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Clinical and Genetic studies in Chronic Myeloid Leukaemia

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Abstract

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This thesis explores strategies to enhance deep molecular response (DMR) rates and treatment-free remission (TFR) eligibility in chronic myeloid leukaemia (CML), investigates factors linked to treatment milestone failures, describes tyrosine kinase inhibitor (TKI) discontinuation outcomes in a population-based cohort, and examines TFR probabilities after a second TKI discontinuation. In paper I we examined data from the Swedish CML registry on 128 CML patients in chronic phase with a reported TKI discontinuation of ≥ 1 month due to DMR. Findings indicate that patients discontinuing a 2nd generation TKI had a higher probability of remaining treatment-free, and 11% of patients with a diagnosis of CML in chronic phase were treatment free by last follow-up. Paper II involved a long-term follow-up of 40 patients treated initially with a 2nd generation TKI, dasatinib, combined with a low dose of pegylated interferon $\alpha 2b$ as part of the phase II study NordCML007. The combination had an acceptable toxicity profile, and the occurrence of late dasatinib-related adverse events was not increased compared with previous studies of single treatment with dasatinib. The proportion of patients achieving major and DMR were high in comparison with historical cohorts of patients treated with dasatinib. In paper III, an interim analysis was conducted on CML patients attempting a second TKI discontinuation within the DAsTop2 study, after a prior molecular relapse. After a median 27 months from the second discontinuation attempt, 50% had re-initiated TKI therapy, and TFR rate after 12 months was 56%. Those with a short (< 6 months) TFR duration after the first discontinuation attempt were more likely to experience a molecular relapse after the second discontinuation attempt. Paper IV retrospectively analysed 20 patients newly diagnosed with CML in chronic phase and primary refractory to TKI treatment without BCR::ABL1 kinase domain mutations. Diagnostic samples were analysed for pathogenic variants in a panel of 54 genes recurrently mutated in myeloid neoplasms. Pathogenic variants were seen in 50% with AXL1 being the most frequently affected gene. All patients with truncating ASXL1 variants exhibited resistance to multiple TKIs. Overall, this thesis highlights the potential of TKI discontinuation in selected CML patients, the promising combination of dasatinib and pegylated interferon α in achieving high DMR rates, and the importance of genetic profiling in understanding TKI resistance.

Keywords: Haematology, Chronic Myeloid Leukemia

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List of Papers

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

- I. Flygt H., Sandin F., Dahlén T., Dreimane A., Lübking A., Markevärn B., Myhr-Eriksson K., Olsson K., Olsson-Strömberg U., Sjölander A., Söderlund S., Wennström L., Wadenvik H., Stenke L., Höglund M., Richter J. (2021) **Successful tyrosine kinase inhibitor discontinuation outside clinical trials – data from the population-based Swedish CML registry.** *British journal of Haematology*, 2021;193(5):915-21
- II. Flygt H., Söderlund S., Stentoft J., Richter J., Koskenvesa P., Majeed W., Mustjoki S., Lübking A., Dreimane A., Markevärn B., Stenke L., Myhr-Eriksson K., Gjertsen B., Gedde-Dahl T., Dimitrijevic A., Udby L., Olsson-Strömberg U., Hjorth-Hansen H. (2021) **Long-term tolerability and efficacy after initial PegIFN- α addition to dasatinib in CML-CP – Five-year follow-up of the NordCML007 study.** *European journal of Haematology*, 2021; 107(6):617-623
- III. Flygt H., Söderlund S., Richter J., Saussele S., Koskenvesa P., Stenke L., Mustjoki S., Dimitrijevic A., Stentoft J., Majeed W., Roy L., Wolf D., Dreimane A., Gjertsen B., Gedde Dahl T., Ahlstrand E., Markevärn B., Hjorth Hansen H., Janssen J., Olsson-Strömberg U. (2024). **Treatment-free remission after a second TKI discontinuation attempt in patients with Chronic Myeloid Leukemia re-treated with dasatinib - interim results from the DAsTop2 trial.** *Leukemia*, 2024; Epub ahead of print.
- IV. Ayoola-Gustafsson K., Flygt H., Arngården L., Hermansson M., Olsson-Strömberg U., Baliakas P., Cavalier L. (Manuscript) **Enrichment of pathogenic ASXL1 variants among patients with primary refractory chronic myeloid leukemia.** *Manuscript*

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Abbreviations

ABL1	ABL proto-oncogene 1
ACA	Additional chromosomal abnormalities
Allo-SCT	Allogeneic stem cell transplantation
AP	Accelerated phase
BM	Bone marrow
BP	Blast Phase
BCR	Breakpoint cluster region
CCyR	Complete cytogenetic response
CD	Cluster of differentiation
CML	Chronic myeloid leukaemia
CP	Chronic phase
CTL	Cytotoxic T lymphocyte
CYP3A4	Cytochrome p450 3A4 enzyme
CyR	Cytogenetic response
ddPCR	digital droplet polymerase chain reaction
DMR	Deep molecular response
ELN	European LeukemiaNet
FGFR	Fibroblast growth factor receptor
FISH	Fluorescence in-situ hybridization
IFN- α	Interferon α
LSC	Leukemic stem cell
MMR	Major molecular response
NCCN	National comprehensive cancer network
NGS	Next generation sequencing
NK cell	Natural killer cell
Peg	Pegylated
PB	Peripheral blood
PDGFR	Platelet derived growth factor receptor
Ph	Philadelphia chromosome
qRT-PCR	Reverse transcriptase quantitative polymerase chain reaction
TFR	Treatment-free remission
TKI	Tyrosine kinase inhibitor
VEGFR	Vascular endothelial growth factor receptor
WHO	World health organization

Introduction

Chronic myeloid leukaemia (CML) is a haematological neoplasm resulting in massive proliferation of myeloid precursors in both the peripheral blood (PB), spleen and bone marrow (BM). Through the years, CML has often acted as a model disease when exploring the mechanisms behind cancer in general, and hematopoietic malignancies in particular. While leukaemia as an entity was described and given its current name (from leukämie, or “white blood”) by pathologist Rudolf Virchow in the 1840s, it took another century before these diseases were more thoroughly explored, and CML was defined (1, 2). A milestone in the characterization of CML occurred in the early 1960s when Peter Nowell and David Hungerford, by improved chromosome visualization techniques, discovered a small unusual chromosome in malignant leukocytes in patients with CML, consequently named the Philadelphia (Ph) chromosome from the city in which it was discovered (3). The Ph chromosome was not present in non-leukemic leukocytes, leading the authors to suggest that the aberration led to a growth advantage in affected cells. Although previous studies had shown sporadic abnormalities in chromosomes in other malignancies, this was the first such chromosomal aberration that seemed pathognomonic, and provided the first solid evidence that cancer originates from genetic alterations. Later studies showed that the Ph chromosome was the result of a reciprocal translocation between the long arms of chromosome 9 and 22 ($t(9;22)(q34;q11)$), fusing the ABL proto-oncogene 1 (*ABL1*) on chromosome 9 to the breakpoint cluster region (*BCR*) on chromosome 22 resulting in a fusion gene, *BCR::ABL1* (4-6). The subsequent discovery of the *BCR::ABL1* mRNA and the resulting fusion protein capable of inducing a myeloproliferative disease in mice established *BCR::ABL1* as the key oncogenic factor in CML (7-9). These discoveries paved the way for the development of targeted therapies against the *BCR::ABL1* fusion protein tyrosine kinase in the early 2000s (10). In analogy with previous CML discoveries, tyrosine kinase inhibitor (TKI) development pioneered the current targeted therapy revolution in cancer. As the use of TKI has led to dramatic improvements in survival for CML patients, the focus of clinical CML research has shifted towards enabling TKI discontinuation in those with the best TKI responses, and further elucidating mechanisms behind treatment resistance in those with poor responses to TKI. The aims of the current thesis were to explore ways to increase TKI discontinuation eligibility in CML by improving the rate of deep molecular

responses (DMR), to evaluate the outcome after TKI cessation and to examine potential mechanisms behind failure to attain an optimal treatment response. Figure 1 illustrates the papers included in the current thesis.

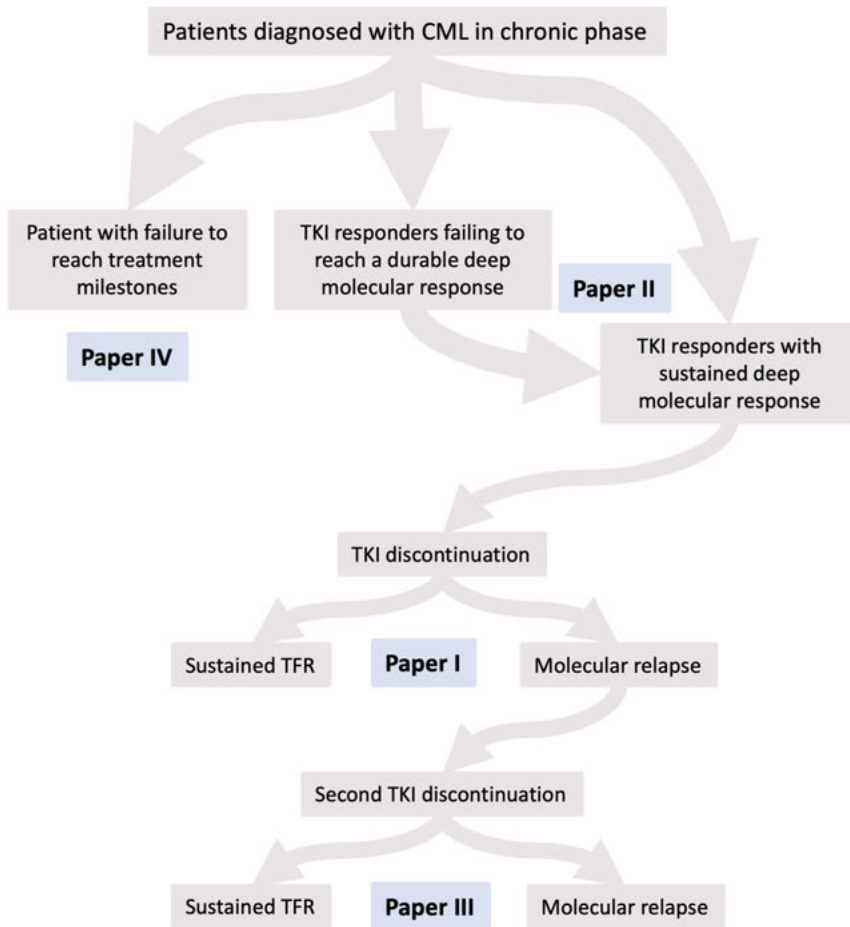


Figure 1. Schematic outline of the scope of the thesis. In **Paper I** we describe the incidence and outcome of tyrosine kinase inhibitor (TKI) discontinuation on a population-based level. In **Paper II** we examine the feasibility and safety of an initial treatment combination of interferon α (IFN- α) and dasatinib as a way to improve treatment responses. In **Paper III** we report on the outcome after a second TKI discontinuation attempt. In **Paper IV** we investigate genetic pathogenic variants in a cohort of patients refractory to TKI and without other common genetic aberrations associated with a lack of response. TFR = Treatment-free remission. TKI = Tyrosine kinase inhibitor.

Chronic myeloid leukaemia

Clinical characteristics and diagnosis

In the majority of instances CML is diagnosed during its chronic phase (CP), a period when the disease often remains unnoticed and is typically found incidentally upon routine blood sampling that reveals elevated white blood cell counts. With time, individuals may develop symptoms such as fatigue, sense of abdominal fullness due to enlargement of the spleen, night sweats, anaemia and bleeding (11). More rarely, the high white blood cell count can lead to hyper-viscosity or leukostasis symptoms including headache, dyspnoea, dizziness, blurred vision and priapism (11). The PB count is characterized by a marked increase in granulopoiesis at different stages of maturation, usually including an increase in basophils and more rarely eosinophils. The BM is likewise hypercellular with myeloid cells at different stages of maturation and dysplastic megakaryocytes (Figure 2) (12). Although a typical clinical presentation and BM morphology as described above may lead the clinician to suspect CML, the presence of the Ph chromosome and/or the *BCR::ABL1* fusion gene is obligate for the diagnosis of CML. The presence of the Ph chromosome can be detected by cytogenetic testing, including karyotyping or fluorescence in-situ hybridization (FISH). In addition to cytogenetic analysis, a reverse transcriptase polymerase chain reaction (RT-PCR) analysis of the *BCR::ABL1* fusion transcript should always be performed to identify the type of transcript. The karyotyping is performed on cultured BM cells and a minimum of 20 metaphases should be analysed using G-banding. In addition to the detection of the Ph chromosome, karyotyping also allows for the detection of other non-t(9;22) abnormalities that are of prognostic significance. Although the typical Ph chromosome is detected in the majority of CML patients, approximately 5% of patients have atypical translocations where the use of FISH is necessary to detect the fusion gene. While qualitative RT-PCR may be used to detect the presence of *BCR::ABL1*, quantitative RT-PCR (qRT-PCR) is commonly used to monitor residual disease.

