweden	This study
Jäger et al	202	47 (23.3)	88 (43.6)	67 (33.2)	182 (45.0)	222 (55.0)	Italy	[147]
Krahling et al	400	111 (27.8)	188 (47.0)	101 (25.2)	410 (51.3)	390 (48.7)	Hungary	[148]
Total	1358	325 (23.9)**	653 (48.0)**	380 (28.1)**	1303 (48.0)**	1413 (52.0)**		
		AA vs AC+CC	AC vs AA+CC	CC vs AA+AC		C vs A		
P- value (* vs **)		<0.001	0.106	<0.001		<0.001		

OR and *P*- values generated using Chi-square test. "From Shandong area; #From Shanghai and Guangzhou areas

4.2.2 *TERT* SNP rs2736100_C is a risk factor for myeloproliferative neoplasms in males

Due to different genotype distributions in the healthy populations, we chose to analyze the Chinese and Swedish MPN patients separately. Both Chinese and Swedish MPN patients had a higher frequency of the rs2736100_C allele compared to their corresponding controls (both $P = 0.004$, table 8). The higher frequency of CC genotypes and C alleles was seen in all MPN subtypes, with no difference between them. Further analysis revealed that the higher frequency of the CC genotype and C allele was only present in male MPN patients, whereas no difference was seen between healthy women and women with MPNs. There was no difference in allele distributions between healthy men and women.

Table 8. Genotypes of TERT rs2736100 in MPN patients compared to healthy controls

rs2736100 genotype	Sweden				China			
	Control	MPN			Control	MPN		
	n. (%)	n. (%)	OR (95% CI)	*P value*	n. (%)	n. (%)	OR (95% CI)	*P value*
All	756 (100)	126 (100)			101 (100)	101 (100)		
Alleles								
A	711 (47.0)	94 (37.3)	1.0 (ref)		116 (57.4)	86 (42.6)	1.0 (ref)	
C	801 (53.0)	158 (62.7)	1.49 (1.13-1.96)	*0.004*	86 (42.6)	116 (57.4)	1.82 (1.23-2.70)	*0.004*
Genotypes								
AA	167 (22.1)	15 (11.9)	1.0 (ref)		33 (32.7)	17 (16.8)	1.0 (ref)	
AC	377 (49.9)	64 (50.8)	1.89 (1.05-3.41)	*0.034*	50 (49.5)	52 (51.5)	2.02 (1.00-4.08)	0.057
CC	212 (28.0)	47 (37.3)	2.47 (1.33-4.57)	*0.003*	18 (17.8)	32 (31.7)	3.45 (1.52-7.85)	*0.005*
AA+AC	544 (72.0)	79 (62.7)	1.0 (ref)		83 (82.2)	69 (68.3)	1.0 (ref)	
CC	212 (28.0)	47 (37.3)	1.53 (1.03-2.27)	*0.044*	18 (17.8)	32 (31.7)	2.14 (1.11-4.14)	*0.033*
AC+CC	589 (77.9)	111 (88.1)	1.0 (ref)		68 (67.3)	84 (83.2)	1.0 (ref)	
AA	167 (22.1)	15 (11.9)	0.48 (0.27-0.84)	*0.009*	33 (32.7)	17 (16.8)	0.42 (0.21-0.81)	*0.014*

OR and *P*- values generated using Fishers' exact test.
MPN, Myeloproliferative Neoplasms; OR, Odds ratio; CI, Confidence interval
Significant *P*- values are shown in italic

4.2.3 TERT mRNA expression and telomere length in patients with myeloproliferative neoplasms carrying different *TERT* rs2736100 genotypes

Patients bearing the *TERT* rs2736100_CC genotype displayed the highest TERT mRNA expression, with a significant difference between the AC and CC carrying patients ($P = 0.024$) (figure 12). The difference between the CC and AA variant was not significant, likely due to the small number of patients. No correlation was seen between TL and *TERT* rs2736100 genotype (figure 12).

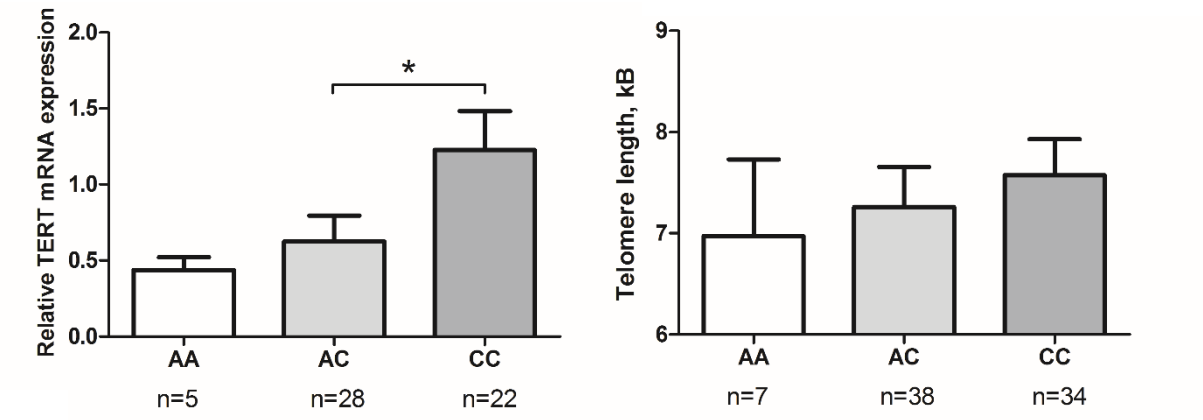

Figure 12. *TERT mRNA expression and TL in Swedish MPN patients with different TERT rs2736100 genotypes. TERT mRNA expression was measured with qRT-PCR and TL was assessed using flow-FISH. * P <0.05*

4.2.4 Discussion

A number of research groups have recently reported an association between the *TERT* rs2736100_C allele and the risk of MPNs[119,120]. Previous studies were exclusively based on Caucasian populations, and in this study we found that the same association is also present in the Chinese population. The frequency of the C allele was found to be higher in the healthy population in Sweden compared to China, which is corroborated by previously published studies from Europe and China. Interestingly, the incidence of MPNs is higher in Europe than in China, which might partly be explained by a difference in *TERT* rs2736100 variants. In this study, the risk of MPNs was associated with the C allele only in males, which has not been described before. Interestingly, female MPN patients have a better prognosis than men[149,150]. Some authors report that the shorter overall survival in men is caused by a higher frequency of secondary MF and AML transformation[150], but others report that the increased risk of developing a secondary malignancy is not gender related[151]. Therefore, further studies are needed to determine if there is an association between the *TERT* rs2736100 C allele and MPN transformation to MDS/AML.

We found that patients with the CC genotype had a higher expression of TERT, which most likely results in a higher telomerase activity. The rs2736100 C allele has previously been linked to higher TERT expression and longer telomeres in both normal- and tumor cells[146]. Our results indicate that patients bearing the C allele may be able to better compensate for the telomere erosion seen in MPNs, an ability which potentially could help to maintain genetically stable cells. However, TERT also has other functions apart from telomere

lengthening[55,57], and has been implicated in cancer development and progression, and could therefore also potentially promote disease transformation in MPNs.

4.3 PAPER III

4.3.1 A unique patient with a very late relapse of acute promyelocytic leukemia

A 42-year-old woman presented with fever, spontaneous bruising and fatigue in April 1994. She was diagnosed with APL by blood/BM analysis which showed the typical translocation (15;17)(q22;q12) and received ATRA and one course of cytarabine and daunorubicin as induction treatment, resulting in CR. After two subsequent consolidation courses of cytarabine and daunorubicin, she remained in good health until 2011, when she again presented with fatigue and bruising. She was again diagnosed with APL and obtained CR after induction treatment with ATRA, idarubicin and cytarabine. She received two consolidation courses with the same agents followed by two additional courses with ATRA and arsenic trioxide (ATO).

