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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-RAR α 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-RAR α -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- <i>trans</i> -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 proteins¹. 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 sequence². The telomere and its associated proteins form protective caps on human chromosome ends and prevents them from being recognized as double strand breaks³. They are thus essential for maintaining genomic stability and integrity.

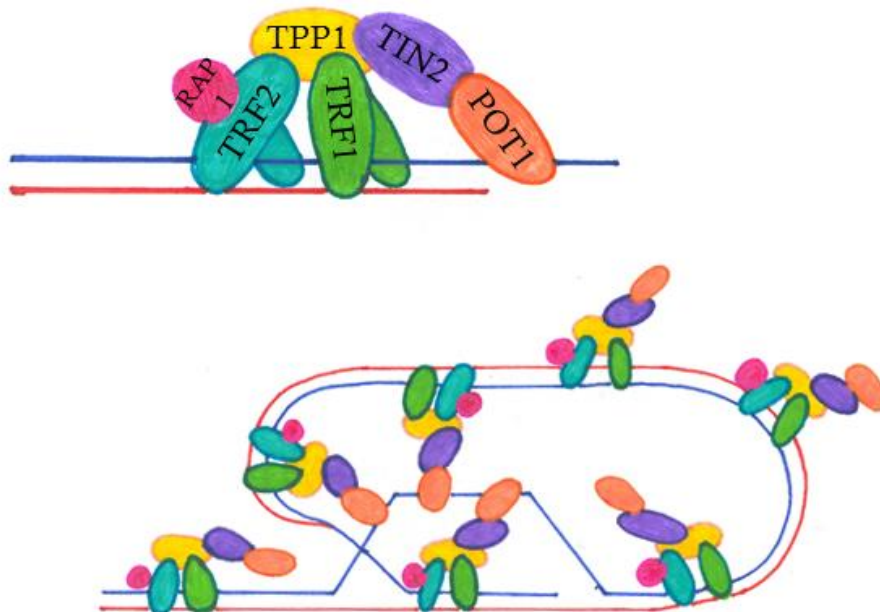


Figure 1. *Telomere structure. Upper picture show how the telomere binding proteins interact with the telomeric DNA in an open state. Lower picture shows a closed telomeric structure that is hold together by the telomere binding proteins.*

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 strand⁴ (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

factors⁷. 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 tissues⁸. The telomere length (TL) in normal human cells varies between 5-15 kb⁹. Many factors influence the TL and telomere maintenance is a delicate dynamic process of lengthening and erosion.

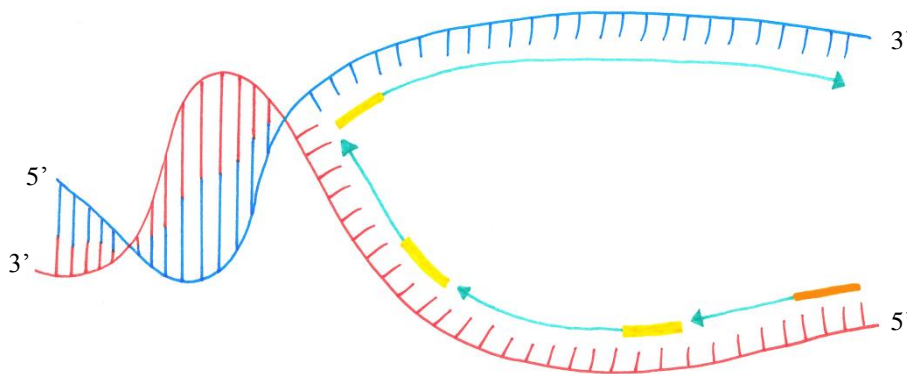


Figure 2. Illustration of the end replication problem. Replication of the lagging strand occurs stepwise. The primers (yellow) required for initiation of replication by polymerase are degraded and the gap filled in. The space where the outermost primer binds (orange) cannot be filled in and the telomere 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 sequence². TRF1 and TRF2 bind directly to the double stranded telomeric sequence whereas POT1 binds directly to the single stranded overhang of telomeres¹⁰. 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 pathways¹¹. 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 lengthening¹². In contrast to POT1, TPP1 is essential for recruiting telomerase to the telomeres, and certain mutations in TPP1 result in excessive telomeric loss¹³.

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 TER¹⁴. Telomerase is very tightly regulated and only active in cells in need of extensive proliferation potential, such as activated lymphocytes, stem cells and embryonic tissues¹⁵. The main regulator of telomerase activity is the expression of TERT¹⁵. Ectopic expression of TERT is enough to induce telomerase activity, suggesting that TERT is the rate limiting component of telomerase¹⁶.

The positive relationship between cellular life-span and telomere length and telomerase expression has been well established¹⁷. Stem/progenitor cells with great proliferation potential have a higher expression of telomerase to be able to compensate for the telomere loss during cell divisions¹⁸. 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 cells¹⁹.

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* promoter 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 Menin²¹. *TERT* transcription is highly influenced by the general transcription factor SP1 that binds to the TATA binding motif²¹. Interestingly, no TATA box has been found in the *TERT* promoter, yet mutations affecting SP1 binding sites can attenuate or even eliminate *TERT* promoter activity²².

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 phosphorylated²⁵. 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 cancers²⁸. 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 demethylation²⁹, 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 histones³²⁻³⁴. Apart from TERT regulation, histone modifications and methylation of the subtelomeric region have also been shown to play a role in regulating TL³⁵.

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 telomerase⁴⁰. ALT typically causes a great variation in TL within an individual cell, ranging from atypically long to undetectably short⁴¹. 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 repressed⁴².