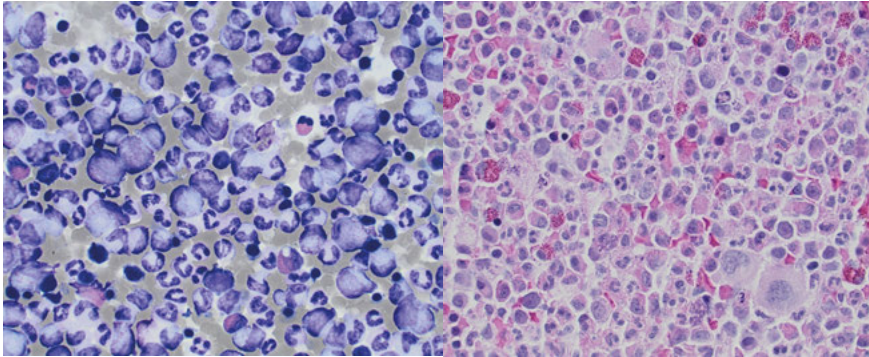


Figure 2. The bone marrow in CML. Courtesy of Rose-Marie Amini.

Epidemiology

The incidence of CML according to most population-based studies is approximately 0.9-1.2 cases per 100 000 per year, including data from the Swedish registry (13). However, some studies have reported incidences of 1.6-1.8 cases per 100 000 per year (14-16). The incidence increases with age, and men are slightly more likely to be affected than women at a ratio of 1.2-1.5:1 (13). The median age at diagnosis is 60 years in Sweden, similar to most western countries. The only known risk factor associated with the development of CML is exposure to high doses of ionizing radiation, and there are consequently no proven hereditary risk factors (17). The incidence does not seem to change over time, although the prevalence of patients with the disease constantly increases due to better survival and a gradual increase in the mean age in the general Swedish population (13).

Pathophysiology

The characteristic translocation $t(9;22)(q34;q11)$ described above causes the juxtaposition of the *BCR* and *ABL1* genes. This rearrangement combines the 5' end of the *BCR* gene, and the 3' end of the *ABL* gene, although the exact location of the breakpoint varies. In the most common variants, the break in the *BCR* gene is located in intron 13 or 14, which is then recombined with a 140 kb region between exons 1b and 2 of the *ABL1* gene (18). This gives rise to the two most common *BCR::ABL1* transcripts, e13a2 and e14a2, also referred to as b2a2 and b3a2 respectively. These transcripts result in a 210 kilo Dalton (kDa) protein called *BCR::ABL1* major or p210^{*BCR::ABL*}. In rare cases of CML, but in most cases of patients with Ph⁺ acute lymphoblastic leukaemia an alternative breakpoint in *BCR* gives rise to the e1a2 transcript resulting in a 190 kDa protein called *BCR::ABL1* minor or p190^{*BCR::ABL*}. A third transcript variant rarely seen in CML is the result of a fusion between *BCR* exon 19 and *ABL* exon 2 (e19a2) which gives rise to a 230 kDa protein known as

BCR::ABL micro or p230^{BCR::ABL}. In cells not affected by CML, ABL1 is a non-receptor tyrosine kinase found in most tissues responsible for signal transduction from growth factors and adhesion receptors and regulates cytoskeletal structure (19, 20). BCR is in itself also a signalling protein (21). The translocation of *BCR* adjacent to *ABL1* affects several intracellular processes, notably by augmenting the tyrosine kinase activity of ABL1. This increase promotes cell proliferation and survival, and downregulate proteins which normally inhibits proliferation and cell survival (22). *BCR::ABL1* positive cells are also prone to acquire additional genetic aberrations due to increased genetic instability, explaining the progression to more advanced disease phases (6, 23, 24).

Disease phases

As described, CML is generally detected in its CP with high cell proliferation, but with low levels or no blast cells in the BM or PB. Traditionally, the course of CML has been divided into three disease phases. If untreated, the CP usually lasts for 3-5 years, and then progresses to blast phase (BP), usually through an intermediate stage known as the accelerated phase (AP) (25, 26). In the Swedish CML population approximately 94%, 4% and 2% have been assigned to the CP, AP and BC entities at diagnosis respectively, and cumulative progression to advanced phase disease is approximately 4% two years after diagnosis using the 2016 World Health Organization (WHO) definition of AP (13, 27). There are two major sets of definition criteria when staging the disease; the WHO criteria, last updated in 2022 (28-30) and the European LeukemiaNet (ELN) criteria (31, 32). The WHO criteria have historically been stricter in their definition of the more advanced phases, defining them by a percentage of blasts in the BM or PB of $\geq 10\%$ and $\geq 20\%$ for AP and BC respectively compared with $\geq 15\%$ and $\geq 30\%$ blasts according to the ELN criteria. In Sweden, the WHO criteria have generally been applied, although the ELN criteria are favoured in clinical trials involving BC. The prognostic significance of AP at CML diagnosis has been shown to be highly dependent on the initial treatment response and there is a clear difference in outcome between those with de novo AP and those who progress to AP from CP during TKI treatment (33, 34). This fact is reflected in the latest update of the WHO classification criteria from 2022, where the term AP is no longer included and high-risk features related to TKI resistance are emphasized instead (30). The implications of this reclassification for CML treatment strategies remain to be fully understood, especially as many treatment guidelines and drug approvals are based on the distinction between CP and AP. Table 1 describes CP, AP and BP CML as defined by the 2016 and 2022 WHO criteria as well as the ELN criteria. A BP can present as a myeloid, lymphoid or mixed phenotype. Almost 70% present as myeloid phenotype, almost 30% as lymphoid phenotype, and a small percentage as mixed phenotype (35).

Table 1. CML staging criteria

Chronic Phase	
WHO 2016 criteria	PB and BM blasts<10% and no criteria for AP fulfilled
ELN criteria	PB and BM blasts<10% and no criteria for AP fulfilled
WHO 2022 criteria	PB and BM blasts <20%
Chronic phase with high progression risk	
WHO 2022 criteria	High ELTS score 10-19% blasts in BM and/or PB ≥20% basophils in PB ACA in Ph+ cells at diagnosis. * Clusters of small megakaryocytes with increased reticulin and/or collagen fibrosis. Failure to achieve complete haematological response on first TKI Resistance to two sequential TKI Development of ACA Occurrence of compound mutations in <i>BCR::ABL1</i> during TKI therapy
Accelerated phase	
WHO 2016 criteria	Blasts in PB or BM 10-19% Basophils in PB ≥20% Persistent thrombocytopenia (<100 x10(9)/l) unrelated to treatment Persistent thrombocytosis (>1000 x10(9)/l) unresponsive to treatment Persistent or increasing splenomegaly, unresponsive to treatment Persistent or increasing high white blood cell count (>10 x10(9)/l), unresponsive to therapy ACA in Ph+ cells at diagnosis†. Any new ACA in Ph+ cells that occurs during therapy Provisional criteria Haematological resistance to the first TKI (failure to achieve complete haematological response) Any haematological, cytogenetic or molecular indications of resistance to 2 sequential TKIs Occurrence of 2 or more mutations in <i>BCR::ABL1</i> during TKI therapy
ELN criteria	Blasts in PB or BM 15-29% or blasts plus promyelocytes in PB or BM >30% with blasts <30% Basophils in PB ≥20% Persistent thrombocytopenia (<100 10(9)/l) unrelated to treatment ACA in Ph+ cells on treatment. ‡
WHO 2022 criteria	Accelerated phase not recognized

Table 1 continued.

Blast Phase	
WHO 2016 criteria	Blasts in PB or BM $\geq 20\%$ and/or presence of extramedullary blast proliferation §
ELN criteria	Blasts in PB or BM $\geq 30\%$ or presence of extramedullary blast proliferation §
WHO 2022 criteria	$\geq 20\%$ myeloid blasts, presence of extramedullary blast proliferation or presence of increased lymphoblasts in PB or BM.

*Including +Ph, -7, i(17q), complex karyotype or 3q26.2 rearrangements. †Including +Ph, +8, i(17q), +19, complex karyotype or 3q26.2 rearrangements. ‡Including +Ph, +8, i(17q) or +19. §Apart from liver or spleen. ACA=Additional chromosomal abnormalities. PB=Peripheral blood, BM=Bone marrow

Response definitions

Sampling of blood and differential counts are initially recommended once every 1-2 weeks until a complete haematological response is achieved (table 2). Disease monitoring is thereafter performed by repeated qRT-PCR analyses. The National Comprehensive Cancer Network (NCCN), ELN and Swedish guidelines recommend molecular monitoring every third month after starting frontline TKI, at least until the achievement of a major molecular response (MMR), after which Swedish and NCCN guidelines under certain circumstances permit qRT-PCR every 6 months (36-38). Based on studies of factors determining long-term outcomes, Swedish and ELN guidelines categorize responses as “optimal”, “warning” or “failure”. Table 2 summarizes the definitions of responses, and Table 3 shows response criteria for “optimal”, “failure” and “warning” (36, 38). Patients with an optimal response have excellent long-term treatment results and a life expectancy comparable with the general population (39, 40), whereas failure in the absence of medication non-adherence is associated with TKI resistance and dismal outcomes (41, 42). Guidelines currently recommend closer monitoring for patients with warning criteria, and therapy switch in combination with *BCR::ABL1* kinase domain (KD) mutation analysis for patients with failure. As *BCR::ABL1* measurements by qRT-PCR have surpassed cytogenetics as the gold standard for measuring treatment response in CML, a standardized method with lab-specific conversion factors was introduced to harmonize *BCR::ABL1* monitoring and express results on an international scale (IS) (43). The discovery that a proportion of patients with very low levels of *BCR::ABL1* transcripts can discontinue TKI treatment with sustained molecular remission led Cross et al. to propose a definition of deeper molecular responses (MR)(44). According to Cross et al. MMR, MR⁴, MR^{4.5} and MR⁵ correspond to *BCR::ABL1* $\leq 0.1\%$ IS, $\leq 0.01\%$ IS, $\leq 0.0032\%$ IS and $\leq 0.0001\%$ IS respectively. Moreover, in case of undetectable *BCR::ABL1* transcripts, MR-levels are determined by the

number of copies of a control gene (generally *ABL1*, *BCR* or *GUSB*), i.e., the sensitivity of the analysis.

Table 2. Response definitions

Modality	Response	Definition
Haematology	Complete Haematological Response (CHR)	Normal peripheral blood counts including differential count and normal spleen size
Cytogenetics	Minimal CyR	Ph+ 66-95%
	Minor CyR	Ph+ 36-65%
	Partial CyR	Ph+ 1-35%
	Complete CyR	Ph+ 0%
Molecular genetics	Major molecular response (MMR)	$BCR::ABL^{IS} \leq 0.1\%$
	MR ⁴	$BCR::ABL^{IS} \leq 0.01\%$
	MR ^{4.5}	$BCR::ABL^{IS} \leq 0.0032\%$
	MR ⁵	$BCR::ABL^{IS} \leq 0.001\%$

CyR = Cytogenetic response. MR = Molecular response

Table 3. Response criteria according to ELN 2020 guidelines

	Optimal response	Warning	Treatment failure
Baseline	NA	High risk ACA* in Ph+ cells or high risk ELTS score	NA
3 months	$BCR::ABL1 \leq 10\%$	$BCR::ABL1 IS > 10\%$	$BCR::ABL1 IS > 10\%$ if confirmed within 1-3 months
6 months	$BCR::ABL1 \leq 1\%$	$BCR::ABL1 IS 1-10\%$	$BCR::ABL1 IS > 10\%$
12 months	$BCR::ABL1 \leq 0.1\%$	$BCR::ABL1 IS > 0.1-1\%$	$BCR::ABL1 IS > 1\%$
At any time	$BCR::ABL1 \leq 0.1\%$	$BCR::ABL1 IS > 0.1-1\%$	$BCR::ABL1 IS > 1\%$, resistance mutations, high risk ACA* in Ph+ cells

* Including +8, +Ph, i(17q), +19, -7/del7q, 11q23 or 3q26.2 rearrangements. ACA = Additional chromosomal abnormalities.

Prognostic scoring

Sokal Score

The first risk score developed to predict the prognosis of CML patients was the Sokal Score. It was introduced in 1984 by Sokal et al. and divided patients into three groups; low risk (LR) intermediate risk (IR) and high risk (HR). The score was designed to predict survival in patients treated with chemotherapy,

most commonly busulfan, in combination with hydroxyurea and immunotherapy, but it has also proven to be of prognostic value for patients treated with imatinib (45, 46). Factors included in the model are age, spleen size, platelet count and the percentage of blasts present at diagnosis. In a Swedish context, approximately 30% of CML patients are in the high-risk group (13).

