Clinical and Immunological Studies in Chronic Myeloid Leukaemia

STINA SÖDERLUND
Chronic myeloid leukaemia (CML) is characterised by the constitutively active tyrosine kinase BCR-ABL. Standard treatment with tyrosine kinase inhibitors (TKI) in the chronic phase (CP) of CML conveys excellent long-term prognosis but is associated with side effects and costs. Treatment free remission (TFR) is possible in a proportion of patients discontinuing treatment after obtaining deep treatment responses but it is not fully known how to select the right patients for stopping attempts. Treatment of accelerated phase (AP) and blast crisis (BC) is more complicated and the prognosis more dismal. In this thesis, we have studied factors of importance for outcome in CML patients with focus on immunological factors and clinical management.

In a cohort of 32 newly diagnosed CP-CML patients, evidence of active immune escape mechanisms were found. These declined with the course of TKI treatment and at the same time, effector lymphocyte responses were elicited. These anti-leukaemia immune responses might help in the long-term control of CML. Multiple plasma protein markers were also measured with three multiplex platforms in a smaller cohort of patients (n=14). Inflammatory cytokines and other plasma proteins were affected by TKI treatment and multiplexing seems useful for finding potential biomarkers with biologic or prognostic significance in CML.

Patients progressing to AP/BC were studied in a population-based material from the Swedish CML register. Approximately 4% of TKI-treated CP-CML patients transformed to AP/BC within 2 years of diagnosis. Monitoring of treatment responses was suboptimal in 1/3 of these patients and the median survival was 1.4 years after diagnosis of AP/BC. Thus, minimising the risk of disease progression through strict adherence to guidelines for monitoring and treatment is essential.

In a cohort of patients (n=50) discontinuing TKI treatment within a large European trial, musculoskeletal pain was reported by 30% of patients, starting within 1-6 weeks of TKI discontinuation and spontaneously resolving over time in most cases. Patients (n=56) were also evaluated with a multiplex platform with a total of 162 inflammation- and cancer-related plasma proteins. No predictive protein biomarkers for successful TKI discontinuation could be found. However, profound effects of TKI-treatment were seen and plasma proteomics could be useful for understanding effects of long-term TKI-treatment.

Keywords: chronic myeloid leukaemia, accelerated phase, blast crisis, tyrosine kinase inhibitor, immunology, myeloid-derived suppressor cells, cytokines, proteomics, TKI discontinuation, population-based

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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.


III. Stina Söderlund, Lisa Christiansson, Inger Persson, Henrik Hjorth-Hansen, Johan Richter, Bengt Simonsson, Satu Mustjoki, Ulla Olsson-Strömberg, Angelica Loskog (2016) **Plasma proteomics in CML patients before and after initiation of tyrosine kinase inhibitor therapy reveals induced Th1 immunity and loss of angiogenic stimuli.** *Leukemia Research* 50, 95-103

IV. Stina Söderlund, Torsten Dahlén, Fredrik Sandin, Ulla Olsson-Strömberg, Maria Creignou, Arta Dreimane, Anna Lübking, Berit Markevärn, Anders Själander, Hans Wadenvik, Leif Stenke, Johan Richter, Martin Höglund (2017) **Advanced phase chronic myeloid leukaemia (CML) in the tyrosine kinase inhibitor era – a report from the Swedish CML register.** *European Journal of Haematology* 98(1); 57-66

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## Contents

Introduction ..................................................................................................... 9  
Chronic myeloid leukaemia........................................................................ 9  
  Epidemiology and aetiology ........................................................................ 10  
  Pathophysiology ..................................................................................... 10  
  The leukaemic stem cell ....................................................................... 11  
  Phases of the disease ........................................................................... 11  
  Diagnosis and definitions .................................................................... 12  
  Prognostic factors and scores ............................................................... 14  
  Treatment ............................................................................................. 14  
Immunosurveillance .................................................................................. 21  
  T-cells ....................................................................................................... 22  
  NK cells .................................................................................................... 23  
  The ‘cancer-immunity cycle’ ................................................................ 23  
Imunoediting and immune inhibitory mechanisms ................................... 25  
  Tumour antigen presentation and activation of cytotoxic T-cells (Figure 3, steps 2 and 3) ............................................................................ 26  
  T-cell tumour infiltration (Figure 3, steps 4 and 5) ............................. 27  
  Recognition of tumour cells by T-cells (Figure 3, step 6) ................... 27  
  Cytotoxic killing of tumour cells (Figure 3, step 7) ............................ 27  
CML, Tyrosine kinase inhibitors and the immune system ....................... 30  
  Flow cytometry and multiplexing ............................................................ 31  
  The Swedish CML register .................................................................... 32  
Aims of the doctoral project ......................................................................... 34  
  Paper I ...................................................................................................... 34  
  Paper II ..................................................................................................... 34  
  Paper III .................................................................................................... 34  
  Paper IV ................................................................................................... 34  
  Paper V ..................................................................................................... 34
Materials and methods ................................................................. 35
  Paper I .......................................................................................... 35
  Papers II and III ........................................................................... 35
    Patient samples ........................................................................... 35
    Flow cytometry .......................................................................... 36
    Plasma analyses ......................................................................... 36
    Statistical analyses .................................................................... 37
  Paper IV .......................................................................................... 37
  Paper V .......................................................................................... 38