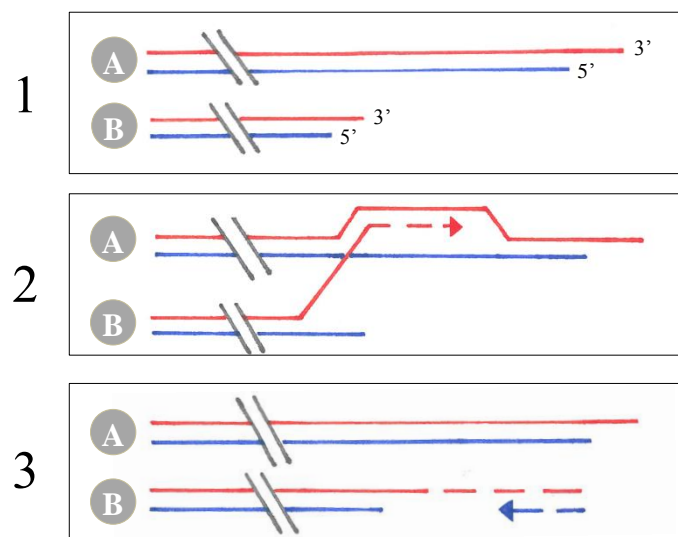


Figure 3. Illustration of alternative lengthening of telomeres. (2) The 3' end of telomere B places itself alongside telomere A and is elongated, using telomere A as a template. (3) This elongation of the B telomere's 3' end enables the synthetization of its complementary strand.

1.1.6 Telomeres and telomerase in human diseases

Telomere dysfunction have been shown to play a role in many different diseases, from depression⁴³ to rheumatoid arthritis⁴⁴, 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 types⁴⁵⁻⁴⁷. The other 10% are thought to elongate their telomeres by ALT or other similar mechanisms⁴⁰. Telomerase activation is thus necessary for the development of several cancer types⁴⁸⁻⁵⁰, 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 telomeres⁵³. 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).

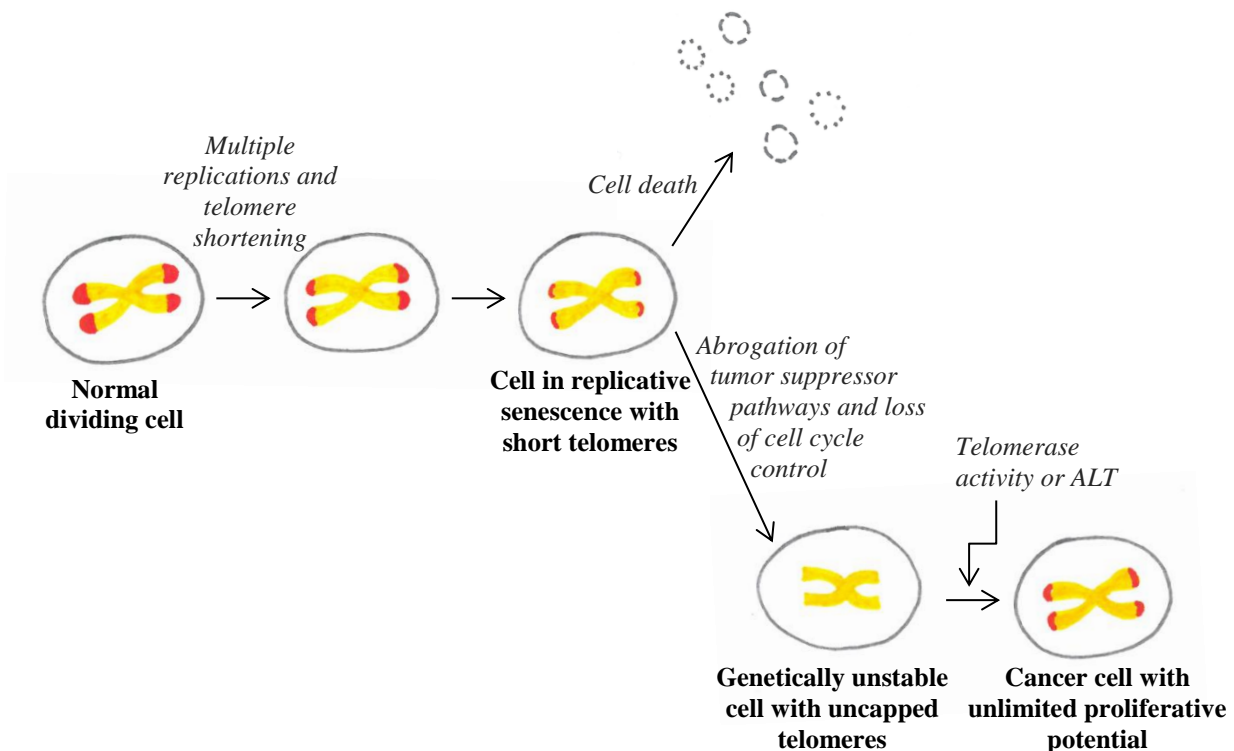


Figure 4. *Illustration of telomere shortening and how malignant transformation can occur when cells manage to bypass replicative senescence. Italic text describes events whereas bold text describes the state of the cell.*

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 telomeres⁵³. 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 melanoma⁵⁸⁻⁶⁰, and mutations in *TPP1* have recently been shown to cause bone marrow failure including aplastic anemia⁶¹.

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 blushing⁶⁷. 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 counts⁶⁷. 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).