ELTS score

The EUTOS long term survival score (ELTS) was conceived to predict the outcome for CML patients in the TKI era, but with a focus on predicting the risk of dying due to CML. An older age, more PB blasts, larger spleen, and low platelets were associated with increased risk of dying of CML. Consequently, the ELTS score is based on the same variables as Sokal Score, but with each factor weighted in a different way. It divides patients into low, intermediate and high-risk groups. In the study by Pffirmann et al. approximately 60% of patients were in the low-risk group, 27% in the intermediate risk group and 12% in the high-risk group. The cumulative 5-year probability of dying due to CML in a validation cohort was only 3% in the ELTS low-risk group compared with 15% in the high-risk group (47).

Hasford (EURO) score and EUTOS score

The Hasford score is based on Sokal Score but was modified to predict the outcome of patients treated with interferon α (IFN- α). It was introduced by Hasford et al. in 1998 and in addition to the factors included in the Sokal Score it also takes into account the percentage of basophils and eosinophils in the blood at diagnosis, and it divides patients into high, intermediate and low risk groups (48). The European Treatment and Outcome study score, or EUTOS score was the first risk score developed after the introduction of tyrosine kinase inhibitors, and aimed at predicting the probability of achieving CCyR at 18 months, and progression-free survival after 5 years. Only percentage of basophils in PB and spleen size at diagnosis are used to discriminate between high risk and low risk patients (49). Some subsequent studies have been able to reproduce the EUTOS score's ability to predict treatment results and survival in imatinib treated patients (50-53). However, when applied on the population-based Swedish CML registry, the EUTOS score had no statistically significant ability to predict 5-year survival (13). Consequently, the Hasford and EUTOS scores are not widely used in clinical routine.

The treatment of CML

After the characterization of leukaemia in the 1900's, unfruitful attempts to treat the condition began, starting with arsenic due to its documented ability to lower the white blood cell count (54). During the 1920's radiation therapy of the spleen for symptomatic relief was introduced, but it was not until the

advent of busulfan and subsequently hydroxyurea that an effective method to control white blood cell counts was established (1, 55). In the mid 1970's the first allogeneic stem cell transplantations (allo-SCT) were performed on CML patients, with the disappearance of the Ph chromosome in some cases, offering for the first time a potential cure for CML (56). Subsequently, IFN- α was shown to induce cytogenetic responses in some patients, and became the therapy of choice for patients not eligible for an allo-SCT (57).

Tyrosine kinase inhibitors

Imatinib

Imatinib was the first BCR::ABL1 targeted tyrosine kinase inhibitor developed, and was approved in 2001 in Sweden. It was initially evaluated in a phase I trial by Druker et al. which provided evidence of its tolerability and significant antileukemic effect (10). Imatinib functions through competitive inhibition at the ATP-binding site of the BCR::ABL1 protein, leading to inhibition of tyrosine phosphorylation. Apart from BCR::ABL1 inhibition, imatinib has a high affinity for PDGFR and KIT tyrosine kinases (58). Subsequent clinical trial IRIS confirmed the early positive results and showed imatinib superiority when compared with IFN- α in combination with cytarabine (59). Imatinib is approved for the treatment of all disease phases, and the standard dose is 400 mg once daily for CP-CML and 600 mg daily for AP- or BP-CML. Long term follow-up of imatinib treated patients from the IRIS trial and French and German imatinib trials show excellent five, 10 and 15-year overall survival of approximately 90%, 85% and 85% respectively. Although most patients respond well to imatinib treatment, approximately 35-50% of patients discontinue first line imatinib therapy within the large studies for various reasons, but most importantly due to lack of efficacy for approximately 10-15% of patients, or side effects for approximately 5-10% of patients (46, 60-63). Adverse events are generally limited to grade I-II, and include peripheral and periorbital oedema, muscle cramps, fatigue, gastrointestinal symptoms (including nausea, pain and diarrhoea) and rash (64).

Dasatinib

Dasatinib is a 2nd generation TKI approved in 2007 in Sweden and is approximately 325 times as potent at inhibiting BCR::ABL1 in vitro compared with Imatinib. It was initially developed as a Src inhibitor, and consequently binds to Src as well as PDGFR and KIT (65, 66). Dasatinib is approved for first line treatment of patients with CP-CML, and as second line treatment for patients with AP or BP with previous resistance or intolerance. The standard dose of dasatinib is 100 mg once daily for CP-CML, and 140 mg once daily for patients in AP or BP-CML.

Efficacy and adverse effects

The Dasatinib Versus Imatinib Study in Treatment-Naïve Chronic Myeloid Leukaemia Patients Trial (DASISION) compared dasatinib treatment with imatinib in CP-CML patients. The trial showed higher rates of MMR compared with imatinib. In the dasatinib arm, 46% and 76% reached MMR by 12 months and 5 years respectively, compared with 28% and 64% in the imatinib arm. Similarly, the cumulative incidence of MR^{4.5} was 42% for dasatinib treated patients, and 33% for imatinib treated patients after 5 years (63). Interestingly, other studies of dasatinib in CP-CML patients have shown significantly better response rates than DASISION (67-70). In DASISION, haematological toxicity was more common in dasatinib treated patients compared with imatinib, and the most common non-haematological AEs were abdominal pain, diarrhoea, fatigue, headache, myalgia, peripheral oedema and skin rash, with occurrences similar to imatinib. However, 28% of patients in the dasatinib arm of DASISION experienced pleural effusions compared with 0.8% in the imatinib arm. Another rare but potentially fatal complication of dasatinib is the development of pulmonary hypertension in some patients, although it is at least partly reversible in most instances upon dasatinib cessation (63, 71). Mechanistically, both pleural effusion and pulmonary hypertension have been shown to be correlated with dasatinib-induced production of reactive oxygen species in rats and in *in vitro* experiments (72, 73). However, studies of dasatinib-treated patients with concurrent lymphocytosis found a high incidence of autoimmune phenomena, including pleural effusions, with clonal lymphocytes present in the pleural fluid, suggesting an underlying immunological mechanism (74). A study from MD Anderson Cancer Center has shown excellent response rates with a daily dose of only 50 mg dasatinib as first line treatment for CP-CML, and with only 6% of patients experiencing pleural effusion, and a French study demonstrated a significant decrease in the incidence of pleural effusions using a dose optimization strategy based on repeated measurements of dasatinib (C)-min values (67, 75).

Dasatinib and the immune system

Alterations in the immune system at CP-CML diagnosis include impaired cytotoxicity and antigen presentation and an increased number and activity of myeloid-derived suppressor cells (MDSCs) and regulatory T-cells (Tregs) hampering anti-tumour immune responses (76, 77). In general, TKI treatments, including dasatinib have been shown to normalize the immune system through the reduction of MDSCs and enhanced NK-cell and T-cell function (77, 78). Early *in vitro* studies showed that dasatinib inhibited the T-cell receptor and T-cell and NK-cell proliferation, as well as reduced the cytotoxic T-lymphocyte (CTL) and NK-cell function, suggesting an immune suppressive effect (79-81). In contrast, some patients treated with dasatinib exhibit a clonal expansion of CTLs and NK cells (74, 77), and dasatinib induces a

decreased expression of the inhibitory NK-cell receptor NKG2A which correlates with higher NK-cell activity and a shorter time to MMR, mechanisms seen primarily after dasatinib, but not imatinib or nilotinib treatment (82, 83). Moreover, dasatinib appears to inhibit Tregs *in vivo* and persistent lower levels of Tregs after dasatinib treatment were seen in patients achieving DMR (defined as MR⁴ or better) compared with those who did not reach DMR (74, 84). As described here and in the section on *Treatment-free remission*, reduction of MDSCs and increased NK-cell function among other factors are not only correlated with better responses, they are also predictive of a better chance of achieving a treatment-free remission (TFR).

Nilotinib

Nilotinib is a 2nd generation TKI and was first approved in 2007 in Sweden. Similar to imatinib it competitively binds to the ATP binding site of the BCR::ABL1 tyrosine kinase, but has an approximately 20-fold higher affinity than imatinib. It also binds and inhibits PDGFR A and B and KIT (85). It is approved for the first line treatment of patients with CP-CML, and second-line treatment for patients with CP- or AP-CML with imatinib resistance or intolerance. As a first line treatment, the standard dose of nilotinib is 300 mg twice daily, and 400 mg twice daily as second line treatment for patients with previous imatinib resistance or intolerance. Similar to dasatinib, cumulative rates of MMR and deeper responses have been superior to imatinib in the registration trial ENESTnd (86, 87). The most common non-haematological AEs reported were rash, headache, elevated liver enzymes and oedema. In addition, hypertension, elevated cholesterol levels and hyperglycaemia were more frequent in nilotinib treated patients (87, 88). More importantly, the risk of cardiovascular events (ischemic heart disease, ischemic cerebrovascular events or peripheral artery disease) was significantly higher in the nilotinib arms (7.5% and 13.4% for nilotinib 300 mg BID and 400 mg BID respectively) than in the imatinib arm (2.1%). This increased risk for cardiovascular events was mainly seen in patients with baseline intermediate or high risk of cardiovascular events according to the Framingham general cardiovascular risk scores (87, 89). Due to its cardiovascular risk profile, international and Swedish guidelines do not recommend nilotinib in patients with high, or very high risk of cardiovascular events or established cardiovascular disease (37, 90).

Bosutinib

Second generation TKI bosutinib is a Src/ABL inhibitor and similar to the other 2nd generation TKIs inhibit BCR::ABL1 more potently than imatinib *in vitro* (91). It was initially approved in 2013 in Sweden for patients with resistance to one or more previous TKIs. Since 2018 it is also approved for first line treatment of CML patients in CP, AP or BP. The standard dose is 400 mg once daily as a first line treatment for patients in CP-CML, and 500 mg once

daily in patients with previous TKI failure or in AP- and BP-CML. Results from randomized trials show superior response rates at 12 months compared with imatinib (92, 93). The most common adverse events on bosutinib are diarrhoea and liver toxicity with elevated ASAT and ALAT levels, and these are significantly more common than in imatinib treated patients (92, 93). While diarrhoea is generally grade 1-2 and manageable, elevated liver enzymes leads to treatment discontinuation in approximately 5% of patients, and \geq grade 3 liver function test AEs occurred in as many as 24% (93).

Ponatinib

Ponatinib, a third generation TKI, was specifically developed for the treatment of patients with multi-TKI resistance, most importantly the T315I mutation, and was first approved in 2013 in Sweden. The phase II PACE trial evaluated ponatinib 45 mg once daily in 449 patients with Ph+ ALL or treatment resistant CML in all stages of the disease, and more importantly, provided evidence of ponatinib's efficacy in patients with the T315I mutation (94). Consequently, ponatinib is currently approved in CML patients with resistance or intolerance to dasatinib or nilotinib, or patients with the T315I mutation. Ponatinib has also been evaluated as first line treatment in the phase III EPIC trial. However, the study was terminated due to the high proportion of vascular side effects on ponatinib reported in other studies, and frontline ponatinib treatment has yet to be evaluated (94, 95). Indeed, cumulative incidence of arterial occlusive disease was 25%, with serious events in 20% of patients (94). In order to evaluate different doses of ponatinib, a study comparing regimens with 45 mg, 30 mg and 15 mg once daily was performed (96). Current treatment guidelines emphasize careful consideration of the patient's cardiovascular risk profile before the initiation of ponatinib treatment, and dose reduction to the lowest efficacious dose upon CCyR is recommended (37). Other common side effect on ponatinib include rash, dry skin, and abdominal pain (97).

Asciminib

Asciminib is the latest TKI approved for the treatment of CML. While previous TKIs bind to the ATP-binding site on the BCR::ABL1 protein, asciminib binds to a different part of the protein, the myristoyl-pocket. Based on an open label randomized comparison of asciminib and bosutinib, asciminib is currently approved for the treatment of CML patients with ≥ 2 previous TKI treatments (98). The ASCEMBL study included patients previously treated with ≥ 2 TKI, and showed superior efficacy and safety compared with bosutinib (99). Importantly, a phase I trial has shown that in a small cohort of patients with the T315I mutation, 47% achieved MMR on asciminib (100). Studies of asciminib as the first line treatment in CP-CML are currently ongoing.