4.3.2 Evolution of the patient's acute promyelocytic leukemia-clone

In order to clarify whether the patient had suffered a relapse, another *de novo* APL, or a secondary APL, we performed array-CGH and WGS on BM samples from 1994 and 2011. The array-CGH revealed genetic aberrations in both samples, with clear differences between the two (figure 13). The cells from the 1st diagnosis had losses in chromosome 19, 3 and 20 together with gain of material at chromosome 21. The cells at the 2nd diagnosis had loss of a large part of the long arm of chromosome 9 and gain at chromosome 16. WGS showed two different *PML/RARα* fusion sites (Chr17:38489469-Chr15:74316176 and Chr15:74316160-Chr17:38489139) in both APL samples with a domination of the first fusion in both samples. Importantly, different mutations in *FLT3* were seen in the two samples (*FLT3-ITD* and *FLT3-D835* respectively).

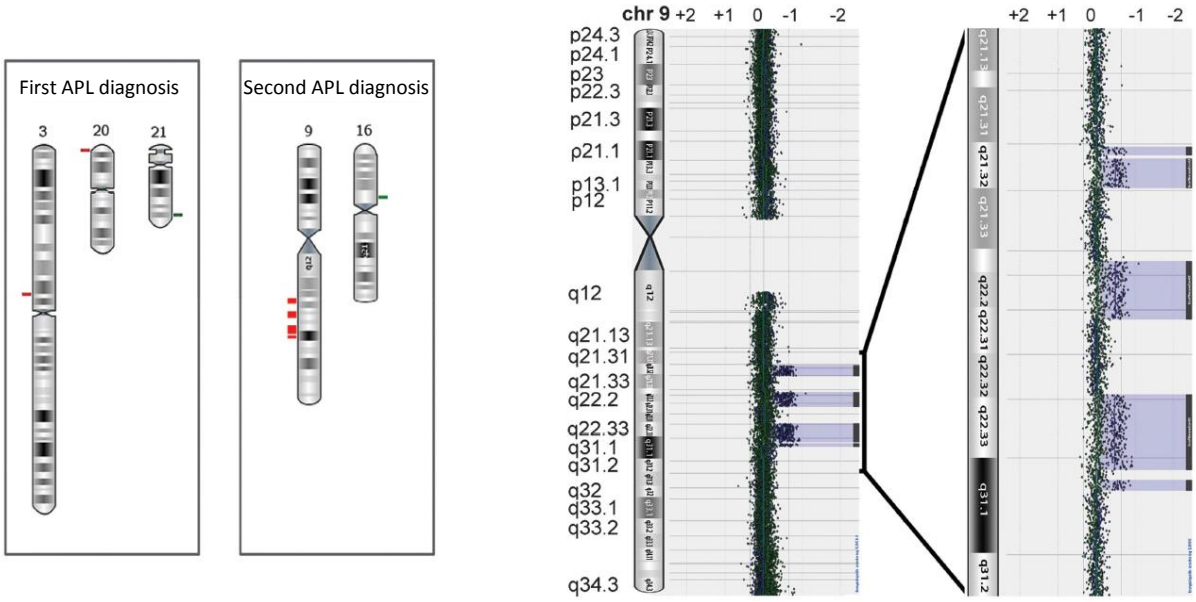

Figure 13. *Array-CGH revealing different genetic aberrations at 1st and 2nd diagnosis. Left panel: regions with genetic loss and gains are presented in red and green respectively.*

4.3.3 Telomere length at diagnosis of acute promyelocytic leukemia and in complete remission

TL was measured using Southern blot in samples from the 1st and 2nd diagnosis and from the first CR. The average terminal restriction fragment length at 1st diagnosis and 2nd diagnosis was shorter (3.40kb, 3.27kb respectively) than that in CR (5.75 kb) (figure 14).

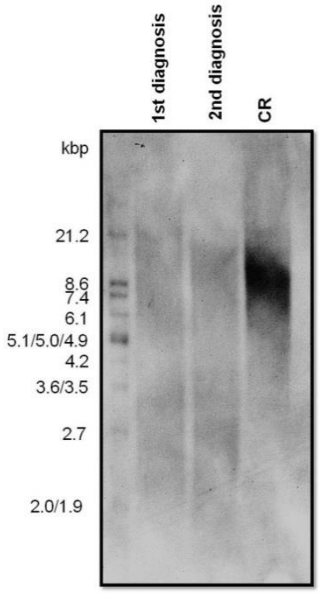

Figure 14. *Southern blot analysis of terminal restriction fragments, showing shorter telomeres at both acute promyelocytic leukemia diagnoses compared to when the patient was in complete remission.*

4.3.4 Discussion

The APL clone at second diagnosis had many genetic similarities with the clone at first diagnosis, such as identical gene fusion for the *PML/RARα* gene. However, the 1st and 2nd samples had significant differences in genetic aberrations and different mutations of *FLT3*. Based on these results, we believe that the 2nd diagnosis was a true relapse from the initial APL. We hypothesize that the patient harbored the *PML/RARα* fusion gene, generating pre-leukemic clones that were not transformed into an APL until the acquisition of the FLT3-ITD mutation. The leukemic clones were successfully eradicated with chemotherapy, whereas the ancestral PML/RARα bearing clone survived. Seventeen years later that clone gained a *FLT3-D835* mutation, leading to a second onset of APL (figure 15). To completely cure APL it is crucial to eradicate the leukemic stem cells (LSC). Evolution of non-eradicated LSC has been reported and relapse after conventional chemotherapy is associated with larger, more complex and more heterogeneous LSC populations[152].

ATO has been demonstrated to target the LSC in APL and thereby prevent relapses[153]. The patient received ATO when she obtained her second APL diagnosis and is currently in CR, but still under close molecular monitoring. The short telomeres seen in her tumor cells at 1st and 2nd diagnosis are coherent with previous findings about critically short telomeres in AML[154]. It would be of importance to define the role of telomere maintenance in pre-leukemic clones and whether this can affect the occurrence of further genetic aberrations causing the development of AML.

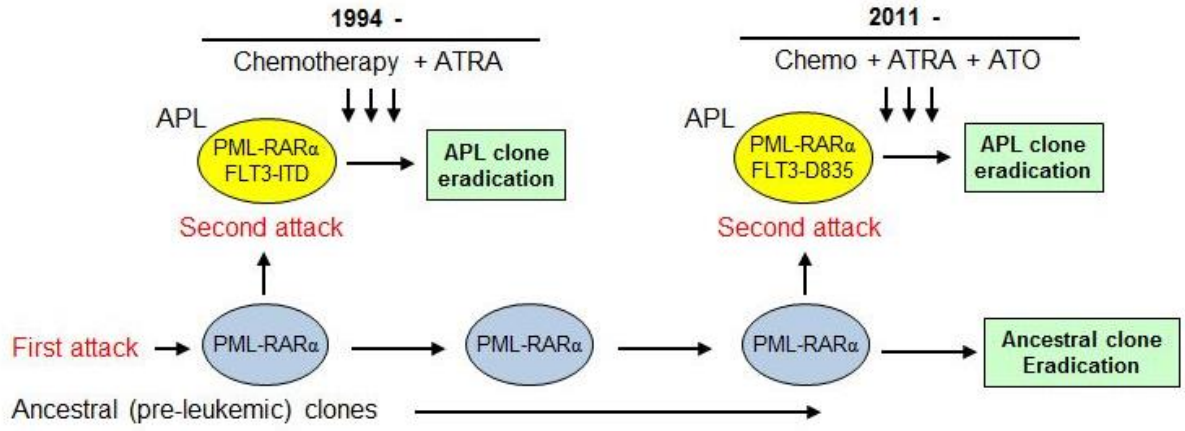

Figure 15. *Proposed evolution of the pre-leukemic clone in the patient.*

4.4 PAPER IV

4.4.1 Reduced cell numbers and viability of HEL cells with JAK2 and telomerase inhibition

Treatment with the JAK2 inhibitor LY2784544 initially reduced the cell number and viability in the erythroleukemia cell line HEL. However, the cells recovered after several weeks of treatment and after 9 weeks of treatment the viability was similar to that of control cells (figure 16). A similar pattern was seen for apoptosis, with a transient increase in apoptotic cells. Treatment with the telomerase inhibitor GRN163L also reduced the number of cells and their viability, but without any recovery over time. Importantly, a combination of LY2784544 and GRN163L reduced the number of cells and their viability more than each treatment by itself, and no recovery was seen over time (figure 16).