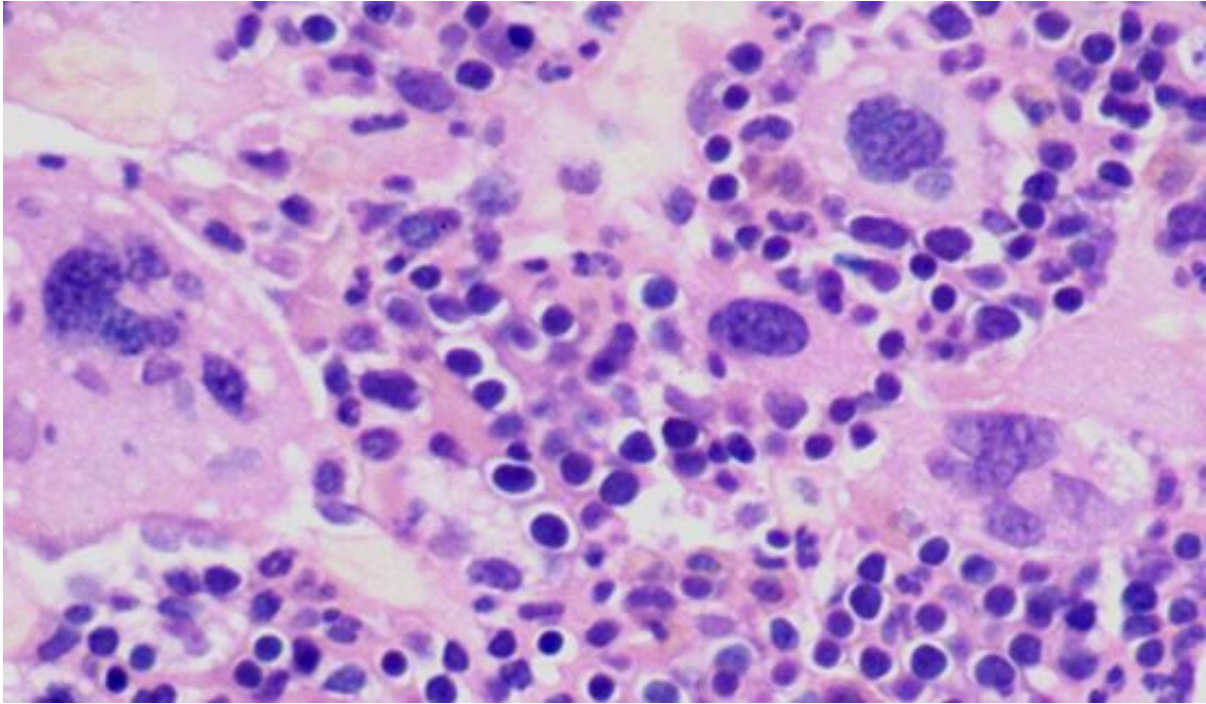


Figure 5. Bone marrow from a patient with polycythemia vera showing a dominating erythropoiesis and large megakaryocytes.

Table 1. The 2008 WHO classification system for diagnosis of PV⁶⁹.

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 ($>450 \times 10^9/L$), 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 ($>1,500 \times 10^9/L$)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 thrombopoietin receptor *MPL* or *CALR* (see chapter 1.2.4).

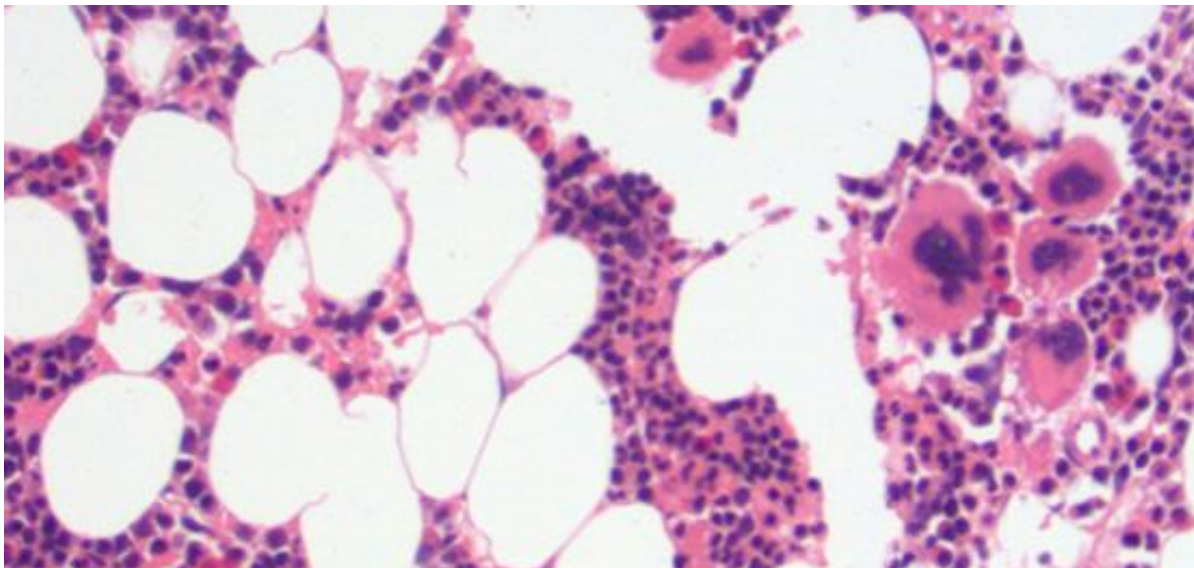


Figure 6. Large mature megakaryocytes in the bone marrow from a patient with essential thrombocythemia.

Table 2. The 2008 WHO classification system for diagnosis of ET⁶⁹.

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

Major criteria

1. Platelet count $>450 \times 10^9/L$
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 Sweden⁶⁵. 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 years⁷².

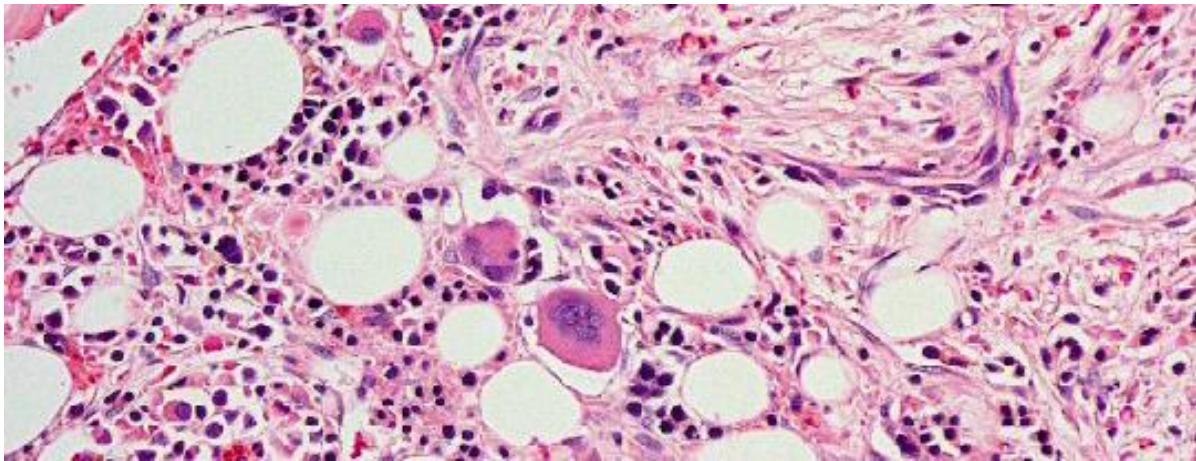


Figure 7. Fibrosis in the bone marrow from a patient with primary myelofibrosis.