Interferon- α

Mechanism of action

Until the advent of tyrosine kinase inhibitors for the treatment of CML, IFN- α , a type 1 IFN, was standard of care in patients not eligible for allo-SCT. Type 1 IFNs affect the expression of several hundred genes, illustrating the complexity of IFNs mechanism of action (101, 102). In addition to an antiviral effect, IFN- α was found to have an antiproliferative effect on granulocyte-macrophage progenitors and malignant hematopoietic cells (103, 104), leading to the development of IFN- α therapy in myeloproliferative diseases and subsequently CML (105, 106). The antitumoral effect of IFN- α in CML is not completely understood, but immune modulation and effects on leukemic stem cells (LSCs) are likely mechanisms. In contrast to the effect of TKIs, IFN- α acts slower and appear to exercise an effect on LSCs by activating quiescent stem cells and inducing differentiation, potentially making them susceptible to anti-cancer immunity (107-110). In most CML patients who attain a durable response from IFN- α treatment, CTLs specific for the PR1 peptide have been found, while not present in patients who relapse, suggesting that PR1 peptide is an important leukaemia-associated antigen in CML, and that anti-leukemic immune responses play a significant role for the IFN- α effect (111-113). PR1 peptide in turn is a degradation product from neutrophil elastase and proteinase-3 (PR3) found in myeloid granules, both of which are overexpressed by CML cells (114). IFN- α has been demonstrated to elicit an anti PR1 CTL response to a higher degree than imatinib (115). However, it has also been shown that an abundance of PR3 induce apoptosis of high avidity PR1-specific T-cells while allowing for the survival of low-avidity PR1-specific T-cells, possibly providing an immune escape mechanism for leukemic cells (116). In summary, the ability of TKIs to swiftly reduce the leukemic burden, allow for resuscitation of the immune system and prevent disease progression in combination with the immunomodulatory and LSC effects of IFN- α in theory makes this an appealing treatment combination.

Clinical experience of the combination of TKI and IFN- α

In the pre-TKI era, IFN- α induced a complete hematologic response in 70% of patients, a cytogenetic response in 10-40% of patients, and in rare cases prolonged CCyR (117, 118). IFN- α treatment has been associated with acute toxicity including flu-like symptoms like headache, myalgia, but also later adverse events such as anorexia, fatigue, weight loss, thrombocytopenia, anaemia, autoimmune disease and depression/anxiety (119). Consequently, an optimal dose regimen in CML patients was difficult to establish, but comparison of high dose regimens (5 MIU/m²/day) with lower dose regimens (3 MIU/m²/day) showed no clear advantage in the high dose group (120). Pegylated forms of IFN- α administered as subcutaneous injections (Peg-IFN- α 2a and PegIFN- α 2b) have been developed in order to allow for a less frequent

administration, generally once every week (121). Combinations of imatinib and IFN- α have been evaluated in several clinical trials (122-125). In a study of the combination of imatinib and PegIFN- α 2b by the Italian GIMEMA group, patients were divided into three PegIFN- α dose cohorts, 50 μ g/week, 100 μ g/week and 150 μ g/week. In all three cohorts, a majority of patients discontinued PegIFN- α treatment during the first year due to toxicity, and substantial dose reductions to 50 μ g/week were done in 28-55% of patients. This resulted in similar median PegIFN- α doses delivered in all three cohorts, and led to imatinib dose reductions in the highest dose group (124). In the German CML IV trial, patients were randomized to 800 mg/d imatinib, 400 mg/d imatinib or 400 mg/d imatinib with the addition of non-pegylated IFN α . In this study, no discernible treatment effect on overall survival was observed in the IFN- α group compared with imatinib alone (126). The French SPIRIT trial similarly compared imatinib 400 mg/d, imatinib 600 mg/d and combinations of imatinib 400 mg and PegIFN- α 2a or cytarabine, in which the imatinib+PegIFN- α group had a higher rate of DMR compared to all other treatment groups, with a positive correlation between combination therapy duration and molecular response, suggesting that a PegIFN- α treatment duration of 12 months is desirable. Importantly, a substantial reduction in toxicity without loss of efficacy was seen in those treated with 45 μ g/w compared with patients treated with 90 μ g/w (60, 127). Similarly, combinations of lower-dose PegIFN- α and 2nd generation TKIs have shown promising results and larger phase III trials are ongoing (128-132)

Allogeneic stem cell transplantation

In the pre-TKI era, allo-SCT was the treatment of choice in patients with CML in CP, given that there was a suitable donor. Since the introduction of TKIs, allo-SCT is mainly used as a salvage treatment in patients with poor response to second line treatment, in patients with the T315I mutation, and for patients with AP-CML who do not respond optimally to TKI treatment. In addition, all patients in BP-CML who have a suitable donor and respond to TKI and/or chemotherapy treatment are eligible for allo-SCT (38, 133).

Current treatment recommendations

First line

Current CML treatment guidelines recommend treatment with a TKI for all patients (except when diagnosis is made during pregnancy). In the most recent treatment recommendations from ELN, there is no strict advice on the choice of first line TKI (38). Rather, the choice of first line treatment is based on CML risk factors, cardiovascular risk factors, prior and concomitant illnesses and renal function. However, ELN highlights the cost effectiveness (due to

the availability of generic alternatives) and relatively mild toxicity profile in favour of imatinib as first line treatment in most patients. Likewise, Swedish guidelines also highlight the effectiveness of all currently approved TKIs for first line treatment. Due to the more recent update of the Swedish guidelines (May 2023), the availability of generic dasatinib has influenced treatment recommendations (37). To summarize, the preferred initial treatment is imatinib 400 mg once daily, with the exception of patients who have a high or intermediate risk ELTS score, no prior lung disease and are younger than 70 years of age. For these individuals, dasatinib 100 mg once daily is recommended.

Second line

The choice of second-line TKI is dependent on whether the change of TKI is due to intolerance or failure/resistance. In case of resistance, both ELN and Swedish guidelines stress the importance of *BCR::ABL1* KD mutation analysis (37, 38). In case of KD mutations, the choice of second-line TKI is highly dependent on the type of mutation as discussed under *TKI resistance*. This is especially true in the presence of the T315I mutation, where ponatinib is the only potentially efficacious TKI treatment (Table 4). In case of resistance, imatinib is not recommended as a second line treatment, and higher doses for nilotinib and bosutinib are recommended, 400 twice daily and 500 mg once daily respectively. In the absence of KD mutations or if the change is due to intolerance, the choice of second-line treatment is instead dependent on patient specific factors, such as type of toxicity, age and concomitant risk factors (37).

Treatment-free remission

Long-term follow-up of patients from the pivotal IRIS trial of imatinib showed that a growing proportion of patients attained a state of undetectable residual disease as measured by qRT-PCR, then designated as complete molecular response (CMR) (64). The hypothesis that TKI treatment might be discontinued in these patients was first tested in a small pilot study followed by the Stop Imatinib (STIM) trial, where patients with maintained undetectable residual disease for more than two years were enrolled (134, 135). Results showed that approximately 40% remained in CMR and attained a TFR. More importantly, patients with a molecular relapse quickly regained a low *BCR::ABL1* IS level upon imatinib rechallenge, and no disease progressions or haematological relapses were seen. In addition, long-term follow-up revealed that 80% of molecular relapses occurred within the first 6 months (134, 136). New definitions for molecular responses were subsequently introduced and the According to Stop Imatinib (A-STIM) trial established loss of MMR as a safe criterion for restarting TKI therapy after attempting TKI discontinuation (44, 137). Following trials of TKI discontinuation after treatment with imatinib, 2nd

generation TKIs, or a combination of the two have shown TFR rates between 33-77% depending on treatment duration, follow-up duration and TKI re-initiation criteria, without any clear advantage for patients treated with 2nd generation TKI (138-145). The largest TKI discontinuation trial to date is the European Stop Kinase Inhibitor (EURO-SKI) trial, which enrolled 821 patients from 11 European countries (146). This study allowed for patients with sustained *BCR::ABL1* levels $\leq 0.01\%$ IS to be enrolled (i.e. patients with detectable *BCR::ABL1* levels corresponding to MR⁴ or DMR) and loss of MMR was set as the criterion for TKI re-initiation. Despite less stringent criteria for TKI discontinuation, results were similar to STIM1. Most molecular recurrences occurred in the first six months after TKI discontinuation, no progressions to advanced phases were seen and the TFR rate at 6 months was 60% (146). Detectable, but stable levels of *BCR::ABL1* transcripts are commonly seen in patients discontinuing TKI treatment, challenging previous ideas that the leukemic stem cells need to be eradicated in order to maintain a durable remission. This has led to the idea of a “functional” cure in CML (147). TKI discontinuation has been implemented in CML guidelines in recent years. Guidelines from ELN and European Society for Medical Oncology recommend a minimum of five years of imatinib, or four years of 2nd generation TKI therapy and stable MR⁴ or better for more than two years before attempting TKI discontinuation (38, 148). In addition, ELN emphasizes the availability of high-quality qRT-PCR monitoring, that the patient had no prior advanced stage disease, treatment failure or rare fusion transcript, and that the patient is motivated and agrees to more close monitoring (qRT-PCR every month initially). Swedish updated clinical guidelines as well as those issued by the National Comprehensive Cancer Network (NCCN) contain similar advice (36, 37). While clinical trials have not reported on any disease progressions after TKI discontinuation, rare cases have since been reported (149).

Factor associated with the achievement of a treatment-free remission

Clinical factors

The initial STIM1 study found Sokal risk group and duration of imatinib treatment prior to discontinuation to be correlated to the chance of maintaining TFR, a finding supported by a subsequent smaller study (134, 150). However, EURO-SKI did not find that Sokal or EURO score were predictive of maintaining TFR, whereas previous IFN- α treatment, duration in DMR and, to a lesser extent, TKI treatment duration were the only factors predictive of TFR maintenance (146). In fact, DMR duration and TKI treatment duration are the two clinical factors consistently found across studies to be predictive of TFR success, and these factors are closely intertwined (151). EURO-SKI reported no difference in TFR rates between those in MR⁴ vs those in MR^{4.5} (152).

However, the definition of MR levels in case of unmeasurable *BCR::ABL1* transcripts is dependent on the number of control gene copy numbers which determines the tests sensitivity, and a patient with unmeasurable transcripts assigned to MR⁴ could actually be in MR⁵ (44). Indeed, other studies have found that with higher qRT-PCR sensitivity (minimum MR^{4.5}), unmeasurable transcripts correlate to a better chance of maintaining TFR (153, 154). This has led to the evaluation of digital droplet PCR (ddPCR) as a method to increase precision when measuring very low-level residual disease, thereby better identifying patients with the highest chances of TFR (155, 156). Indeed, ddPCR appears better than qRT-PCR at sorting out those with the lowest *BCR::ABL1* transcripts and selecting patients with a better chance of TFR (155). The DESTINY trial introduced an innovative strategy involving de-escalation to half of the standard dose of TKI for one year before TKI discontinuation (144). Interestingly, this approach led to a 3-year TFR rate of 72% in patients with DMR, which is better than almost all previous TKI stopping trials. The reason for this favourable outcome after TKI de-escalation is not completely understood. Additionally, *BCR::ABL1* halving time measurements have also been able to predict the likelihood of achieving MMR or DMR in clinical trials (157, 158). Similarly, *BCR::ABL1* halving time upon TKI treatment initiation was highly predictive of the chance of maintaining TFR, where 75% of those with less than 10 days halving time, and only 12% of those with more than 40 days halving time maintained TFR at 12 months respectively (159). Furthermore, patients with the e14a2 transcript have also been observed to have better chance of sustaining TFR (160-163)

Immunological factors

The translocation t(9;22)(q34;q11) occurs in primitive CD34+, CD38- hematopoietic stem cells and the persistence of LSCs insensitive to TKI treatment is likely the mechanism behind molecular recurrence after TKI cessation (164-166). A quiescent subpopulation of these LSCs has been identified to endure despite TKI treatment and undetectable *BCR::ABL1* transcripts in PB (167, 168). Furthermore, the LSC population is noted for its heterogeneity, which evolves with TKI treatment (169). Several immunological markers have been linked to TFR probability, including elevated counts of natural killer cells (170-173), a reduced presence of plasmacytoid dendritic cells expressing the T-cell inhibitory receptor ligand CD86 (174), diminished levels of FoxP3 positive Tregs and monocytic myeloid-derived suppressor cells (173) and increased number of CTLs (175, 176). Taken together, the patient's immune response likely affects persistent LSCs ability to induce a molecular relapse. The mechanisms through which TKIs modulate these immunological conditions in certain patients but not in others remain to be fully understood, and points to a complex interplay between TKI treatment and the immune system's ability to control LSCs.

A second TKI discontinuation

Since most patients who experience a molecular relapse after TKI discontinuation regain DMR, it has been hypothesized that a second discontinuation attempt might be successful in a proportion of these patients. A retrospective observational study involving patients who had not succeeded in their first TKI discontinuation attempt but later regained MR^{4,5} with TKI therapy, reported on seventy patients attempting a second TKI discontinuation after a median of 32 months from TKI resumption. The TFR probabilities at 6 and 12 months were 66% and 48% respectively, suggesting that a second discontinuation could be viable for some. Nevertheless, late onset relapses were seen in this study, with TFR decreasing to 42% and 35% at 24 and 36 months respectively (177). Furthermore, a Canadian group recently reported their results from a study of a second TKI discontinuation in 35 patients re-treated with dasatinib (178). In short, all but 3 patients (91%) experienced a molecular relapse defined as either loss of MMR at one occasion, or loss of MR⁴ in two consecutive samples. Another small French prospective study of a second TFR attempt after re-treatment with nilotinib reported a molecular relapse in 12 of 22 patients (54%) (179). Interestingly, the median time to molecular relapse in that study was 8 months, and 33 % of relapses occurred after 12 months. These findings suggest that while a second attempt at TKI discontinuation is plausible, outcomes are mixed, and larger studies of a second TKI discontinuation are warranted.