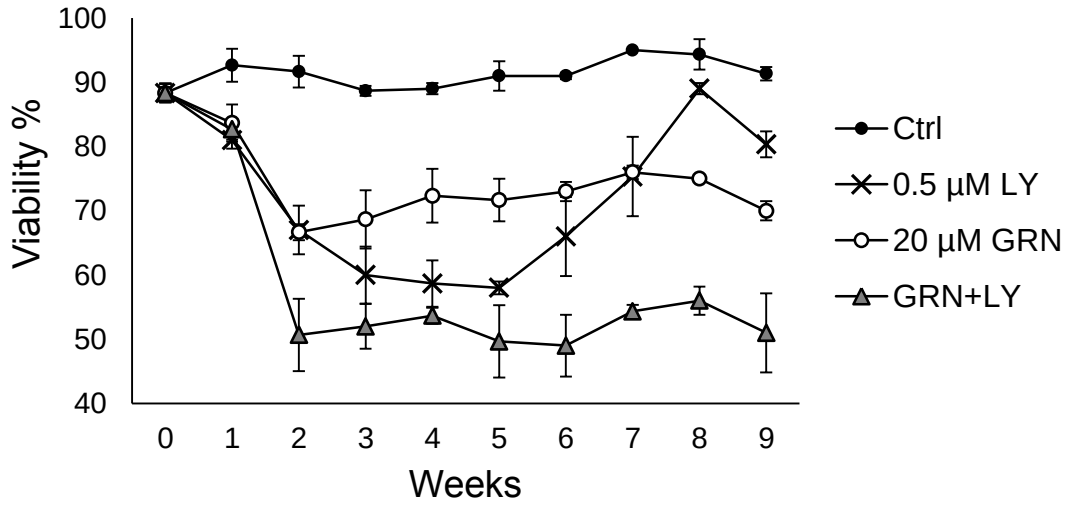

Figure 16. *Viability in HEL cells after treatment with LY2484544 and/or GRN163L.*

4.4.2 Accumulation of CD34 positive HEL cells following JAK2 inhibition

The fraction of CD34 positive HEL cells was drastically increased after treatment with LY2784544, ranging from a few percent to >85% after 9 weeks of incubation (figure 17). In contrast, treatment with GRN163L reduced the number of CD34 positive cells, and even more importantly, a combination of LY2784544 and GRN16L did not increase the CD34 positive fraction. A colony formation assay confirmed that the increased fraction of CD34 positive cells after LY2784544 also gave rise to more and larger colonies.

HEL cells were also separated into CD34 negative and positive fractions before treatment with LY2784544. Here we found a lower viability and higher percentage of apoptotic cells after LY2784544 treatment in the CD34 negative population than in the positive population. An increase in the fraction of CD34 positive cells was seen after LY2784544 treatment in the CD34 negative population.

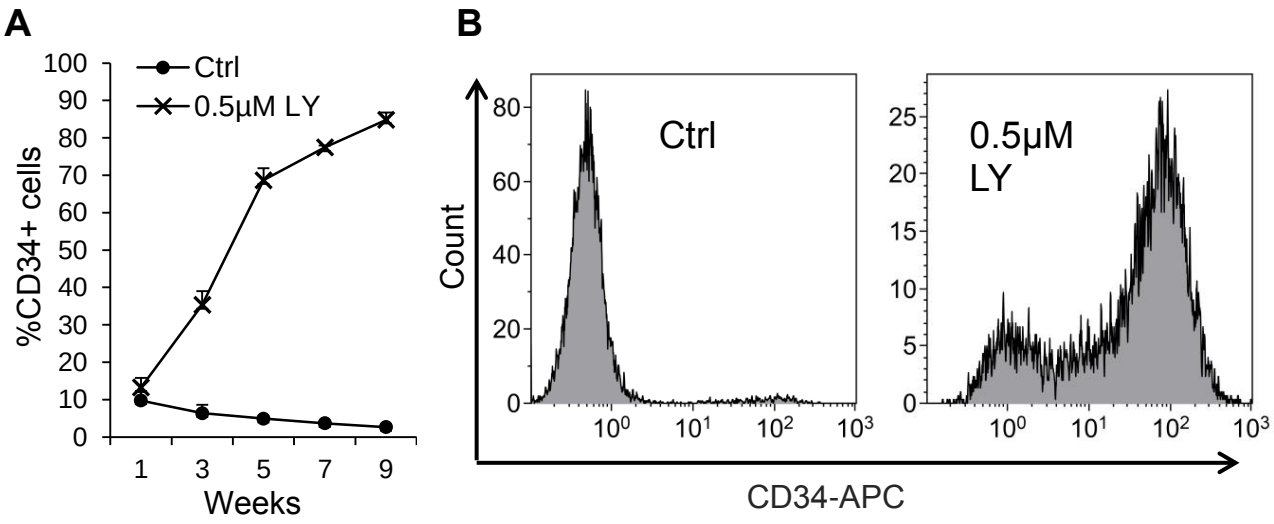

Figure 17. A) *Fraction of CD34 positive HEL cells after LY2784544 treatment.* B) *Histograms of CD34 in cells after 7 weeks of incubation without (left) and with (right) LY2784544 treatment.*

4.4.3 Silencing KLF4 expression attenuated the LY2784544 mediated increase of CD34 positive cells

An Affymetrix whole-transcript analysis was performed to find the mechanism underlying the accumulation of CD34 positive cells after JAK2 inhibition. Several genes were chosen for conformational PCR and WB. From these genes, KLF4 was chosen for further analysis, as it is known to regulate stem cells features. An increased expression of KLF4 after LY2784544 treatment was confirmed both at the mRNA and protein level. Interestingly, KLF4 mRNA expression was significantly lower after treatment with a combination of LY2784544 and GRN163L compared to LY2784544 alone (figure 18A).

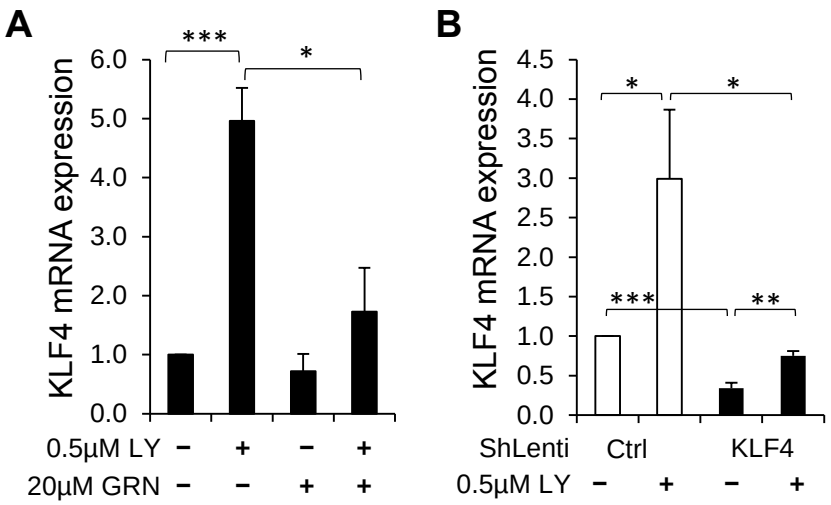

Figure 18. *KLF4 mRNA expression in:* A) *HEL treated with LY2784544 and/or GRN163L* B) *HEL transduced with ShKLF4 or scrambled control treated with LY2784544. * indicates P-value <0.05, **<0.01, ***<0.001.*

KLF4 was silenced using lentiviral transfection with ShKLF4 and the effect was confirmed by PCR and WB. The KLF4 mRNA expression was still increased in the ShRNA KLF4 lentiviral transduced cells following LY2784544, but at much lower levels compared to the cells transduced with the scramble control ShRNA (figure 18B). Silencing KLF4 expression attenuated the LY2784544 mediated increase in CD34 positive cells, but did not block it completely.