Table 3. The 2008 WHO classification system for diagnosis of PMF⁶⁹.

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 simultaneously⁷³⁻⁷⁵. 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 signal⁷⁶. 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 $JAK2^{V617F}$ is present in 95% of PV-patients and 60% and 50% of ET- and PMF patients, respectively⁷⁷. 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 patients⁷⁹. The reports regarding the clinical role of $JAK2^{V617F}$ in PMF are somewhat contradictory, where some authors report a reduced overall survival when the mutation is present⁸⁰, and others report that a low allele burden has been associated with shorter survival⁸¹.

$JAK2^{V617F}$ 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 lineage⁸²⁻⁸⁴. About 5% of ET and PMF patients harbor activating mutations in the thrombopoietin receptor *MPL* (W515L or W515K), resulting in cytokine-independent growth mediated through the JAK/STAT signaling cascade⁸⁵.

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 $JAK2^{V617F}$ mutation⁸⁸. 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, respectively⁹³⁻⁹⁵. Most frequent abnormalities are shared with other hematological malignancies, but +9 and del(13q) mainly occur in MPNs⁹⁶.

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 ($>1,500 \times 10^9/L$) 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 treatment⁹⁷. 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 pathway⁸⁹. 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 ET¹⁰¹.

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 ET¹⁰². Patients with PMF have the highest risk of transformation, 8 - 20% during a 10 year observation¹⁰². 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 treatment¹⁰².

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 fibrosis¹¹¹. The most common mutation in MPNs, $JAK2^{V617F}$, has not been linked to an increased risk for transformation into AML¹¹². However an association between $JAK2^{V617F}$ allele burden in PV and ET and the transformation to MF has been reported¹¹³. 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 MPN¹¹⁴.

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 individuals¹¹⁵⁻¹¹⁷. Authors have also reported that short telomeres in BM cells could predict progression of MPN¹¹⁵. 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.

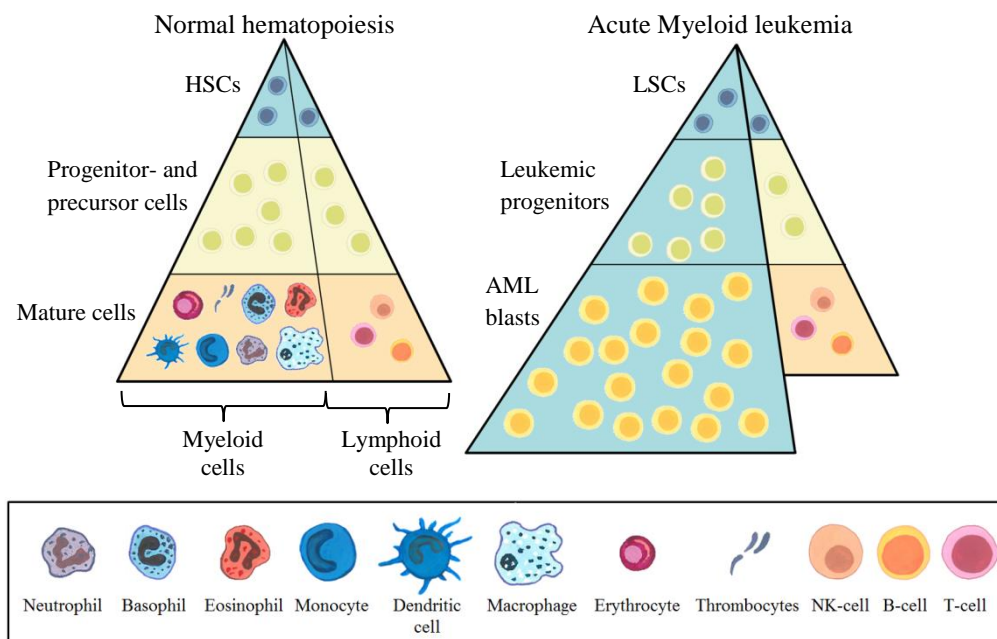


Figure 8. Normal and leukemic hematopoiesis. Hematopoietic stem cells (HSCs) give rise to progenitor and precursor cells that are committed to specific hematopoietic lineages. Those cells later differentiate to become mature blood cells. Leukemic stem cells (LSCs) generate leukemic progenitors and the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

Diagnostic criteria for AML are the presence of $\geq 20\%$ myeloblasts in the BM or even less if characteristic genetic aberrations are detected⁶⁸. Historically, AML has been classified using the FAB-classification system described in table 4¹²². Today the WHO 2008 classification is used, which is based on the presence of genetic aberrations and is shown in table 5⁶⁸. 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 Sweden¹²³. The median age at diagnosis is approximately 70 years and AML is slightly more common among men than among women¹²¹.