TKI resistance

A subset of patients treated with TKI experience primary resistance, defined as a lack of effect from the start of TKI therapy, or develop secondary resistance, which can occur at any time after an initial achievement of a response to treatment. Resistance can further be characterized as lack or loss of haematological, cytogenetic or molecular response (table 2). Long-term follow-up (>5 years) of clinical trial data of TKI treatment show that approximately 15% discontinue imatinib due to lack of response, the vast majority during the first 4 years, while the same is true for 11% and 13% of patients treated with dasatinib and nilotinib respectively (61, 63, 64, 180). Other real-world data suggest that as much as 28% of patients diagnosed in CP-CML switch from first line treatment due to resistance, and 16% exhibit failure on second line treatment (181). Failure to reach early milestones (table 3) and loss of response are linked with an increased risk of progression to advanced stage disease and increased mortality (63, 64, 87).

Chromosomal abnormalities and clonal evolution

The emergence of the *BCR::ABL1* fusion gene is known to render genetic instability, leading to additional chromosomal abnormalities in Ph⁺ cells (ACA) that follow a non-random pattern (182, 183). The most commonly observed ACA in patients with advanced stages of CML include trisomy 8 (+8), a second Ph chromosome (+Ph), trisomy 19 (+19) and partial trisomy of the long arm with partial monosomy of the short arm of chromosome 17 (isochromosome (17)(q10) or i(17q)). These aberrations, identified as “major-route” of karyotypic evolution, are seen in approximately 60% of patients in advanced-stage disease (25, 182, 184). Less common ACA in advanced stages include t(3;12), -7, -17, +17 and +21 and -Y, and are considered as “minor-route” aberrations (184). At diagnosis, approximately 7% have any ACA and 1-2 % have major-route ACA (185). Of note, the classification of major route ACA was based on how common the different ACAs were, and not on their impact on treatment results. While some studies in the TKI era have demonstrated an inferior progression-free survival in patients with all types of major route ACA and no impact on survival of minor route ACA at diagnosis (185, 186), more recent studies have shown that +8 and +Ph when occurring alone and without other characteristics of AP are not associated with a worse overall survival (187, 188). Conversely, patients with minor route ACA -7/del(7q), 3q26.2 and 11q23 rearrangements respond poorly to TKI and have a dismal prognosis (188-190). Moreover, when comparing groups of patients, those with i(17q), -7/del(7q) and 3q26.2 rearrangements showed significantly poorer responses to TKI therapy compared to patients with +8, +Ph and -Y (188). Consequently, the term “high-risk” ACA is more frequently used, although not meticulously defined, and often include major route ACA and above mentioned -7/del(7q), 3q26.2 and 11q23 rearrangements. Especially -7/del(7q), 3q26.2 rearrangements and i(17q) confer a high risk of imminent BP transformation (191). The overall adverse prognostic value of high-risk ACA development during treatment is acknowledged in the ELN guidelines where baseline high risk ACA fulfils the “warning”-criteria, while acquisition of high-risk ACA during treatment signifies “failure” (table 3) (38, 192).

BCR::ABL1 kinase domain mutations

Point-mutations within *BCR::ABL1* KD are the most well documented TKI resistance mechanism. Initial investigations of patients with primary and secondary resistance in all stages of CML using Sanger Sequencing found *BCL::ABL1* KD mutations in 20-45% of patients with primary resistance and in 50-70% of patients with secondary resistance (193-195). Most early studies on *BCR::ABL1* KD mutations during TKI treatment have been performed using Sanger Sequencing, but while 23% and 10% of patients with first line failure and warning respectively have *BCR::ABL1* mutations by Sanger

Sequencing, these numbers jumped to 47% and 34% respectively when more sensitive Next Generation Sequencing (NGS) methods were applied to the same cohort of patients (196). To date, researchers have identified more than 90 different *BCR::ABL1* KD mutations, affecting more than 55 amino acids (197). Nonetheless, a few amino acid substitutions are responsible for the majority of these mutations. The most common mutations in patients treated with imatinib are T315I, G250E, M244V, M351T and E255K/V (195). Among patients experiencing failure on second-line 2nd generation TKI treatment, the predominant mutations are T315I, F317L, V299L for dasatinib and E255K/V, T315I, F359C/V, Y253H for nilotinib respectively (198, 199). Similarly, for patients treated with first line dasatinib emerging mutations were all T315I, F317L, F317I or V299L and for patients with first line nilotinib T315I, E255K/V, F359C/V and Y253H (200, 201). The most common mutations are summarized in table 4. The emergence of *BCR::ABL* KD mutations during TKI treatment increase the risk of treatment failure, disease progression and adversely affects overall survival (202-205). For patients treated with imatinib, the T315I mutation along with mutations in the ATP binding-loop (P-loop, residues 245-255) of *BCR::ABL1* KD have been associated with a significantly shorter progression-free survival and overall survival (206-208). Notably, the T315I mutation confers resistance to all current TKIs except ponatinib and possibly asciminib. Despite this, with the broader application of *BCR::ABL1* KD sequencing at the time of treatment failure and a wider use of 2nd and 3rd generation TKIs, a more recent study found no clear negative prognostic significance of the T315I and P-Loop mutations on survival compared with non T315I and P-Loop mutations (209). Indeed, studies of CML patients with pre-existing *BCR::ABL1* KD mutations after imatinib treatment show that nilotinib and dasatinib are efficacious against many of the imatinib resistant mutant clones (198, 199). Mutations that fall below the detection threshold of Sanger Sequencing (15-20%) are referred to as low-level mutations and can be detected using more modern techniques (see *Next generation sequencing* below). Early detection of these mutations may be crucial when choosing the subsequent TKI in order to mitigate the risk of expansion of resistant clones, and improving patient outcomes (196). Additionally, multiple mutations arising in the same molecule (compound mutations) are seen in approximately 30% of patients with BP-CML, and present a significant treatment challenge (210, 211).

Table 4. The most common *BCR::ABL1* KD mutations with poor sensitivity to tyrosine kinase inhibitors (TKI). Adapted from Soverini et al. (197)

Mutations poorly sensitive to imatinib	M244V, G250E, Q252H, Y253F, Y253H, E255K, E255V, D276G, F311L, T315I, F317L, M351T, E355G, F359V, L384M, L387F, H396R, H396P, E459K, F486S
Mutations poorly sensitive to dasatinib	V299L, T315I, T315A, F317L, F317V, F317I, F317C
Mutations poorly sensitive to nilotinib	Y253H, E255K, E255V, T315I, F359V, F359I, F359C
Mutations poorly sensitive to bosutinib	E255V, E255K, V299L, T315I
Mutations poorly sensitive to ponatinib	T315M, T315L

Non *BCR::ABL1* pathogenic variants

The exact contribution of mutations in other genes than *BCR::ABL1* as resistance drivers in CML is not completely understood. In a broader context, clonal haematopoiesis with somatic mutations without evidence of any haematological disease has been identified in 10-20% of people at the age of 65 with prevalence increasing with age and a higher prevalence found with more comprehensive methods (212-214). However, while the risk of developing haematological cancer in individuals with clonal haematopoiesis is approximately 0.5-1% per year, the relative risk of developing haematological cancer and overall mortality is substantially increased in this group compared with patients without clonal haematopoiesis (213, 215). Somatic mutations in epigenetic regulators, notably *DNMT3A*, *ASXL1* and *TET2*, are among the most seen in clonal haematopoiesis (212, 213). Studies on CML patients progressing to BP show that apart from the acquisition of *BCR::ABL1* KD mutations and cytogenetic clonal evolution, mutations in other cancer associated genes are commonly seen. Recurrent mutations in BP have been found in genes such as *RUNX1*, *IKZF1*, *ASXL1*, *WT1*, *TET2*, *CBL*, *IDH1*, *NRAS*, *KRAS*, and *TP53* (216, 217). In a study facilitating an integrated approach of whole exome sequencing and RNA-sequencing in CML patients, those with an optimal response to TKI therapy were compared with patients developing BP (217). A number of variants were commonly seen already at diagnosis of CP-CML in patients who later developed BP, particularly in genes like *ASXL1*, *IKZF1*, *SETD1B* and *RUNX1*. In this study, 58% of patients had *BCR::ABL1* KD mutations at BP diagnosis. However, for patients with a prior mutation analysis

available, 62% had cancer gene variants that predated the *BCR::ABL1* KD mutations (217). Another study found that somatic mutations at CML diagnosis in genes associated with epigenetic regulation, with *ASXL1* mutations being most common, are associated with a significantly worse outcome with regards to molecular response independent of other prognostic factors (218). In these studies, the median age at diagnosis was 55 and 47 years old (range 37-82) for patients harbouring an *ASXL1* variant (217, 218). The presence of somatic mutations in genes associated with epigenetic modulation has also been correlated with worse event-free survival in imatinib treated patients, though no impact on survival was observed in patients treated with 2nd generation TKI (219). Interestingly, in a small cohort of paediatric patients with CML, somatic variants in *ASXL1* were found in 30% of patients (220). *ASXL1* variants are commonly found in other myeloid malignancies and are associated with poor prognosis in these diseases (221), and it has recently been discovered that common *ASXL1* mutations occur in exon 12 resulting in truncated ASXL1 protein, which by gain-of-function gives rise to more open chromatin and dysregulation of genes responsible for hematopoietic stem cell renewal and differentiation (222). The mechanisms by which truncated ASXL1 confer these effects are likely multifaceted (221). However, an acquired interaction with a bromodomain containing protein, BRD4, and a subsequent enrichment of BRD4 at promotor sites of genes responsible for stem cell, haematopoiesis and myeloid pathways is one possible mechanism (222). BRD4 in turn exerts its effects by inducing more open chromatin, and enhancing transcription via RNA polymerase II (223).

Additional factors

Several studies have shown considerable variability on peak plasma concentrations between patients treated with imatinib (224, 225). Imatinib as well as 2nd generation TKIs nilotinib, dasatinib, bosutinib and 3rd generation TKI ponatinib are all mainly degraded through cytochrome p450 isoenzyme 3A4 (CYP3A4), and interindividual variations in CYP3A4 concentrations and activity, as well as drugs that inhibit or induce CYP3A4 activity can affect plasma TKI concentrations (226, 227). BM fibrosis (228, 229) and higher *BCR:ABL1* expression (230, 231) have been shown to have a negative impact on treatment outcome. Moreover, several studies have shown that patients with the e14a2 transcripts exhibit better molecular responses upon imatinib treatment than patients with the e13a2 transcript, but whether this has any impact on survival remains unclear (232-237). Interestingly, the e14a2 transcript also seems to be predictive of a better chance of maintaining TFR (160, 161). The effect of TKI is dependent on the intracellular concentration of the drug, and upregulation of major transmembrane efflux transporters, or downregulation of influx transporters of TKI have also been implicated in TKI resistance (238).

Next generation sequencing

The founding DNA sequencing method was developed by Sanger et al. in the 1970s and uses the principle of chain-terminating nucleotides that lack a 3'-OH group (dideoxy ribonucleoside triphosphates or ddNTPs). The ddNTPs were initially radioactively and later fluorescently labelled for detection, and with improved methodology and automation the method has remained the gold standard in DNA sequencing (239, 240). Consequently, conventional Sanger sequencing has been the recommended method for mutation screening in CML, but has the limitation of not being able to detect mutations expressed in less than 15-20% of transcripts, and cannot discriminate between multiple mutations in different clones (polyclonal mutations) or in the same clone (compound mutations) (241). NGS, also referred to as second generation sequencing, is a collective term including varying methods to sequence thousands or millions of oligonucleotide molecules simultaneously (242). Depending on the type of DNA templates used when sequencing, analysis can be referred to as whole genome sequencing, whole exome sequencing, epigenome sequencing or targeted sequencing. Similarly, RNA can be analysed through whole transcriptome sequencing, mRNA sequencing and small RNA sequencing. The most widely used NGS methods use the sequencing by synthesis (SBS) method, and are based on Sanger sequencing principles but differ in some important aspects. Generally, they produce shorter reads (approximately 300-500 bases), but utilize parallel sequencing to produce millions or billions short DNA sequence reads, subsequently analysed to create a consensus sequence (243).

Illumina technology

Illumina sequencing offers the most widely used NGS methods (244). In short, the methods rely on the preparation of a DNA library of short, approximately 500 bp fragments. The fragments are ligated with adapters, which are then bound to complementary oligonucleotide sequences on a glass slide. The slide can harbour millions of adjacent DNA molecules, and amplification creates clusters of clonal oligonucleotide strands in order to produce strong fluorescent signals. Fluorescently labelled oligonucleotides with a reversible terminator are incorporated into the growing strands and thereby terminate the synthesis. Direct imaging measures the fluorescent colour after each round of amplification. After each round of synthesis, the labelled oligonucleotide is then unblocked by removing the terminator, to allow for the binding of the next oligonucleotide. These steps are repeated approximately 300 times resulting in billions of short reads with a length corresponding to the number of sequencing cycles. The reads are then aligned to create a consensus sequence. The read depth or coverage at a given position in the sequence corresponds to the number of reads at that position. The consensus sequence is aligned and

compared with a reference sequence, and variants are simply deviations from the reference sequence. The variant allele frequency (VAF) of a given variant is the proportion of the reads on that position that represent the variant (242). NGS analysis for the detection of resistance mutations in *BCR::ABL1* KD frequently utilizes Illumina technology (197).