4.4.4 JAK2 inhibition down-regulates TERT expression and telomerase activity but elongates the telomeres in HEL cells

A lower TERT mRNA expression and telomerase activity was seen in HEL cells following JAK2 inhibition with LY2784544 (figure 19A). Despite a lower TERT expression and telomerase activity, a significant increase in TL was seen after 7 and 9 weeks of incubation (figure 19B). In order to clarify if the longer telomeres observed after LY2784544 treatment were secondary to the increase in CD34 positive cells, TL was determined in CD34 negative- and positive populations, respectively. Indeed, the CD34 positive cells harbored significantly longer telomeres, but significantly longer telomeres were also observed in CD34 positive cells following 8 weeks of treatment with LY2784544 (figure 19D). Telomerase activity was reduced in both CD34 negative and positive populations at 4 and 8 weeks of treatment with LY2784544 (figure 19C).

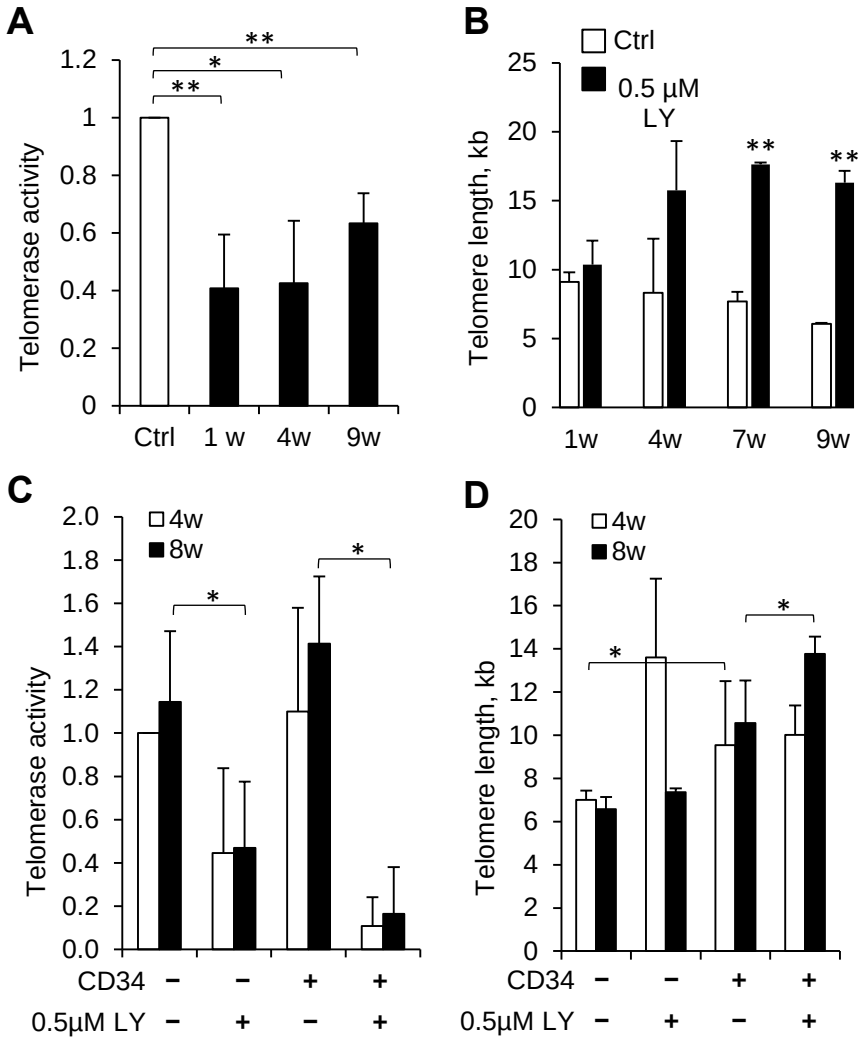

Figure 19. A) *Telomerase activity in HEL cells treated with LY2784544.* B) *Telomere length in HEL cells treated with LY2784544.* C) *Telomerase activity in CD34-sorted HEL cells treated with LY2784544.* D) *Telomere length in CD34-sorted HEL cells treated with LY2784544.*

4.4.5 Discussion

Despite their effect on clinical symptoms JAK2 inhibitors have been reported to be unable to eradicate the disease clone in MPN and to have a minimal effect on $JAK2^{V617F}$ allele burden in most patients[99,155]. Our findings support the notion that immature cells are less sensitive to JAK2 inhibition, resulting in an enrichment of these cells. This could explain the drugs' incapacity to eliminate the disease-initiating clone. Our data also indicated that JAK2 inhibition can induce a more premature state among the cells. The accumulation of CD34 positive cells following treatment with LY2784544 can be of future importance in assisting us to understand potential side effects and clinical responses to this drug.

Interestingly, simultaneous treatment with GRN163L did not increase the number of CD34 positive cells and had a larger effect on cell viability than any of the drugs used alone. The therapeutic effect of telomerase inhibition in MF is promising, but the treatment can also induce severe dose-dependent myelodepression in humans[100]. Targeted therapy in combination could therefore be beneficial by allowing lower doses of GRN163L with the same efficiency. Our data suggests that a combination of JAK2- and telomerase inhibition might be effective in the treatment of MPNs.

KLF4 is one of the four transcription factors that together can be manipulated to generate induced pluripotent stem cells[156]. The role of KLF4 in hematopoiesis is not completely understood, but its ability to contribute to dedifferentiation supports a role in the maintenance of tissue-specific stem cells. Silencing KLF4 expression attenuated the LY2784544-mediated enrichment of CD34 positive cells, indicating that KLF4 upregulation is one mechanism affecting this enrichment. However, the enrichment was not completely blocked by KLF4 repression, indicating that other unknown factors also play a role in this process. Other authors have previously demonstrated that the presence of $JAK2^{V617F}$ downregulates KLF4 in MPN patients[157]. Collectively, this data suggests KLF4 as a potential therapeutic target in MPNs.

In this study we found that JAK2 inhibition reduces telomerase activity. Other authors have previously reported that JAK/STAT signaling is a positive regulator of TERT by the direct binding of STAT3 and STAT5 to the TERT promoter[158]. It is possible that the general activation of the JAK/STAT signaling pathway in MPNs results in an increased TERT expression with a higher telomerase activity as a consequence. Previous studies have shown a higher telomerase activity in hematopoietic stem cells from MPN patients compared to healthy individuals[115]. Despite the lower telomerase activity, longer telomeres were seen after LY2784544 treatment, suggesting that they were elongated by a telomerase-independent mechanism. Further studies are needed to assess whether JAK2 inhibition results in telomere elongation *in vivo* in MPN cells and if so, its potential effect on MPN progression needs to be determined.

5 SUMMARY AND CONCLUSIONS

Overall, the studies included in this thesis support that telomeres and telomerase play a role in the pathogenesis of MPNs and AML. More specifically:

I. Patients with MPNs have shorter telomeres and dysregulation of shelterin proteins compared to healthy controls. Specifically, two shelterin proteins negatively regulating TL were overexpressed and two telomere lengthening shelterins were repressed in MPNs. This may be one of the mechanisms contributing to the telomere shortening seen in MPNs. TERT expression was not higher in granulocytes from MPN patients, suggesting that telomerase is not generally activated in MPN cells.

II. The frequency of the TERT rs2736100 A and C allele is higher and lower, respectively, in the Han Chinese population than in the European population. This may contribute to the lower MPN incidence seen in China compared to that in Europe. The TERT rs2736100_CC genotype is associated with an increased risk of MPNs in both the Chinese and Swedish populations. This association was only seen in males, and the potential role of TERT rs2736100_CC genotype on disease progression needs to be elucidated. The TERT rs2736100_CC is associated with a higher TERT expression in MPN patients, but does not influence TL.

III. We compared the genomic landscape of tumor cells from a patient's 1st APL diagnosis to that of tumor cells from her 2nd diagnosis of APL, after 17 years in CR. The identical breakpoints at the PML-RARα fusion in two APL samples suggest that the patient most likely suffered a relapse of her initial APL. However, different mutations in FLT3 and different genetic aberrations showed how the PML-RARα-carrying pre-leukemic clone evolved while the patient was in complete remission, which provides profound insights into AML development.