Table 4. French-American-British classification of acute myeloid leukemias	Table 5. WHO 2008 classification of acute myeloid leukemia
<p>M0: Acute myeloblastic leukemia, minimally differentiated</p> <p>M1: Acute myeloblastic leukemia, without maturation</p> <p>M2: Acute myeloblastic leukemia, with granulocytic maturation</p> <p>M3: Acute promyelocytic leukemia</p> <p>M4: Acute myelomonocytic leukemia</p> <p>M5: Acute monoblastic leukemia</p> <p>M6: Acute erythroid leukemias</p> <p>M7: Acute megakaryoblastic leukemia</p>	<ul style="list-style-type: none"> • 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 acquired¹²⁴. 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 development¹²⁵. 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 AML¹²¹. 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-RAR α 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 erythroleukemia¹²⁹. 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 × 10⁶/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 JAK2¹³². 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%

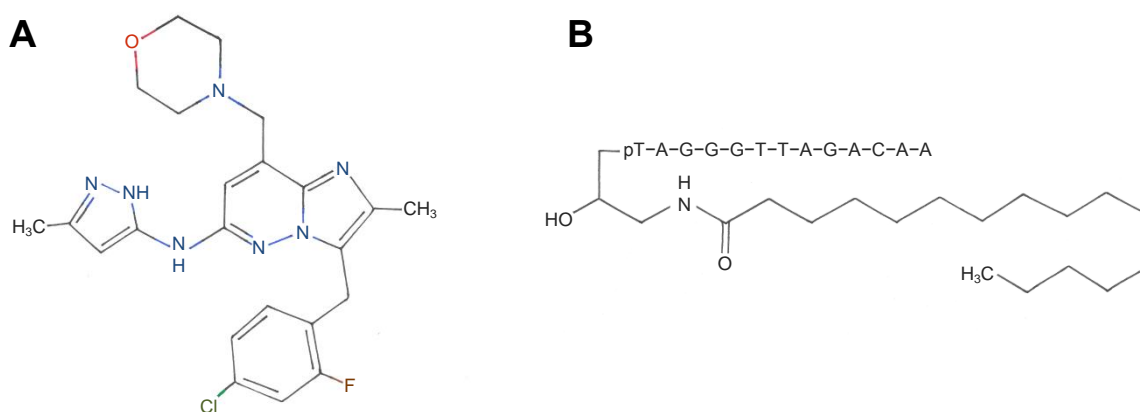


Figure 9. Chemical structure of: A) LY2784544, B) GRN163L.

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 al¹³⁵ 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:

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

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.

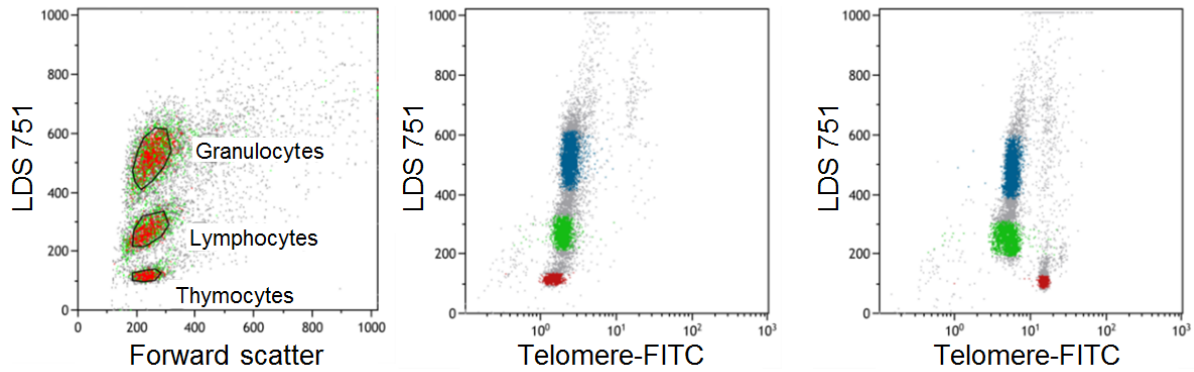


Figure 10. Flow-FISH assessment of telomere length in blood cells from MPN patients.

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 Real-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 substrate¹³⁶.

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-RAR α , the intrachromosomal translocation analysis of paired-end sequence data of initial and second APL samples was performed using BreakDancer¹³⁸. First, all Maq-mapped reads within 3000bp of chromosomal locations of PML and RAR α were extracted by SAMtools¹³⁹. 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 phosphatase (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% CO₂. 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 apoptosis¹⁴⁰. 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 staining¹⁴¹. 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 cells¹⁴².

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 \pm SD: 7.27 kb \pm 2.11 vs 8.66 kb \pm 1.82, $P < 0.001$; figure 11). As previously reported¹⁴³, 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 \pm SD	67 \pm 13	52 \pm 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 \pm SD	6.6 \pm 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

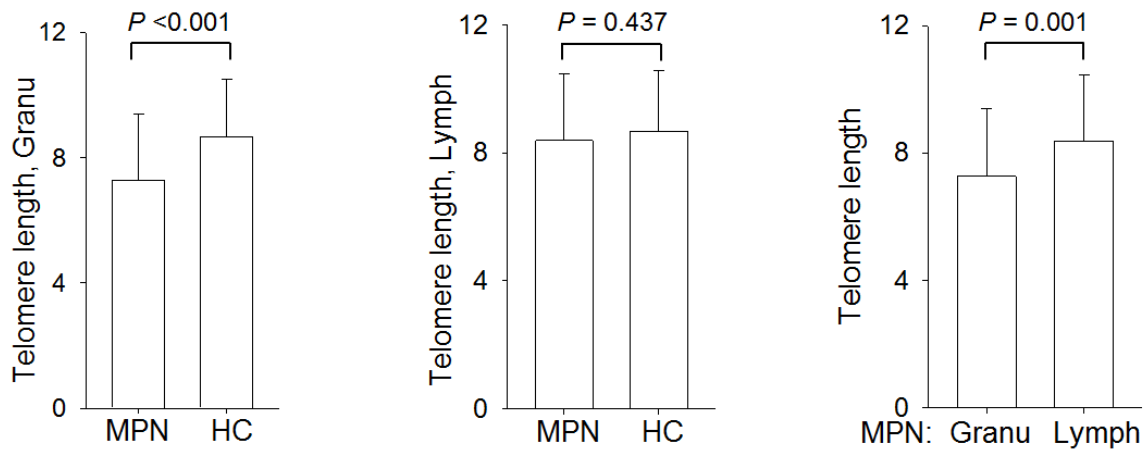


Figure 11. Telomere length (kb) in granulocytes (*Granu*) and lymphoid cells (*Lymph*) derived from patients with MPNs and healthy controls (HC).