Pacific Biosciences technology

Sometimes referred to as third generation sequencing technology, long-read sequencing generates oligonucleotide sequences of >10 kilobases (kb). The major players providing long range sequencing techniques are Pacific Biosciences (PacBio) and Oxford Nanopore Technologies. PacBio have developed Single Molecule, Real Time (SMRT) sequencing which require a single-stranded, circular DNA referred to as a SMRTbell template. This circular DNA molecule is generated by ligating hairpin adapters to a template DNA molecule. Similar to other NGS sequencing methods, the sequencing procedure takes place in micropores on a chip. Each cell contains an individual DNA polymerase enabling the sequencing of a single SMRTbell template. Fluorescently labelled nucleotides are used to determine the sequence, and due to its' circularity, sequencing of the template can continue past the adapter sequences. Each read of a DNA-strand is referred to as a pass, and multiple passes provides multiple reads of the same template. By discarding the adapter sequences a DNA template is obtained, referred to as a subread. The multiplicity of the long read creates improved accuracy over single reads where random errors affect the results (244, 245). PacBio technology is also in clinical use for the detection of resistance mutations in the *BCR::ABL1* KD (246, 247).

The Swedish CML registry

The Swedish CML registry was originally created in 2002 by the Swedish CML study group in collaboration with the Regional Cancer Centres (RCC) of Sweden. It covers a population of 10 million people and >95% of newly diagnosed patients with CML are reported to the registry (13, 14). Patients ≥ 18 years of age with a diagnosis of CML in all stages of the disease are reported to the registry electronically. Follow-up data are reported at 1, 2, 5 and 10 years after diagnosis. The registry has been utilized in several publications including studies on outcome in CML patients (14, 40), secondary malignancies in CML patients (248, 249), cardiovascular events in TKI treated patients (250), characterization of CML patients in advanced stages (27), and allo-SCT in the TKI era (133).

U-CAN

U-CAN is a collaboration run by Uppsala University, Umeå University, Stockholm University, and KTH Royal Institute of Technology and in close collaboration with the University Hospitals in Uppsala and Umeå, and a number of regional hospitals. It was initiated in 2010 with the aim to prospectively gather clinical data and biological samples from patients with a range of different types of cancer, including haematological malignancies. The ambition is to generate a longitudinal biobank and database to gain a better understanding of the cause, treatment and evolution of cancer. Stored biobank samples are made available to researchers after an application process (251).

Research aims

The general aims of this thesis were to explore novel ways to increase TFR eligibility in CML by improving the rate of DMR, to evaluate the outcome after TKI cessation and to explore potential mechanisms behind failure to attain an optimal treatment response. The specific aims were as follows:

Paper I

To examine the incidence and outcome of TKI treatment discontinuation on a population-based level.

Paper II

To examine the long-term safety and efficacy after the initial addition of PegIFN- α to 2nd generation TKI dasatinib in patients with CP-CML.

Paper III

To examine the feasibility of attempting a second TKI discontinuation after re-treatment with 2nd generation TKI dasatinib in patients with a previous failed TFR attempt.

Paper IV

To examine the prevalence of non-*BCR::ABL1* pathogenic variants in a cohort of patients with suboptimal response to TKI and no resistance mutations in the *BCR::ABL1* KD.

Patients and methods

Paper I

Data sources

Paper I constitutes a retrospective, population-based study utilizing the comprehensive Swedish CML registry, which is detailed further in the background section of this thesis. This registry encompasses over 95% of all newly diagnosed CML patients in Sweden, identifying 584 individuals with a confirmed CP-CML diagnosis from January 1, 2007, to December 31, 2012. Within this web-based registry, a questionnaire was developed to inquire from clinicians about any TKI interruptions exceeding one month. Following data extraction, information regarding TKI interruptions was available for 548 patients (representing 94% of the cohort). Among these, 235 patients reported a TKI interruption of more than one month, with 128 of these interruptions attributed to a DMR. For these patients, further details on TKI discontinuation were collected. The date of the last follow-up ranged from March 2018 to November 2019. For instances where the most recent TKI was not documented, the Swedish Prescribed Drug Registry was used to identify the last prescribed TKI (252).

Methods

For all patients experiencing a TKI discontinuation of at least one month due to DMR, information was gathered using the specially designed questionnaire. This included the date of TKI discontinuation, the *BCR::ABL1* IS percentage prior to discontinuation, whether the patient resumed TKI treatment, the reason and date for restarting TKI therapy, whether the discontinuation was part of a clinical trial, date of achievement of DMR and the most recent TKI used before stopping. Additionally, pre-existing data from the Swedish CML registry, such as age, date of CML diagnosis, gender, Sokal score and initial treatment were also extracted for each patient.

Statistics

Median values and ranges were used as a measure of variability. The cumulative incidence of resuming TKI therapy was evaluated using the Kaplan-Meier

method, with the log-rank test applied to examine the hypothesis of no difference between groups. A p-value less than 0.05 was deemed statistically significant. The Mann-Whitney U test was utilized to assess the hypothesis of no difference in the duration from diagnosis to TKI interruption among different groups. Cox proportional hazard models calculated hazard ratios with 95% confidence intervals for resuming TKI therapy, considering factors such as age, gender, Sokal risk score, initial treatment with imatinib versus a second-generation TKI, treatment just before discontinuation, and duration in MR⁴ or better before stopping treatment. P-values were determined using the likelihood ratio test, and a minimal model was selected by using the likelihood ratio test.

Paper II

Data sources

Paper II presents a 5-year follow-up of the NordCML007 study, initiated by the Nordic CML Study Group. This study recruited 40 newly diagnosed patients with CP-CML from February 2013 to May 2014 across 14 university or regional hospitals in Sweden, Norway, Finland, and Denmark. Eligible patients were aged 18-70 years and had received no previous CML treatment, except for hydroxyurea, which was allowed for up to 30 days.

Methods

Patients were treated up-front with dasatinib 100 mg. After three months, patients started receiving 15 micrograms of PegIFN- α per week via subcutaneous injections. Assuming good tolerance, the dosage was increased to 25 micrograms and maintained through to the end of the 15th month, at which point, treatment continued with dasatinib alone. In the study's initial two years, follow-ups incorporating qRT-PCR and standard laboratory tests occurred monthly for the first six months, and quarterly up to the 24-month mark. Beyond 24 months, follow-ups for clinical assessments, treatment monitoring, and decision-making reverted to standard clinical practice, under the discretion of the investigators (figure 3). Prospective data collection on current treatment, adverse events, and *BCR::ABL1* IS values was conducted annually for all participants using a web-based case report form.

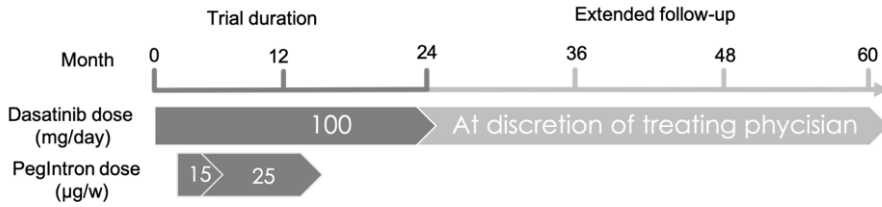


Figure 3. General outline of the NordCML007 study.

Statistics

Treatment response data are reported as the proportion of patients achieving MMR, MR⁴, and MR^{4.5} at specified time points. Patients who were lost to follow-up were excluded from the analysis subsequent to their last recorded follow-up. Deaths were accounted for as a loss of response. Confidence intervals were determined using the Clopper-Pearson exact method. Moreover, cumulative incidence functions for MMR, MR⁴, and MR^{4.5} were calculated and are shown as annual figures after each patient completed their yearly follow-up.

Paper III

Data sources

Paper III features an interim analysis of the DAstop2 clinical trial, which assesses the feasibility of a second attempt at TKI discontinuation in patients who previously failed a TFR attempt, either within the EURO-SKI study or according to its criteria (146). Ninety-four patients were included in the study between October 2017 to December 2021 across 17 university or regional hospitals in Sweden, Norway, Denmark, Finland, the Netherlands, Germany and France.

Methods

We prospectively evaluated the probability of maintaining TFR after a second TKI discontinuation after re-treatment with 2nd generation TKI dasatinib. Only patients with a previous TKI discontinuation attempt within EURO-SKI or according to EURO-SKI criteria were eligible. Upon inclusion in the study, patients were switched to dasatinib 100 mg or 70 mg at the discretion of the investigator. For 24 months patients were monitored with qRT-PCR every month between month 1-6, every 6 weeks between month 7-12 and every 3 months thereafter. Patients with sustained MR⁴ or better for ≥ 1 year were then eligible for a second TKI discontinuation attempt (figure 4).

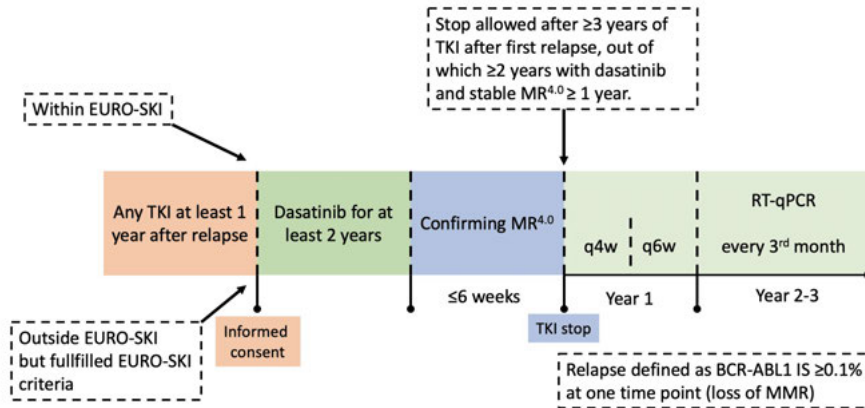


Figure 4. General outline of the DAsTop2 study.

Statistics

Descriptive statistics are presented using either mean or median values, with the range indicating variability. The proportion of patients achieving TFR was determined using the Kaplan-Meier method, accompanied by binomial 95% confidence intervals. The log rank test was employed for comparisons of TFR rates between groups. To compare the timing of molecular relapse post-second TKI discontinuation across groups, the Mann-Whitney U test was utilized.

Paper IV

Data sources

Paper IV conducts a retrospective analysis of patients diagnosed with CML with of primary TKI refractoriness. The study includes patients who exhibited a poor response to first-line TKI therapy and had their PB or BM samples preserved within the UCAN biobank in Uppsala. A poor response was defined as either fulfilling failure criteria (n=17), or persistent warning criteria (n=3) according to ELN guidelines during the first year of treatment, without a previous optimal response. In addition, all patients had performed *BCR::ABL1* KD mutation analysis using PacBio long-read sequencing without the finding of any variants associated with TKI resistance. A total of twenty patients diagnosed between March 2009 and February 2017, identified as having primary refractory CML with available UCAN samples, were included. For comparative analysis, ten patients diagnosed with CP-CML who demonstrated an optimal response to TKI therapy and had available blood samples in the UCAN biobank were selected as a control group.

Methods

Genomic DNA from diagnostic PB or BM samples from patients with primary refractory CML were retrospectively analysed for the presence of pathogenic variants in a panel of genes recurrently mutated in myeloid neoplasms. Mutation analysis was performed using the Trusight Myeloid panel from Illumina covering hotspots or full coding regions of 54 genes. Clinical data was collected including date of diagnosis, age at diagnosis, sex, Sokal risk score, *BCR::ABL1* IS% values from diagnosis to last follow-up, data on whether the patient had undergone an allo-SCT and whether the patient had died and date of death. Peripheral blood mononuclear cells from one patient with a pathogenic *ASXL1* variant, and four patients with wild type *ASXL1* were generated by Ficoli separation, cultured for 24 hours and subsequently treated with 100 nM/L of the bromodomain and extra-terminal motif (BET) inhibitor JQ1 for 24 and 96 hours. Cell viability was measured using the CellTiter-Glo 3D cell viability assay.