IV. LY2784544 treatment of HEL cells caused a transient reduction in the number of cells and their viability. CD34 negative cells are more sensitive to LY2784544, resulting in a dramatic increase in the number of CD34 positive cells. The accumulation of CD34 positive cells can be abolished by simultaneous telomerase inhibition. The increase of the CD34 positive fraction after LY2784544 is mediated by an increased KLF4 expression together with other unknown factors. LY2784544 treatment reduces TERT expression and telomerase activity, but increases TL in HEL cells. Collectively, this study proposes that combining JAK2 and telomerase inhibition may have a therapeutic benefit, and that KLF4 may be a potential therapeutic target in MPNs.

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7 REFERENCES

1. Blackburn EH. Structure and function of telomeres. *Nature*. 1991;350(6319):569-573.
2. de Lange T. Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev*. 2005;19(18):2100-2110.
3. Longhese MP. DNA damage response at functional and dysfunctional telomeres. *Genes Dev*. 2008;22(2):125-140.
4. Levy MZ, Allsopp RC, Futcher AB, Greider CW, Harley CB. Telomere end-replication problem and cell aging. *J Mol Biol*. 1992;225(4):951-960.
5. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. *Nature*. 1990;345(6274):458-460.
6. Vaziri H, Schächter F, Uchida I, et al. Loss of telomeric DNA during aging of normal and trisomy 21 human lymphocytes. *Am J Hum Genet*. 1993;52(4):661-667.
7. Romano GH, Harari Y, Yehuda T, et al. Environmental stresses disrupt telomere length homeostasis. *PLoS Genet*. 2013;9(9):e1003721.
8. Campisi J. Aging and cancer: the double-edged sword of replicative senescence. *J Am Geriatr Soc*. 1997;45(4):482-488.
9. Samassekou O, Gadji M, Drouin R, Yan J. Sizing the ends: normal length of human telomeres. *Ann Anat*. 2010;192(5):284-291.
10. Lei M, Podell ER, Cech TR. Structure of human POT1 bound to telomeric single-stranded DNA provides a model for chromosome end-protection. *Nat Struct Mol Biol*. 2004;11(12):1223-1229.
11. Denchi EL, de Lange T. Protection of telomeres through independent control of ATM and ATR by TRF2 and POT1. *Nature*. 2007;448(7157):1068-1071.
12. Loayza D, De Lange T. POT1 as a terminal transducer of TRF1 telomere length control. *Nature*. 2003;423(6943):1013-1018.
13. Karlseder J. Modern genome editing meets telomeres: the many functions of TPP1. *Genes Dev*. 2014;28(17):1857-1858.
14. Sandin S, Rhodes D. Telomerase structure. *Curr Opin Struct Biol*. 2014;25:104-110.
15. Cifuentes-Rojas C, Shippen DE. Telomerase regulation. *Mutat Res*. 2012;730(1-2):20-27.
16. Bodnar AG, Ouellette M, Frolkis M, et al. Extension of life-span by introduction of telomerase into normal human cells. *Science*. 1998;279(5349):349-352.
17. Counter CM. The roles of telomeres and telomerase in cell life span. *Mutat Res*. 1996;366(1):45-63.
18. Hiyama E, Hiyama K. Telomere and telomerase in stem cells. *Br J Cancer*. 2007;96(7):1020-1024.
19. Ju Z, Jiang H, Jaworski M, et al. Telomere dysfunction induces environmental alterations limiting hematopoietic stem cell function and engraftment. *Nat Med*. 2007;13(6):742-747.
20. Wang Z, Zhu B, Zhang M, et al. Imputation and subset-based association analysis across different cancer types identifies multiple independent risk loci in the TERT-CLPTM1L region on chromosome 5p15.33. *Hum Mol Genet*. 2014;23(24):6616-6633.
21. Kyo S, Takakura M, Fujiwara T, Inoue M. Understanding and exploiting hTERT promoter regulation for diagnosis and treatment of human cancers. *Cancer Sci*. 2008;99(8):1528-1538.
22. Kyo S, Takakura M, Taira T, et al. Sp1 cooperates with c-Myc to activate transcription of the human telomerase reverse transcriptase gene (hTERT). *Nucleic Acids Res*. 2000;28(3):669-677.

23. Yi X, White DM, Aisner DL, Baur JA, Wright WE, Shay JW. An alternate splicing variant of the human telomerase catalytic subunit inhibits telomerase activity. *Neoplasia*. 2000;2(5):433-440.
24. Ulaner GA, Hu JF, Vu TH, Giudice LC, Hoffman AR. Telomerase activity in human development is regulated by human telomerase reverse transcriptase (hTERT) transcription and by alternate splicing of hTERT transcripts. *Cancer Res*. 1998;58(18):4168-4172.
25. Cong YS, Wright WE, Shay JW. Human telomerase and its regulation. *Microbiol Mol Biol Rev*. 2002;66(3):407-425, table of contents.
26. Ulaner GA, Hu JF, Vu TH, Oruganti H, Giudice LC, Hoffman AR. Regulation of telomerase by alternate splicing of human telomerase reverse transcriptase (hTERT) in normal and neoplastic ovary, endometrium and myometrium. *Int J Cancer*. 2000;85(3):330-335.
27. Klapper W, Shin T, Mattson MP. Differential regulation of telomerase activity and TERT expression during brain development in mice. *J Neurosci Res*. 2001;64(3):252-260.
28. Wick M, Zubov D, Hagen G. Genomic organization and promoter characterization of the gene encoding the human telomerase reverse transcriptase (hTERT). *Gene*. 1999;232(1):97-106.
29. Devereux TR, Horikawa I, Anna CH, Annab LA, Afshari CA, Barrett JC. DNA methylation analysis of the promoter region of the human telomerase reverse transcriptase (hTERT) gene. *Cancer Res*. 1999;59(24):6087-6090.
30. Guilleret I, Benhattar J. Demethylation of the human telomerase catalytic subunit (hTERT) gene promoter reduced hTERT expression and telomerase activity and shortened telomeres. *Exp Cell Res*. 2003;289(2):326-334.
31. Guilleret I, Yan P, Grange F, Braunschweig R, Bosman FT, Benhattar J. Hypermethylation of the human telomerase catalytic subunit (hTERT) gene correlates with telomerase activity. *Int J Cancer*. 2002;101(4):335-341.
32. Xu D, Popov N, Hou M, et al. Switch from Myc/Max to Mad1/Max binding and decrease in histone acetylation at the telomerase reverse transcriptase promoter during differentiation of HL60 cells. *Proc Natl Acad Sci U S A*. 2001;98(7):3826-3831.
33. Wang S, Hu C, Zhu J. Transcriptional silencing of a novel hTERT reporter locus during in vitro differentiation of mouse embryonic stem cells. *Mol Biol Cell*. 2007;18(2):669-677.
34. Takakura M, Kyo S, Sowa Y, et al. Telomerase activation by histone deacetylase inhibitor in normal cells. *Nucleic Acids Res*. 2001;29(14):3006-3011.
35. Blasco MA. The epigenetic regulation of mammalian telomeres. *Nat Rev Genet*. 2007;8(4):299-309.
36. Bryan TM, Englezou A, Gupta J, Bacchetti S, Reddel RR. Telomere elongation in immortal human cells without detectable telomerase activity. *EMBO J*. 1995;14(17):4240-4248.
37. Bryan TM, Englezou A, Dalla-Pozza L, Dunham MA, Reddel RR. Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. *Nat Med*. 1997;3(11):1271-1274.
38. Cesare AJ, Reddel RR. Alternative lengthening of telomeres: models, mechanisms and implications. *Nat Rev Genet*. 2010;11(5):319-330.
39. Henson JD, Reddel RR. Assaying and investigating Alternative Lengthening of Telomeres activity in human cells and cancers. *FEBS Lett*. 2010;584(17):3800-3811.
40. Dilley RL, Greenberg RA. ALTernative Telomere Maintenance and Cancer. *Trends Cancer*. 2015;1(2):145-156.