healthy controls (mean \pm SD: 8.38 kb \pm 2.08 vs 8.67 kb \pm 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 healthy 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 \pm SD: 1.85 \pm 0.62 vs 1.15 \pm 0.54, $P < 0.001$) and TIN2 (mean \pm SD: 1.32 \pm 0.35 vs 1.07 \pm 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 \pm SD: 0.69 \pm 0.18 vs 1.01 \pm 0.35, $P < 0.001$ and 0.83 \pm 0.29 vs 1.0 \pm 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 AML¹¹⁵. 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 contention¹⁰⁷. A telomerase inhibitor is in clinical

trials for PMF with promising preliminary results¹⁰⁰. 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		
<i>P</i> -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

<i>rs2736100</i> genotype	Sweden				China			
	Control n. (%)	MPN n. (%)	OR (95% CI)	<i>P</i> value	Control n. (%)	MPN n. (%)	OR (95% CI)	<i>P</i> 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)	<i>0.004</i>	86 (42.6)	116 (57.4)	1.82 (1.23-2.70)	<i>0.004</i>
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)	<i>0.034</i>	50 (49.5)	52 (51.5)	2.02 (1.00-4.08)	<i>0.057</i>
CC	212 (28.0)	47 (37.3)	2.47 (1.33-4.57)	<i>0.003</i>	18 (17.8)	32 (31.7)	3.45 (1.52-7.85)	<i>0.005</i>
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)	<i>0.044</i>	18 (17.8)	32 (31.7)	2.14 (1.11-4.14)	<i>0.033</i>
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)	<i>0.009</i>	33 (32.7)	17 (16.8)	0.42 (0.21-0.81)	<i>0.014</i>

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).

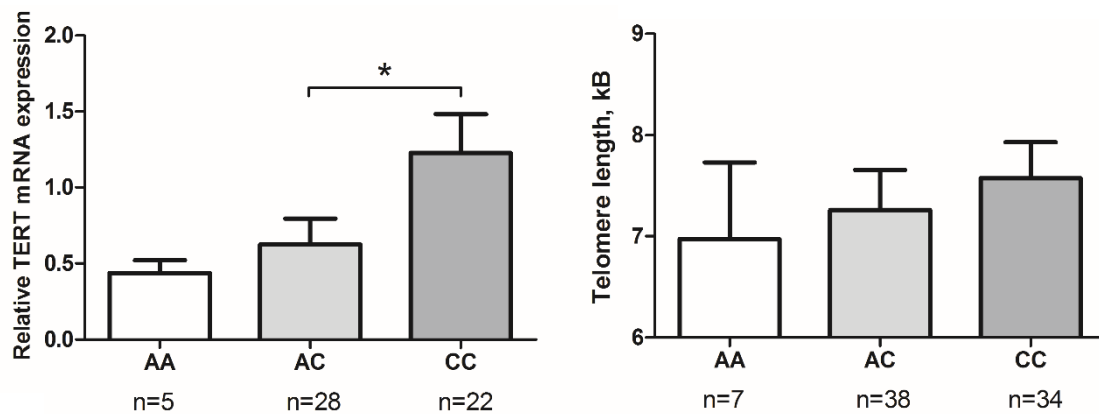


Figure 12. *TERT* mRNA expression and TL in Swedish MPN patients with different *TERT* rs2736100 genotypes. *TERT* mRNA expression was measured with qRT-PCR and TL was assessed using flow-FISH. * $P < 0.05$

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 transformation¹⁵⁰, but others report that the increased risk of developing a secondary malignancy is not gender related¹⁵¹. 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 cells¹⁴⁶. 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/RAR α* 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).

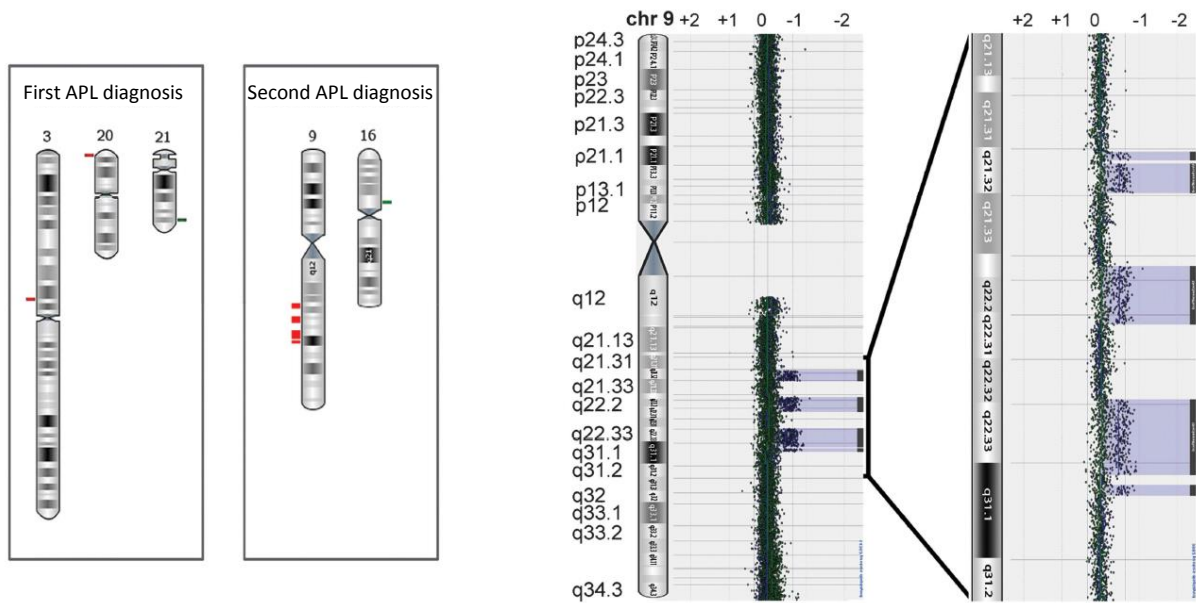


Figure 13. Array-CGH revealing different genetic aberrations at 1st and 2nd diagnosis. Left panel: regions with genetic loss and gains are presented in red and green respectively.

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).