Ethical considerations

All studies presented in this thesis received approval from the Ethical Review Board (references: dnr 2013/069/7, 2012/365, 2016/513, and 2014/233). For **Paper I**, data were sourced from the Swedish CML registry, where all registered patients are informed about their inclusion, and their data are pseudonymized to protect privacy. The analysis conducted for our study utilized aggregated data, eliminating the need for individual informed consent as determined by the ethics committee. While accessing patient information from national registries poses a potential risk to personal privacy, we minimized this risk through the use of pseudonymized data and a stringent data extraction approval process. Although individuals do not receive direct benefits from registry participation, the insights gained can significantly contribute to the improvement of future CML patient care. In **Papers II and III**, participation involved adult patients who provided written informed consent before joining the studies. The collection of data was strictly limited to the parameters defined in the study protocols. Data entered into the web-based case report forms were pseudonymized to ensure confidentiality. For **Paper IV**, included patients had previously consented in writing for their clinical information and PB and/or BM samples to be stored in the UCAN database and biobank. This consent covered the prospective collection of clinical data, periodic blood sampling, and broad participation in cancer research initiatives. Additionally, our study secured specific Ethical Review Board approval for the intended use of this data, negating the need for further consent beyond what was initially provided at the time of UCAN enrolment. All research conducted for this thesis adhered to the principles outlined in the Declaration of Helsinki and observed Good Clinical Practice guidelines, ensuring ethical standards and patient welfare were maintained throughout.

Results and discussion

TKI discontinuation in clinical routine is common and as feasible as when attempted in clinical trials.

In **Paper I**, we discovered that 22% of patients diagnosed with CP-CML between January 2007 and December 2012 attempted TKI discontinuation after a median follow-up of 8.9 years. Notably, 70% of these discontinuation attempts occurred outside clinical trials, despite clinical guidelines at the time advising against such actions. The median duration from diagnosis to TKI discontinuation was 5.3 years, with the period from TKI discontinuation to the last follow-up ranging from 0.1 to 8.1 years (median 2.8 years). During this interval, 49% of patients resumed TKI treatment. Consequently, at the time of the last follow-up, 11.1% of the entire CP-CML cohort diagnosed within the specified period were treatment-free. Reassuringly, 62% of those who discontinued TKI treatment outside clinical trials remained treatment-free by last follow-up, suggesting that treating physicians are effectively identifying patients suitable for TKI discontinuation in routine clinical practice. The Cox proportional hazard model corroborated findings from earlier trials, demonstrating that an extended duration of DMR significantly reduced the likelihood of resuming TKI therapy (HR 0.54, 95% CI 0.32-0.91, $p=0.020$). Additionally, among patients treated with a second-generation TKI at both diagnosis and discontinuation (33% of patients), the probability of restarting TKI therapy was lower compared to those treated with imatinib at both time-points (HR 0.50, 95% CI 0.28-0.90, $p=0.024$). A subset of patients (22%) switched between imatinib and a second-generation TKI between diagnosis and discontinuation, with outcomes similar to those with second-generation TKIs at both time-points. Direct randomized comparisons between imatinib and second-generation TKIs regarding the achievement of TFR are lacking. Comparative analyses of TKI discontinuation studies following imatinib or second-generation TKI treatment yield varying results, with no definitive trend favouring better outcomes with second-generation TKIs. However, differences in study designs complicate direct comparisons.

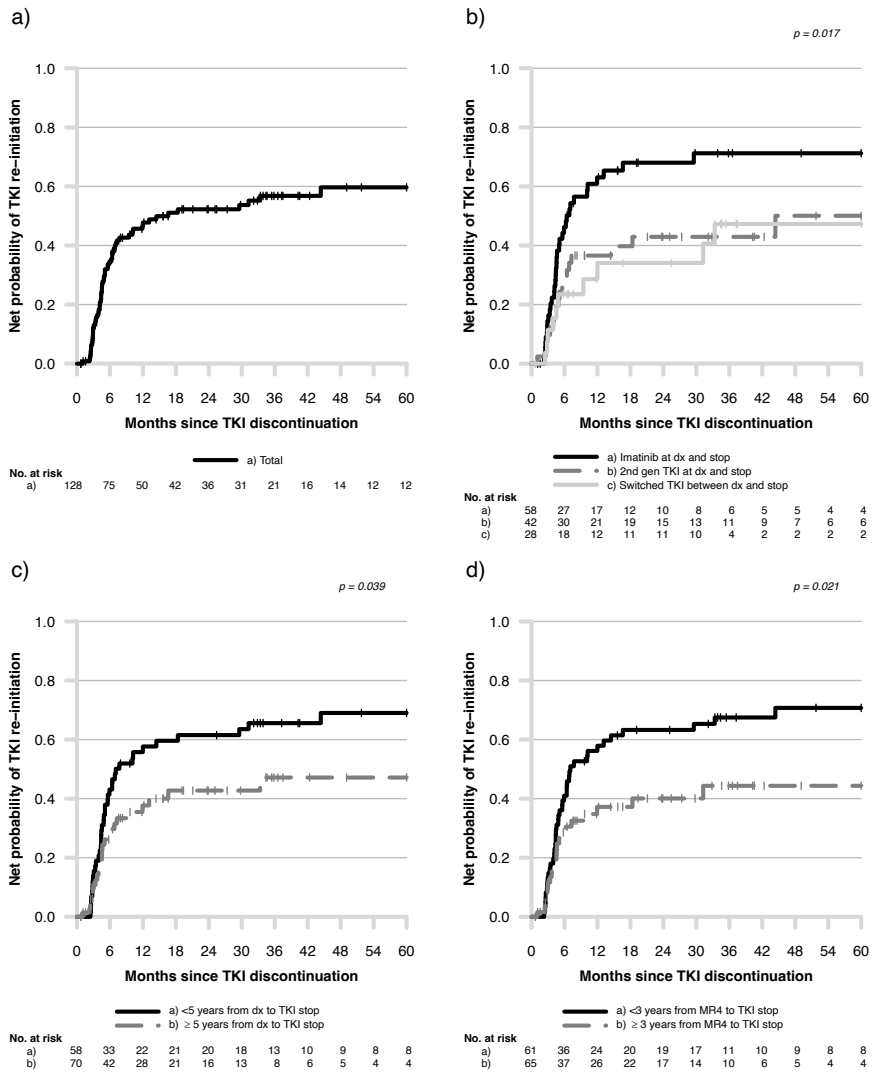


Figure 5. Net probability of tyrosine kinase inhibitor (TKI) re-initiation after TKI discontinuation in patients stopping in deep molecular response (DMR) for; a) all patients stopping in DMR, b) patients with imatinib treatment as first line and at discontinuation, patients with 2nd generation TKI treatment as first line and at discontinuation, and patients who switched between imatinib and 2nd generation TKI between diagnosis and discontinuation, c) patients stopping $<$ or \geq than 5 years from diagnosis, and d) patients with a duration of DMR of $<$ or \geq than 3 years prior to stop.

The addition of PegIFN- α to dasatinib and its role in optimizing treatment response

In **Paper II** we found that despite that only 10% of patients reached MMR by 3 months (before the addition of PegIFN- α) all but two patients had reached MMR at any time-point after five years of follow-up. Moreover, cumulative incidence of DMR was 74% at the end of the study, comparing favourably to results from the registration trial of dasatinib, DASISION (63). At the five-year follow-up, 49% of patients were either eligible for treatment discontinuation, or had already attempted treatment discontinuation. As reported in an earlier publication of the NordCML007 trial, the low dose of PegIFN- α was well tolerated, and 84% of patients were still on the combination treatment after 12 months (129). After 5 years, 15 patients (38%) had discontinued dasatinib due to adverse events (n=9) or other reasons (n=6). Due to the role of IFN- α in immune modulation, the occurrence of dasatinib-induced serosal effusions was of special interest. In total, eight patients (20%) experienced pleural effusion in NordCML007 compared with 28% in DASISION, indicating that the addition of PegIFN- α does not increase the risk of serosal effusions. Furthermore, no previously unknown adverse events and no progressions to AP or BP-CML were reported.

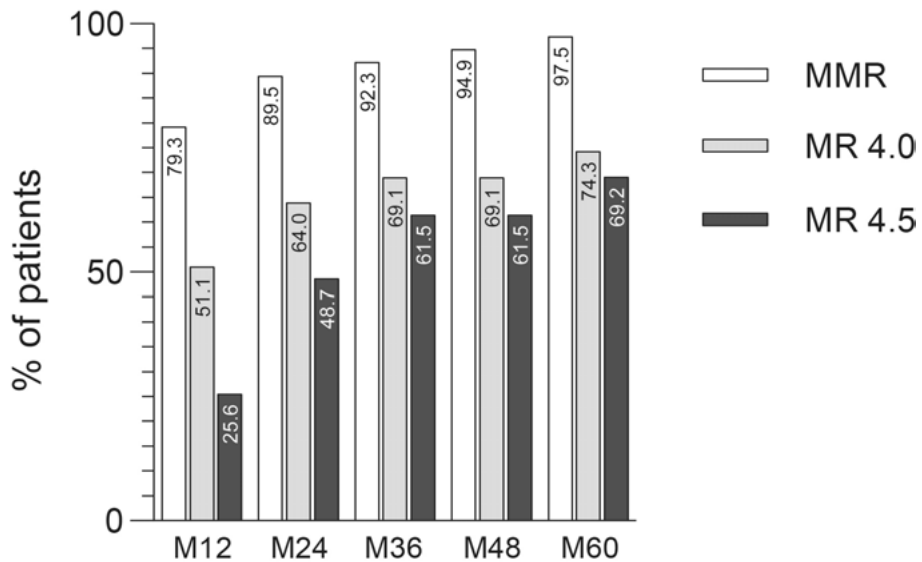


Figure 6. Cumulative incidence of major molecular response (MMR), MR⁴ and MR^{4.5} in the NordCML007 study after the respective yearly follow-up.

A second TKI discontinuation after re-treatment with dasatinib – as successful as a first TKI discontinuation?

In **Paper III**, we observed that the TFR rates after a second attempt at TKI discontinuation in patients with a prior unsuccessful attempt were 61% at 6 months and 56% at 12 months. The median age at diagnosis within this patient cohort was 49 years, with the median age at the first discontinuation attempt being 55 years. The duration to molecular relapse post-first discontinuation ranged from 1 to 65 months, with a median of 4 months. At the time of this interim analysis, 64 patients had surpassed the 24-month mark, and 62 of these were considered suitable for a second attempt at TKI discontinuation. For this subgroup, the median age at the time of the second discontinuation attempt was 60 years (range: 28-88), and the median duration of TKI treatment from the first molecular relapse to the second discontinuation attempt was 65 months (range: 36-97). Analysing the data based on the duration of the first TFR, it was found that patients who had a first TFR lasting ≥ 6 months had a significantly lower likelihood of experiencing a molecular relapse following their second TKI discontinuation, with 12-month TFR rates of 91% versus 35% for the two groups, respectively. However, an higher number of relapses were observed beyond 12 months among those with a longer initial TFR, indicated by a median time to molecular relapse of 18.6 months compared to 3.5 months for those with a shorter initial TFR. Consequently, the difference between the Kaplan-Meier curves lessened over an extended follow-up period, although with few patients at risk beyond 18 months. This phenomenon raises the question of whether a longer initial TFR duration indeed suggests a higher probability of maintaining TFR after a second discontinuation attempt, or if the dynamics of *BCR::ABL1* kinetics differ between the groups.

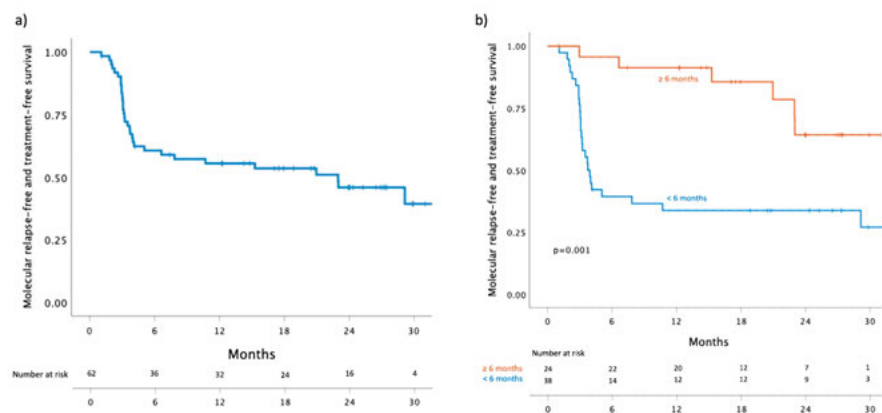


Figure 7. Treatment-free remission (TFR) probabilities after the second tyrosine kinase inhibitor (TKI) discontinuation for a) the whole cohort and b) patients divided by whether the first TFR duration was \geq or $<$ than 6 months.