41. Henson JD, Neumann AA, Yeager TR, Reddel RR. Alternative lengthening of telomeres in mammalian cells. *Oncogene*. 2002;21(4):598-610.
42. Queisser A, Heeg S, Thaler M, von Werder A, Opitz OG. Inhibition of telomerase induces alternative lengthening of telomeres during human esophageal carcinogenesis. *Cancer Genet*. 2013;206(11):374-386.
43. Wei YB, Martinsson L, Liu JJ, et al. hTERT genetic variation in depression. *J Affect Disord*. 2016;189:62-69.
44. Schönland SO, Lopez C, Widmann T, et al. Premature telomeric loss in rheumatoid arthritis is genetically determined and involves both myeloid and lymphoid cell lineages. *Proc Natl Acad Sci U S A*. 2003;100(23):13471-13476.
45. Newbold RF. The significance of telomerase activation and cellular immortalization in human cancer. *Mutagenesis*. 2002;17(6):539-550.
46. Shay JW, Bacchetti S. A survey of telomerase activity in human cancer. *Eur J Cancer*. 1997;33(5):787-791.
47. Prescott J, Wentzensen IM, Savage SA, De Vivo I. Epidemiologic evidence for a role of telomere dysfunction in cancer etiology. *Mutat Res*. 2012;730(1-2):75-84.
48. Elenbaas B, Spirio L, Koerner F, et al. Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. *Genes Dev*. 2001;15(1):50-65.
49. Hahn WC, Counter CM, Lundberg AS, Beijersbergen RL, Brooks MW, Weinberg RA. Creation of human tumour cells with defined genetic elements. *Nature*. 1999;400(6743):464-468.
50. Rich JN, Guo C, McLendon RE, Bigner DD, Wang XF, Counter CM. A genetically tractable model of human glioma formation. *Cancer Res*. 2001;61(9):3556-3560.
51. Morales CP, Holt SE, Ouellette M, et al. Absence of cancer-associated changes in human fibroblasts immortalized with telomerase. *Nat Genet*. 1999;21(1):115-118.
52. Jiang XR, Jimenez G, Chang E, et al. Telomerase expression in human somatic cells does not induce changes associated with a transformed phenotype. *Nat Genet*. 1999;21(1):111-114.
53. Shay JW, Wright WE. Role of telomeres and telomerase in cancer. *Semin Cancer Biol*. 2011;21(6):349-353.
54. Stewart SA, Hahn WC, O'Connor BF, et al. Telomerase contributes to tumorigenesis by a telomere length-independent mechanism. *Proc Natl Acad Sci U S A*. 2002;99(20):12606-12611.
55. Liu Z, Li Q, Li K, et al. Telomerase reverse transcriptase promotes epithelial-mesenchymal transition and stem cell-like traits in cancer cells. *Oncogene*. 2013;32(36):4203-4213.
56. Gorbunova V, Seluanov A, Pereira-Smith OM. Expression of human telomerase (hTERT) does not prevent stress-induced senescence in normal human fibroblasts but protects the cells from stress-induced apoptosis and necrosis. *J Biol Chem*. 2002;277(41):38540-38549.
57. Ci X, Li B, Ma X, et al. Bortezomib-mediated down-regulation of telomerase and disruption of telomere homeostasis contributes to apoptosis of malignant cells. *Oncotarget*. 2015;6(35):38079-38092.
58. Horn S, Figl A, Rachakonda PS, et al. TERT promoter mutations in familial and sporadic melanoma. *Science*. 2013;339(6122):959-961.
59. Shi J, Yang XR, Ballew B, et al. Rare missense variants in POT1 predispose to familial cutaneous malignant melanoma. *Nat Genet*. 2014;46(5):482-486.
60. Robles-Espinoza CD, Harland M, Ramsay AJ, et al. POT1 loss-of-function variants predispose to familial melanoma. *Nat Genet*. 2014;46(5):478-481.

61. Guo Y, Kartawinata M, Li J, et al. Inherited bone marrow failure associated with germline mutation of ACD, the gene encoding telomere protein TPP1. *Blood*. 2014;124(18):2767-2774.
62. Michiels JJ, Berneman Z, Schroyens W, De Raeve H. Changing concepts of diagnostic criteria of myeloproliferative disorders and the molecular etiology and classification of myeloproliferative neoplasms: from Dameshek 1950 to Vainchenker 2005 and beyond. *Acta Haematol*. 2015;133(1):36-51.
63. Hultcrantz M, Andersson TM-L, Landgren OM, et al. A Population-Based Study of Incidence of Myeloproliferative Neoplasms in Sweden Between 2000 and 2012. American society of hematology 57th annual meeting and exposition. Orlando, FL; 2015.
64. http://haodf.health.sohu.com/disease/zhenxinghongxibaozengduozheng/jieshao.htm. Vol. 2016; 2009.
65. Johansson P, Kutti J, Andréasson B, et al. Trends in the incidence of chronic Philadelphia chromosome negative (Ph-) myeloproliferative disorders in the city of Göteborg, Sweden, during 1983-99. *J Intern Med*. 2004;256(2):161-165.
66. Berglund S, Zettervall O. Incidence of polycythemia vera in a defined population. *Eur J Haematol*. 1992;48(1):20-26.
67. Melo JV, Goldman JM. Myeloproliferative disorders. Berlin Heidelberg: Springer; 2007.
68. Vardiman JW, Harris NL, Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. *Blood*. 2002;100(7):2292-2302.
69. Tefferi A, Vardiman JW. Classification and diagnosis of myeloproliferative neoplasms: the 2008 World Health Organization criteria and point-of-care diagnostic algorithms. *Leukemia*. 2008;22(1):14-22.
70. Michiels JJ, Berneman Z, Schroyens W, Finazzi G, Budde U, van Vliet HH. The paradox of platelet activation and impaired function: platelet-von Willebrand factor interactions, and the etiology of thrombotic and hemorrhagic manifestations in essential thrombocythemia and polycythemia vera. *Semin Thromb Hemost*. 2006;32(6):589-604.
71. Abdel-Wahab OI, Levine RL. Primary myelofibrosis: update on definition, pathogenesis, and treatment. *Annu Rev Med*. 2009;60:233-245.
72. Cervantes F, Dupriez B, Passamonti F, et al. Improving survival trends in primary myelofibrosis: an international study. *J Clin Oncol*. 2012;30(24):2981-2987.
73. Kralovics R, Passamonti F, Buser AS, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med*. 2005;352(17):1779-1790.
74. Levine RL, Wadleigh M, Cools J, et al. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell*. 2005;7(4):387-397.
75. James C, Ugo V, Le Couédic JP, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature*. 2005;434(7037):1144-1148.
76. Rawlings JS, Rosler KM, Harrison DA. The JAK/STAT signaling pathway. *J Cell Sci*. 2004;117(Pt 8):1281-1283.
77. Tefferi A, Skoda R, Vardiman JW. Myeloproliferative neoplasms: contemporary diagnosis using histology and genetics. *Nat Rev Clin Oncol*. 2009;6(11):627-637.
78. Vannucchi AM, Antonioli E, Guglielmelli P, et al. Clinical profile of homozygous JAK2 617V>F mutation in patients with polycythemia vera or essential thrombocythemia. *Blood*. 2007;110(3):840-846.
79. Haider M, Gangat N, Lasho T, et al. Validation of the revised International Prognostic Score of Thrombosis for Essential Thrombocythemia (IPSET-thrombosis) in 585 Mayo Clinic patients. *Am J Hematol*. 2016;91(4):390-394.