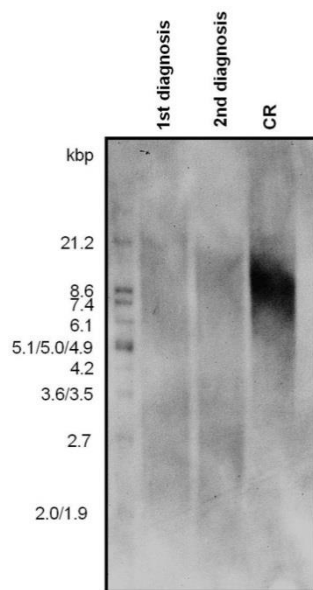


Figure 14. Southern blot analysis of terminal restriction fragments, showing shorter telomeres at both acute promyelocytic leukemia diagnoses compared to when the patient was in complete remission.

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/RAR α* 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/RAR α* 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/RAR α* 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 populations¹⁵².

ATO has been demonstrated to target the LSC in APL and thereby prevent relapses¹⁵³. 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 AML¹⁵⁴. 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.

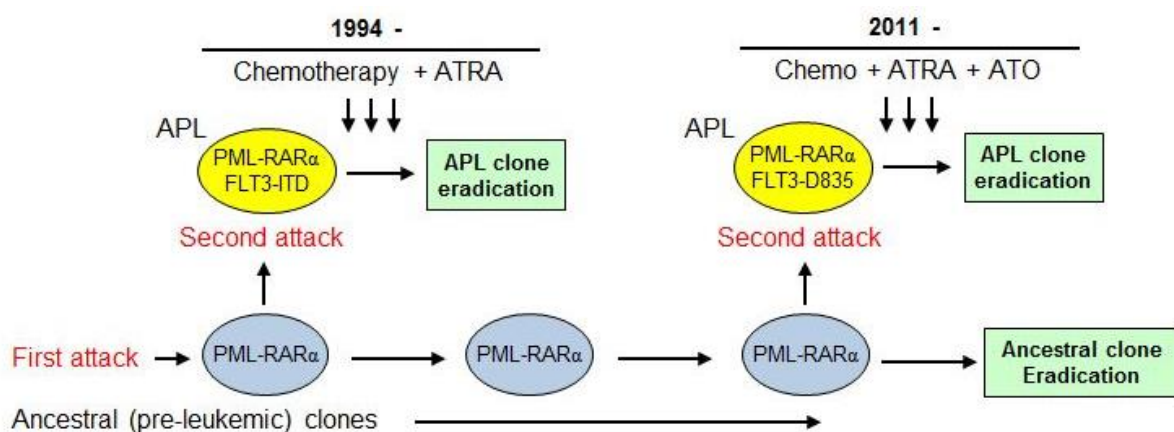


Figure 15. Proposed evolution of the pre-leukemic clone in the patient.

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).

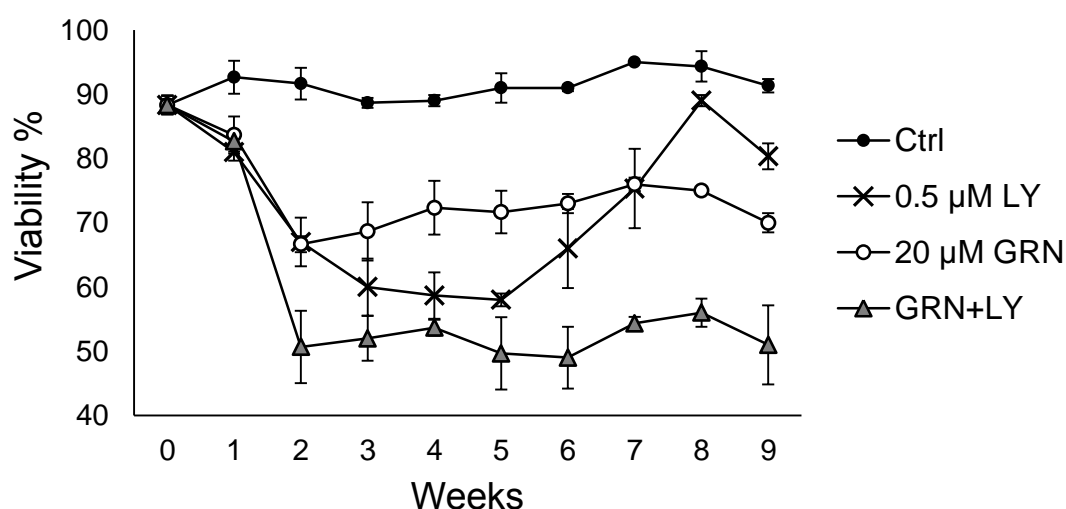


Figure 16. Viability in HEL cells after treatment with LY2484544 and/or GRN163L.

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.