Enrichment of pathogenic variants in *ASXL1* in patients with primary refractory CML

In **Paper IV**, our analysis revealed that 50% of a cohort of patients with primary refractory CML, who did not exhibit *BCR::ABL1* KD mutations, possessed pathogenic variants in genes frequently mutated in myeloid neoplasms. Notably, one patient had more than one pathogenic variant. The most frequently affected gene was *ASXL1*, with pathogenic variants identified in 6 (30%) patients, followed by *DNMT3A* in 2 patients, and *IKZF1*, *GATA2* and *TP53* in one patient each. All detected *ASXL1* variants were located in exon 12, with five out of six being the p.G646fs*12 variant, a prevalent frameshift mutation in *ASXL1* associated with myelodysplastic syndromes, myeloproliferative neoplasms, and acute myeloid leukaemia. This particular mutation has been documented to produce a truncated ASXL1 protein that hampers myeloid differentiation and biases cell differentiation toward the granulocytic/monocytic lineage in mouse models (222, 253, 254). All of the patients with the p.G646fs*12 *ASXL1* variant at diagnosis had poor response to ≥ 3 TKIs and all of them subsequently had an allo-SCT. While truncating *ASXL1* mutations in CML appears to be associated with an inferior outcome in patients with CP-CML in general, its exact role in TKI resistance remains unknown. In a mouse model, truncated ASXL1 but not wild type ASXL1 had the ability to bind BRD4, and BM cells in these mice exhibit a greater sensitivity to BRD4-inhibitors (222). In an initial exploratory study, when cultured PB mononuclear cells from a patient with an *ASXL1* pathogenic variant were treated with the BET inhibitor JQ1, a notable reduction in cell viability was observed using a CellTiter-Glo 3D viability assay, in comparison to cells from four CML patients with wild-type *ASXL1*. This finding suggests a potential therapeutic avenue for patients harbouring *ASXL1* mutations.

Limitations

In **Paper I**, when assessing the potential effect of 2nd generation TKI use on TFR probabilities, and comparing patients inside and outside clinical trials, the long intervals between reporting time-points and design of the CML registry need to be taken into consideration. Only data on TKI treatment at diagnosis and just before discontinuation were available, leaving the possibility of patients switching to another TKI, and then switching back again before discontinuing treatment. In general, data generated from non-randomized trials increases the risk of undiscovered confounding factors and the limited number of data-points in the registry further limits the adjustment for confounders. Patients discontinuing outside clinical trials might be more carefully selected by treating physicians, as illustrated by a higher proportion of Sokal low risk patients, a longer median treatment time and time in MR⁴ prior to discontinuation compared with those discontinuing outside clinical trials. Similarly, the better outcomes for patients treated with a 2nd generation TKI might be due to confounding factors not discovered in the current dataset.

In **Paper II**, data were reported yearly after 24 months. The long interval between reporting increases the risk of underreporting of adverse events. However, this risk is likely higher for mild adverse events, and not grade III-IV adverse reactions, especially those clearly attributable to dasatinib treatment such as pleural effusions or pulmonary arterial hypertension. Moreover, efficacy comparison to historical cohorts must be done with caution, as illustrated by the diverse efficacy outcomes reported by clinical trials of dasatinib.

Paper III is an interim analysis of a study with a planned follow-up duration of 24 months after the second discontinuation attempt. This means that any results regarding factors associated with a successful second discontinuation is exploratory, and a final analysis of all patients included in the study is needed to draw robust conclusions. As not all patients in the study have reached the discontinuation time-point, there is a risk of bias. For example, patients included early in the study could potentially differ from those included later due to different study sites joining the study at different time-points. In addition, the late relapses could increase with a longer follow-up time.

In **Paper IV** patients were selected based on the site of treatment (Uppsala University hospital), their failure to achieve treatment milestones, a lack of *BCR::ABL1* KD resistance mutations and the fact that diagnostic samples were stored in the UCAN database. It is plausible to assume that these patients are not necessarily representative of all CML patients with primary TKI refractoriness and no KD mutations. NGS analysis reveals pathogenic variants in genes recurrently mutated in myeloid neoplasms, but cannot discriminate between variants co-occurring in *BCR::ABL1* positive cells and those sitting in other cell populations. Furthermore, since the only tissue available was PB or BM and longitudinal samples were only available in a few patients and not at specified time-points, it is difficult to draw any robust conclusions on the clonal architecture and thereby the role in TKI resistance of the individual pathogenic variants. While a gender- and age-matched cohort of patients with optimal response to treatment was used as a control when assessing the pathogenic variants, this cohort would need to be further expanded to increase the generalisability. Further, the scarcity of PB or BM from patients with concurrent *ASXL1* pathogenic variants and *BCR::ABL1* only allowed for one single experiment with JQ1 treatment at this time.

Conclusions and future perspectives

Long-term follow-up of TKI clinical trials has demonstrated excellent efficacy, but has also shed light on various detrimental effects of especially the 2nd and 3rd generations of TKI. Moreover, the risk of many of these adverse effects accumulates over time (250, 255). With the knowledge that a substantial proportion of patients with a DMR can discontinue treatment, further efforts to increase the number of eligible patients and shorten the time to DMR achievement are appealing. We show in **Paper II** that an initial combination of a low dose of PegIFN- α and dasatinib is feasible and tolerable, and may provide a way to increase the rate of DMR further. In addition, we show that long-term tolerability is not adversely affected by the initial PegIFN- α addition, as it did not appear to increase late dasatinib toxicity or significantly increase the proportion switching to another TKI. This, in combination with a cumulative DMR rate of 74% after 5 years warrants further randomized clinical trials examining the effect of the addition of a low dose of PegIFN- α to TKI.

The feasibility of TKI cessation in those with a DMR has been established through numerous clinical trials, but it was not until 2019 that recommendations on TKI cessation were included in routine care guidelines. While clinical trials are essential to establish the concept of TFR, real world data are needed to determine how TKI cessation is implemented in non-selected population-based cohorts. In this aspect, the Swedish CML registry provides unique comprehensive real-world data. We conclude in **Paper I** that even before treatment cessation was included in clinical guidelines, 22% of patients diagnosed with CP-CML had attempted a TKI discontinuation and 11% were treatment-free. Encouragingly, those discontinuing outside clinical trials appear to be at least as likely to attain a TFR as those patients who have historically discontinued within clinical trials. It has been hypothesized that the number of patients that could potentially attain a TFR is somewhere between 20-30%. The choice to discontinue TKI treatment is multifaceted and based on a combination of treatment related factors such as treatment duration, duration of DMR, comorbidities and treatment adverse reactions as well as patient and physician attitudes toward treatment discontinuation. Future follow-up studies using the Swedish CML registry are warranted to determine how TKI discontinuation guidelines will be implemented and affect the rate of TFR in the broad CML population.

Furthermore, estimations of how many could attain a TFR have largely been based on studies of a first TKI discontinuation. To what extent patients with a failed TFR attempt are destined to life-long TKI treatment has been largely unknown. In **Paper III** we demonstrate that a second TKI discontinuation is feasible and that the TFR rate is similar to that seen after a first TKI discontinuation. Reassuringly, similar to studies of a first TKI discontinuation, no progressions to AP or BP-CML were seen, and patients quickly regained their previous treatment response upon TKI re-challenge. Similar to a previous retrospective study of a 2nd TFR attempt, we found that a longer TFR duration after the first attempt translates into a better chance of maintaining TFR after the second stop. However, more late molecular relapses were seen in the group with a longer first TFR duration. This raises the question of whether a longer first TFR duration is a marker of different *BCR::ABL1* kinetics. A longer follow-up time is needed and we hope to answer this question in a future final report of the DAsTop2 study. In addition, it is yet unknown why some patients experience a molecular relapse while others don't. Further studies of immunological and genetic alterations following TKI cessation are needed to improve the selection of patients attempting TKI discontinuation, and to find actionable factors that could improve patients' probability of maintaining TFR.

A swift and deep reduction in *BCR::ABL1* transcripts is not only essential to be eligible for a TKI discontinuation, but is also a predictor of excellent long-term survival. In contrast, primary resistance to TKI treatment is associated with dismal outcomes. Although ACA or *BCR::ABL1* KD mutations account for a proportion of primary resistance, in many cases, the reason for treatment resistance remains unknown. We show in **Paper IV** an enrichment of pathogenic variants in *ASXL1* in patients with primary refractoriness and no initial *BCR::ABL1* KD mutations or cytogenetic aberrations, potentially responsible for a proportion of cases with primary resistance. The finding of *ASXL1* in primary refractory CML is interesting for a number of reasons; *ASXL1* is frequently found in other myeloid malignancies including myeloproliferative neoplasms, acute myeloid leukaemia, myelodysplastic syndromes and especially chronic myelomonocytic leukaemia. In addition, patients with *ASXL1* pathogenic variants have been shown to express a truncated ASXL1 protein and the expression of a truncated ASXL1 protein in mice induces myeloid malignancies. This indicates a gain-of-function mechanism and opens the possibility of targeted therapies against truncated ASXL1 or its interacting proteins. A first exploratory attempt in our study to treat cells from a patient with an *ASXL1* pathogenic variant confirmed this finding. While it is difficult to draw any robust conclusion from this single attempt, we hope to expand this experiment in order to shed light on the therapeutic role of BET-inhibitors in CML patients harbouring pathogenic *ASXL1* variants. In addition, single cell sequencing would aid in shedding light on the clonal architecture of somatic variants.

In conclusion, this thesis adds to the evidence showing that TKI discontinuation is safe and feasible for CML patients, and expands the knowledge by prospectively describing the safety and feasibility of a second discontinuation attempt. In addition, it provides a basis for further studies of optimizing TFR eligibility by the combination of PegIFN- α and TKI, and indicates that pathogenic variants in *ASXL1* may provide a treatable mechanism responsible for primary TKI resistance in some CML patients.

Svensk populärvetenskaplig sammanfattning

I den här sammanställningen presenteras resultat från studier om behandling av kronisk myeloisk leukemi (KML). Målet med avhandlingen var att undersöka sätt att öka andelen patienter som kan uppnå djupa molekyllära behandlingssvar och därmed öka andelen som kan vara aktuella för att avsluta sin behandling med tyrosinkinashämmare (TKI). Vidare ville vi beskriva förekomsten och utfallet efter TKI-stopp i den svenska KML-populationen, samt undersöka möjligheten att genomföra ett andra TKI-stopp för patienter som tidigare misslyckats med ett första stoppförsök. Vi ville också utforska genetiska mekanismer bakom behandlingssvikt hos patienter som saknar de mest välkända genetiska orsakerna till behandlingsresistens.

Studie I visar att 22% av patienterna som diagnostiserades med KML i kronisk fas mellan 2007 och 2012 hade gjort försök att avsluta sin TKI-behandling efter en medianuppföljningstid på 8,9 år. Majoriteten (70%) av dessa avbrott skedde utanför kliniska prövningar, trots att detta inte rekommenderades i kliniska riktlinjer vid den här tiden. Nästan hälften (49%) av patienterna behövde återuppta TKI-behandlingen, medan 62% av patienterna som gjort ett stoppförsök utanför kliniska studier var behandlingsfria vid senaste uppföljningen, vilket tyder på att läkare framgångsrikt kan välja ut lämpliga patienter för behandlingsavbrott.

Studie II undersökte effekten av att lägga till en låg dos pegylerat interferon alpha till dasatinibbehandling hos KML-patienter vid diagnos för att optimera behandlingssvaret. Trots att endast 10% uppnådde en viss nivå av sjukdomskontroll (MMR) inom 3 månader, hade nästan alla uppnått MMR efter fem år. Dessutom tolererades låga doser pegylerat interferon α väl, och 49% av patienterna uppfyllde kriterierna för, till eller hade redan gjort ett försök, att avbryta behandlingen efter fem år.

Studie III fokuserade på patienter som gjorde ett andra försök att avsluta TKI-behandlingen efter ett tidigare misslyckat försök. Sannolikheten att vara behandlingsfri var 61% efter 6 månader och 56% efter 12 månader. Studien visar att patienter med en längre duration av behandlingsfrihet under det första avbrottet hade en lägre risk för återfall efter det andra avbrottet.

Studie IV identifierade en anrikning av patogena varianter i genen *ASXL1* hos patienter med primärt behandlingsresistent KML. Dessa varianter har visats vara kopplade till sämre svar på TKI-behandling för KML-patienter och en sämre prognos för patienter med andra myeloida sjukdomar. Patogena varianter i *ASXL1* resulterar dessutom i ett förkortat, men biologiskt aktivt protein som kan agera mål för framtida behandlingar.

Sammanfattningsvis visar den här avhandlingen att TKI-avbrott är möjligt och genomförbart i klinisk vardag, med en betydande andel patienter som förblir behandlingsfria. Tillägget av pegylerat interferon alpha till TKI-behandling kan potentiellt förbättra behandlingsresultaten och göra att fler patienter kan bli aktuella för att avsluta sin TKI-behandling. Därtill visar vi att även en stor andel av de patienter som tidigare misslyckats i ett försök att avsluta sin TKI-behandling framgångsrikt kan göra ett ytterligare stoppförsök. För patienter med behandlingsresistent KML kan genetiska varianter i andra gener än *BCR::ABL1*, såsom i *ASXL1*, erbjuda förklaringar till varför vissa patienter inte svarar på behandling. Dessa insikter bidrar sammantaget till en mer individualiserad behandlingsstrategi för patienter med KML

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