80. Campbell PJ, Griesshammer M, Döhner K, et al. V617F mutation in JAK2 is associated with poorer survival in idiopathic myelofibrosis. *Blood*. 2006;107(5):2098-2100.
81. Vannucchi AM, Pieri L, Guglielmelli P. JAK2 Allele Burden in the Myeloproliferative Neoplasms: Effects on Phenotype, Prognosis and Change with Treatment. *Ther Adv Hematol*. 2011;2(1):21-32.
82. Pietra D, Li S, Brisci A, et al. Somatic mutations of JAK2 exon 12 in patients with JAK2 (V617F)-negative myeloproliferative disorders. *Blood*. 2008;111(3):1686-1689.
83. Scott LM, Tong W, Levine RL, et al. JAK2 exon 12 mutations in polycythemia vera and idiopathic erythrocytosis. *N Engl J Med*. 2007;356(5):459-468.
84. Schnittger S, Bacher U, Haferlach C, et al. Detection of JAK2 exon 12 mutations in 15 patients with JAK2V617F negative polycythemia vera. *Haematologica*. 2009;94(3):414-418.
85. Akpınar TS, Hançer VS, Nalçacı M, Diz-Küçükkaya R. MPL W515L/K Mutations in Chronic Myeloproliferative Neoplasms. *Turk J Haematol*. 2013;30(1):8-12.
86. Klampfl T, Gisslinger H, Harutyunyan AS, et al. Somatic mutations of calreticulin in myeloproliferative neoplasms. *N Engl J Med*. 2013;369(25):2379-2390.
87. Nangalia J, Massie CE, Baxter EJ, et al. Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. *N Engl J Med*. 2013;369(25):2391-2405.
88. Tefferi A, Lasho TL, Tischer A, et al. The prognostic advantage of calreticulin mutations in myelofibrosis might be confined to type 1 or type 1-like CALR variants. *Blood*. 2014;124(15):2465-2466.
89. Santos FP, Verstovsek S. JAK2 inhibitors for myelofibrosis: why are they effective in patients with and without JAK2V617F mutation? *Anticancer Agents Med Chem*. 2012;12(9):1098-1109.
90. Abdel-Wahab O, Pardanani A, Bernard OA, et al. Unraveling the genetic underpinnings of myeloproliferative neoplasms and understanding their effect on disease course and response to therapy: proceedings from the 6th International Post-ASH Symposium. *Am J Hematol*. 2012;87(5):562-568.
91. Gelsi-Boyer V, Brecqueville M, Devillier R, Murati A, Mozziconacci MJ, Birnbaum D. Mutations in ASXL1 are associated with poor prognosis across the spectrum of malignant myeloid diseases. *J Hematol Oncol*. 2012;5:12.
92. Guglielmelli P, Biamonte F, Score J, et al. EZH2 mutational status predicts poor survival in myelofibrosis. *Blood*. 2011;118(19):5227-5234.
93. Hussein K, Huang J, Lasho T, et al. Karyotype complements the International Prognostic Scoring System for primary myelofibrosis. *Eur J Haematol*. 2009;82(4):255-259.
94. Gangat N, Strand J, Lasho TL, et al. Cytogenetic studies at diagnosis in polycythemia vera: clinical and JAK2V617F allele burden correlates. *Eur J Haematol*. 2008;80(3):197-200.
95. Gangat N, Tefferi A, Thanarajasingam G, et al. Cytogenetic abnormalities in essential thrombocythemia: prevalence and prognostic significance. *Eur J Haematol*. 2009;83(1):17-21.
96. Bacher U, Schnittger S, Kern W, Weiss T, Haferlach T, Haferlach C. Distribution of cytogenetic abnormalities in myelodysplastic syndromes, Philadelphia negative myeloproliferative neoplasms, and the overlap MDS/MPN category. *Ann Hematol*. 2009;88(12):1207-1213.
97. Nordic Myeloproliferative Neoplasm Study Group: Nordic guidelines on the diagnosis

and treatment of patients with Myeloproliferative Neoplasms. Sweden 2013.

98. Deininger M, Radich J, Burn TC, Huber R, Paranagama D, Verstovsek S. The effect of long-term ruxolitinib treatment on JAK2p.V617F allele burden in patients with myelofibrosis. *Blood*. 2015;126(13):1551-1554.
99. Angona A, Alvarez-Larrán A, Bellosillo B, Longarón R, Fernández-Rodríguez C, Besses C. Dynamics of JAK2 V617F allele burden of CD34+ haematopoietic progenitor cells in patients treated with ruxolitinib. *Br J Haematol*. 2016;172(4):639-642.
100. Tefferi A, Lasho TL, Begna KH, et al. A Pilot Study of the Telomerase Inhibitor Imetelstat for Myelofibrosis. *N Engl J Med*. 2015;373(10):908-919.
101. Cerquozzi S, Tefferi A. Blast transformation and fibrotic progression in polycythemia vera and essential thrombocythemia: a literature review of incidence and risk factors. *Blood Cancer J*. 2015;5:e366.
102. Björkholm M, Derolf AR, Hultcrantz M, et al. Treatment-related risk factors for transformation to acute myeloid leukemia and myelodysplastic syndromes in myeloproliferative neoplasms. *J Clin Oncol*. 2011;29(17):2410-2415.
103. Mrózek K. Cytogenetic, molecular genetic, and clinical characteristics of acute myeloid leukemia with a complex karyotype. *Semin Oncol*. 2008;35(4):365-377.
104. Milosevic JD, Puda A, Malcovati L, et al. Clinical significance of genetic aberrations in secondary acute myeloid leukemia. *Am J Hematol*. 2012;87(11):1010-1016.
105. Berk PD, Goldberg JD, Silverstein MN, et al. Increased incidence of acute leukemia in polycythemia vera associated with chlorambucil therapy. *N Engl J Med*. 1981;304(8):441-447.
106. Radaelli F, Onida F, Rossi FG, et al. Second malignancies in essential thrombocythemia (ET): a retrospective analysis of 331 patients with long-term follow-up from a single institution. *Hematology*. 2008;13(4):195-202.
107. Björkholm M, Hultcrantz M, Derolf Å. Leukemic transformation in myeloproliferative neoplasms: therapy-related or unrelated? *Best Pract Res Clin Haematol*. 2014;27(2):141-153.
108. Tefferi A, Jimma T, Sulai NH, et al. IDH mutations in primary myelofibrosis predict leukemic transformation and shortened survival: clinical evidence for leukemogenic collaboration with JAK2V617F. *Leukemia*. 2012;26(3):475-480.
109. Abdel-Wahab O, Manshouri T, Patel J, et al. Genetic analysis of transforming events that convert chronic myeloproliferative neoplasms to leukemias. *Cancer Res*. 2010;70(2):447-452.
110. Harutyunyan A, Klampfl T, Cazzola M, Kralovics R. p53 lesions in leukemic transformation. *N Engl J Med*. 2011;364(5):488-490.
111. Mascarenhas J. A Concise Update on Risk Factors, Therapy, and Outcome of Leukemic Transformation of Myeloproliferative Neoplasms. *Clin Lymphoma Myeloma Leuk*. 2016;16 Suppl:S124-129.
112. Rossi D, Deambrogi C, Capello D, et al. JAK2 V617F mutation in leukaemic transformation of philadelphia-negative chronic myeloproliferative disorders. *Br J Haematol*. 2006;135(2):267-268.
113. Shirane S, Araki M, Morishita S, et al. Consequences of the JAK2V617F allele burden for the prediction of transformation into myelofibrosis from polycythemia vera and essential thrombocythemia. *Int J Hematol*. 2015;101(2):148-153.
114. Theocharides A, Boissinot M, Girodon F, et al. Leukemic blasts in transformed JAK2-V617F-positive myeloproliferative disorders are frequently negative for the JAK2-V617F mutation. *Blood*. 2007;110(1):375-379.