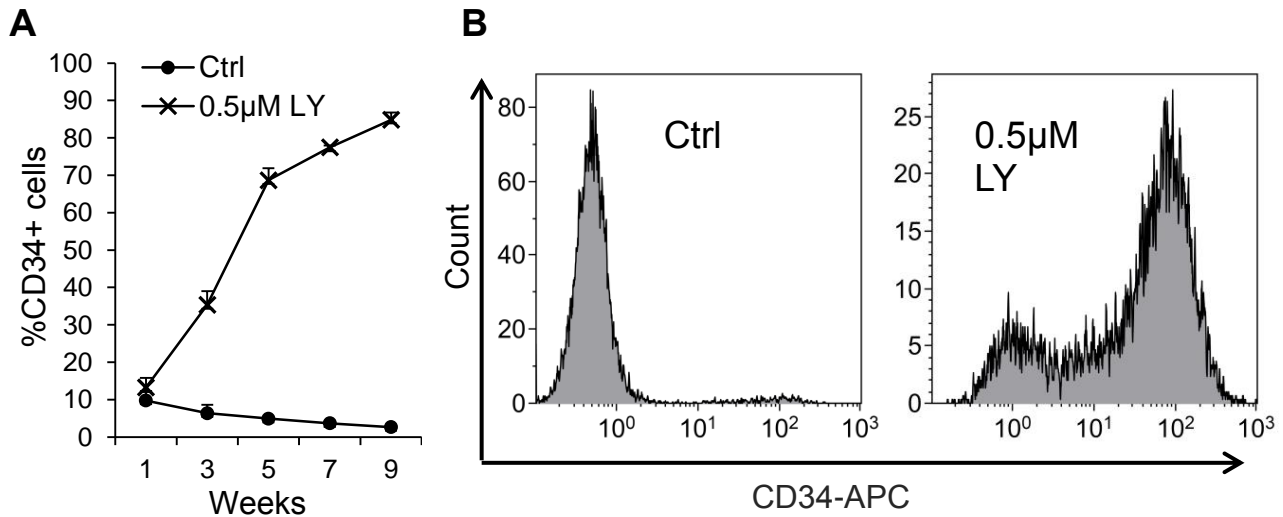


Figure 17. A) Fraction of CD34 positive HEL cells after LY2784544 treatment. B) Histograms of CD34 in cells after 7 weeks of incubation without (left) and with (right) LY2784544 treatment.

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).

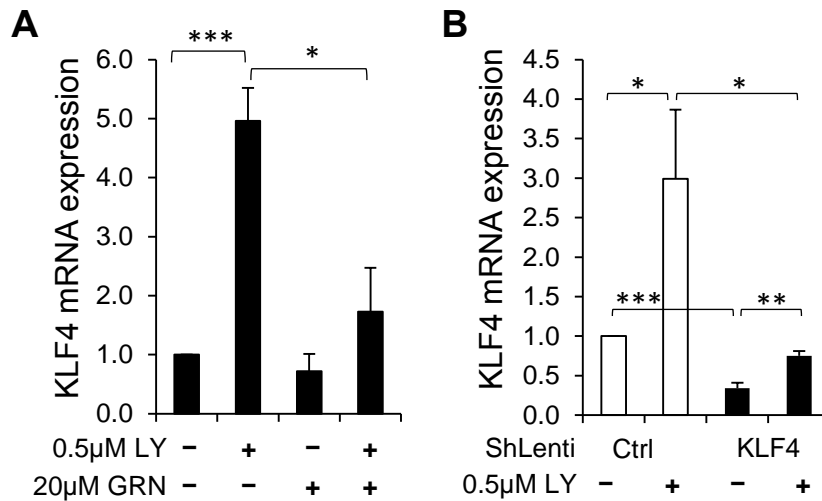


Figure 18. *KLF4* mRNA expression in: A) HEL treated with LY2784544 and/or GRN163L B) HEL transduced with ShKLF4 or scrambled control treated with LY2784544. * indicates *P*-value <0.05, **<0.01, ***<0.001.

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).

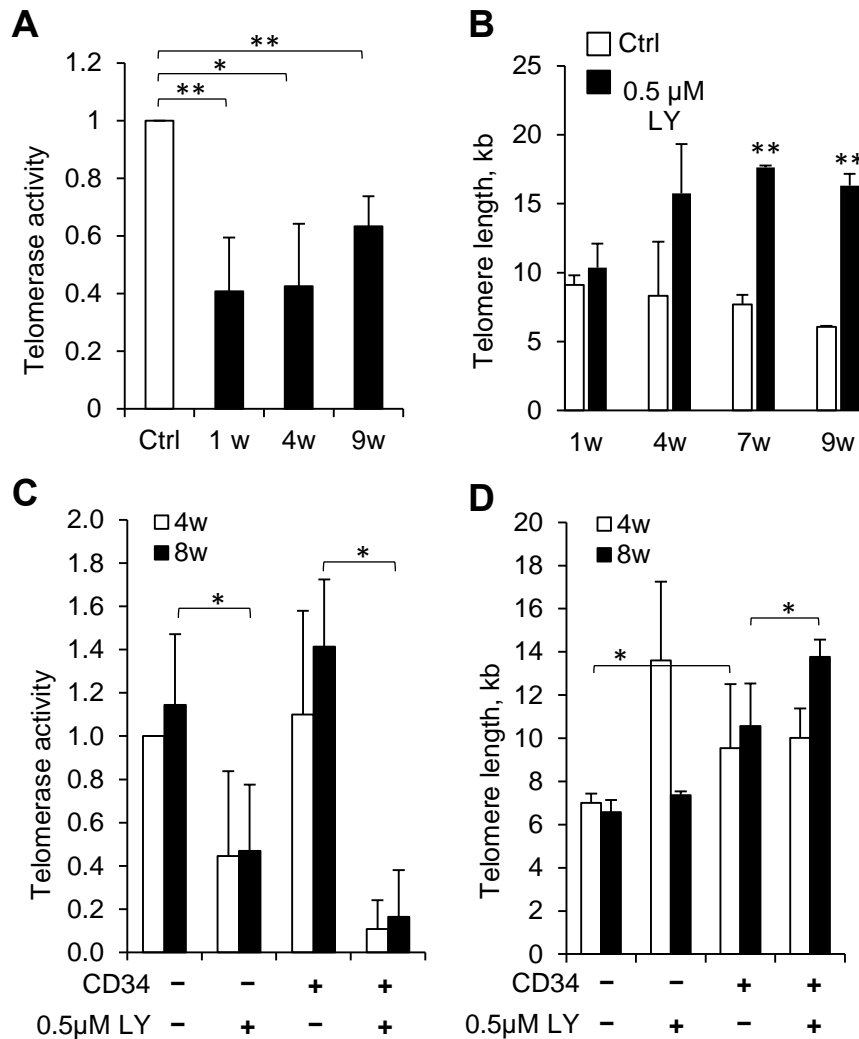


Figure 19. A) Telomerase activity in HEL cells treated with LY2784544. B) Telomere length in HEL cells treated with LY2784544. C) Telomerase activity in CD34-sorted HEL cells treated with LY2784544. D) Telomere length in CD34-sorted HEL cells treated with LY2784544.

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 humans¹⁰⁰. 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 cells¹⁵⁶. 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 patients¹⁵⁷. 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 promoter¹⁵⁸. 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 individuals¹¹⁵. 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-RAR α 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-RAR α -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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