115. Spanoudakis E, Bazdiara I, Pantelidou D, et al. Dynamics of telomere's length and telomerase activity in Philadelphia chromosome negative myeloproliferative neoplasms. *Leuk Res*. 2011;35(4):459-464.
116. Bernard L, Belisle C, Mollica L, et al. Telomere length is severely and similarly reduced in JAK2V617F-positive and -negative myeloproliferative neoplasms. *Leukemia*. 2009;23(2):287-291.
117. Elena C, Rumi E, Portolan M, Della Porta MG, Pascutto C, Passamonti F. Flow-FISH evaluation of telomere length in Philadelphia-negative myeloproliferative neoplasms. *Haematologica*. 2011;96(8):1236-1238.
118. Kim M, Oh B, Kim TY, et al. Elevated telomerase activity in essential thrombocythemia with extreme thrombocytosis. *Clin Biochem*. 2014;47(6):389-392.
119. Oddsson A, Kristinsson SY, Helgason H, et al. The germline sequence variant rs2736100_C in TERT associates with myeloproliferative neoplasms. *Leukemia*. 2014;28(6):1371-1374.
120. Trifa AP, Bănescu C, Tevet M, et al. TERT rs2736100 A>C SNP and JAK2 46/1 haplotype significantly contribute to the occurrence of JAK2 V617F and CALR mutated myeloproliferative neoplasms - a multicentric study on 529 patients. *Br J Haematol*. 2016.
121. Khwaja A, Bjorkholm M, Gale RE, et al. Acute myeloid leukaemia. *Nat Rev Dis Primers*. 2016;2:16010.
122. Bennett JM, Catovsky D, Daniel MT, et al. Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. *Br J Haematol*. 1976;33(4):451-458.
123. Juliusson G, Antunovic P, Derolf A, et al. Age and acute myeloid leukemia: real world data on decision to treat and outcomes from the Swedish Acute Leukemia Registry. *Blood*. 2009;113(18):4179-4187.
124. Takahashi S. Current findings for recurring mutations in acute myeloid leukemia. *J Hematol Oncol*. 2011;4:36.
125. Kelly LM, Gilliland DG. Genetics of myeloid leukemias. *Annu Rev Genomics Hum Genet*. 2002;3:179-198.
126. Shen Y, Zhu YM, Fan X, et al. Gene mutation patterns and their prognostic impact in a cohort of 1185 patients with acute myeloid leukemia. *Blood*. 2011;118(20):5593-5603.
127. Rowley JD, Golomb HM, Dougherty C. 15/17 translocation, a consistent chromosomal change in acute promyelocytic leukaemia. *Lancet*. 1977;1(8010):549-550.
128. Grimwade D, Gorman P, Duprez E, et al. Characterization of cryptic rearrangements and variant translocations in acute promyelocytic leukemia. *Blood*. 1997;90(12):4876-4885.
129. Martin P, Papayannopoulou T. HEL cells: a new human erythroleukemia cell line with spontaneous and induced globin expression. *Science*. 1982;216(4551):1233-1235.
130. Quentmeier H, MacLeod RA, Zaborski M, Drexler HG. JAK2 V617F tyrosine kinase mutation in cell lines derived from myeloproliferative disorders. *Leukemia*. 2006;20(3):471-476.
131. Scott LM, Campbell PJ, Baxter EJ, et al. The V617F JAK2 mutation is uncommon in cancers and in myeloid malignancies other than the classic myeloproliferative disorders. *Blood*. 2005;106(8):2920-2921.
132. Ma L, Zhao B, Walgren RA, et al. Efficacy of LY2784544, a Small Molecule Inhibitor Selective for Mutant JAK2 Kinase, In *JAK2 V617F*-Induced Hematologic Malignancy Models. 52nd Annual Meeting of the American Society of Hematology. Orlando, Florida, USA; 2010.

133. Scherber R, Dueck AC, Johansson P, et al. The Myeloproliferative Neoplasm Symptom Assessment Form (MPN-SAF): international prospective validation and reliability trial in 402 patients. *Blood*. 2011;118(2):401-408.
134. Verstovsek S, Mesa RA, Kloeker Rhoades S, et al. Phase I Study of the JAK2 V617F Inhibitor, LY2784544, in Patients with Myelofibrosis (MF), Polycythemia Vera (PV), and Essential Thrombocythemia (ET). *Blood*. 2011;118(21):2814.
135. Baerlocher GM, Vulto I, de Jong G, Lansdorp PM. Flow cytometry and FISH to measure the average length of telomeres (flow FISH). *Nat Protoc*. 2006;1(5):2365-2376.
136. Xin H. Telomeric repeat amplification protocol: measuring the activity of the telomerase. *Methods Mol Biol*. 2011;735:107-111.
137. Skvortsov DA, Zvereva ME, Shpanchenko OV, Dontsova OA. Assays for detection of telomerase activity. *Acta Naturae*. 2011;3(1):48-68.
138. Chen K, Wallis JW, McLellan MD, et al. BreakDancer: an algorithm for high-resolution mapping of genomic structural variation. *Nat Methods*. 2009;6(9):677-681.
139. Li H, Handsaker B, Wysoker A, et al. The Sequence Alignment/Map format and SAMtools. *Bioinformatics*. 2009;25(16):2078-2079.
140. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J Immunol Methods*. 1995;184(1):39-51.
141. O'Brien MC, Bolton WE. Comparison of cell viability probes compatible with fixation and permeabilization for combined surface and intracellular staining in flow cytometry. *Cytometry*. 1995;19(3):243-255.
142. Schmid I, Uittenbogaart CH, Keld B, Giorgi JV. A rapid method for measuring apoptosis and dual-color immunofluorescence by single laser flow cytometry. *J Immunol Methods*. 1994;170(2):145-157.
143. Sanders JL, Newman AB. Telomere length in epidemiology: a biomarker of aging, age-related disease, both, or neither? *Epidemiol Rev*. 2013;35:112-131.
144. Kim SH, Kaminker P, Campisi J. TIN2, a new regulator of telomere length in human cells. *Nat Genet*. 1999;23(4):405-412.
145. Yuan X, Meng Y, Li P, et al. The association between the TERT rs2736100 AC genotype and reduced risk of upper tract urothelial carcinomas in a Han Chinese population. *Oncotarget*. 2016.
146. Wei R, Cao L, Pu H, et al. TERT Polymorphism rs2736100-C Is Associated with EGFR Mutation-Positive Non-Small Cell Lung Cancer. *Clin Cancer Res*. 2015;21(22):5173-5180.
147. Jäger R, Harutyunyan AS, Rumi E, et al. Common germline variation at the TERT locus contributes to familial clustering of myeloproliferative neoplasms. *Am J Hematol*. 2014;89(12):1107-1110.
148. Krahling T, Balassa K, Kiss KP, et al. Co-occurrence of Myeloproliferative Neoplasms and Solid Tumors Is Attributed to a Synergism Between Cytoreductive Therapy and the Common TERT Polymorphism rs2736100. *Cancer Epidemiol Biomarkers Prev*. 2016;25(1):98-104.
149. Hultcrantz M, Kristinsson SY, Andersson TM, et al. Patterns of survival among patients with myeloproliferative neoplasms diagnosed in Sweden from 1973 to 2008: a population-based study. *J Clin Oncol*. 2012;30(24):2995-3001.
150. Stein B, Spivak JL, Moliterno AR. Gender Is a Core Modifier of Disease Outcomes and Survival in the MPN. American Society of Hematology, 57th annual meeting and exposition. Orlando; 2015.

151. Ravn Landtblom A, Bower H, Andersson TM-L, et al. Increased Risk of Second Malignancies in Patients with Myeloproliferative Neoplasms Diagnosed in Sweden 1973-2009 - A population-based Cohort Study of 9,379 Patients. European Hematology Association 21st Congress. Copenhagen, Denmark; 2016.
152. Ho TC, LaMere M, Stevens BM, et al. Evolution of acute myelogenous leukemia stem cell properties after treatment and progression. *Blood*. 2016;128(13):1671-1678.
153. Mi JQ, Li JM, Shen ZX, Chen SJ, Chen Z. How to manage acute promyelocytic leukemia. *Leukemia*. 2012;26(8):1743-1751.
154. Swiggers SJ, Kuijpers MA, de Cort MJ, Beverloo HB, Zijlmans JM. Critically short telomeres in acute myeloid leukemia with loss or gain of parts of chromosomes. *Genes Chromosomes Cancer*. 2006;45(3):247-256.
155. Wang X, Ye F, Tripodi J, et al. JAK2 inhibitors do not affect stem cells present in the spleens of patients with myelofibrosis. *Blood*. 2014;124(19):2987-2995.
156. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 2006;126(4):663-676.
157. Berkofsky-Fessler W, Buzzai M, Kim MK, et al. Transcriptional profiling of polycythemia vera identifies gene expression patterns both dependent and independent from the action of JAK2V617F. *Clin Cancer Res*. 2010;16(17):4339-4352.
158. Yamada O, Kawauchi K. The role of the JAK-STAT pathway and related signal cascades in telomerase activation during the development of hematologic malignancies. *JAKSTAT*. 2013;2(4):e25256.