Results and discussion ................................................................. 39
  Musculoskeletal pain in patients with chronic myeloid leukaemia
  after discontinuation of imatinib: A tyrosine kinase inhibitor
  withdrawal syndrome? (Paper I) ..................................................... 39
  The Tyrosine kinase inhibitors imatinib and dasatinib reduce myeloid
  suppressor cells and release effector lymphocyte responses (Paper II)... 40
  Plasma proteomics in CML patients before and after initiation of
  tyrosine kinase inhibitor therapy reveals induced Th1 immunity and
  loss of angiogenic stimuli (Paper III) .............................................. 41
  Advanced phase chronic myeloid leukaemia in the tyrosine kinase
  inhibitor era (Paper IV) ................................................................. 42
  Proximity extension assay-based plasma proteomics cannot predict
  relapse in chronic myeloid leukaemia patients stopping treatment with
  tyrosine kinase inhibitors (TKI) but reveal profound effects of long-
  term TKI treatment on plasma protein profiles (Paper V) .............. 43

Conclusions ...................................................................................... 45
  Paper I .......................................................................................... 45
  Paper II .......................................................................................... 45
  Paper III .......................................................................................... 45
  Paper IV .......................................................................................... 45
  Paper V .......................................................................................... 46

Future perspectives ................................................................. 47

Svensk populärvetenskaplig sammanfattning ................................ 48

Acknowledgements ........................................................................ 51

References ....................................................................................... 53
Abbreviations

ALL Acute lymphoblastic leukaemia
Allo-HSCT Allogeneic haematopoietic stem cell transplantation
AML Acute myeloid leukaemia
AP Accelerated Phase
APC Antigen presenting cell
BC Blast crisis
BCR-ABL Breakpoint cluster region-Abelson gene
CD Cluster of differentiation
CML Chronic myeloid leukaemia
CP Chronic Phase
CTL Cytotoxic T-lymphocyte
CTLA Cytotoxic T-lymphocyte-associated protein
DC Dendritic cell
FISH Fluorescent in situ Hybridisation
HLA Human leukocyte antigen
IFN Interferon
IL Interleukin
KD Kinase domain
LSC Leukaemic stem cell
MDSC Myeloid-derived suppressor cell
MHC Major histocompatibility complex
MMR Major molecular response
PD1/PD-L1 Programmed death receptor 1/Programmed death receptor ligand 1
PDGFR Platelet-derived growth factor receptor
Ph Philadelphia chromosome
ROS Reactive oxygen species
TGF Transforming growth factor
TKI Tyrosine kinase inhibitor
TNF Tumour necrosis factor
Treg T-regulatory cell
STAT Signal Transducers and Activators of Transcription
VEGF Vascular endothelial growth factor
Introduction

Chronic myeloid leukaemia

Chronic myeloid leukaemia (CML) is a myeloproliferative neoplasm characterised by uncontrolled proliferation of immature myeloid cells in the bone marrow and other organs such as the spleen. Clinically, CML is characterised by a marked leukocytosis of the blood due to neutrophils in different stages of maturation. Basophilia is also present and eosinophilia is common. There can be a slight increase in blast cells and thrombocytosis is often present. The bone marrow is hypercellular due to granulopoiesis in different stages of maturation (Figure 1).¹ Most patients are diagnosed in chronic phase (CP), but a small proportion are diagnosed in advanced disease phases (accelerated phase, AP; blast crisis, BC).² Patients presenting in CP are often asymptomatic and CML is then suspected when a high white blood cell count is found upon blood sampling for other causes such as routine medical examinations. However, most patients present with slowly increasing symptoms such as fatigue, weight loss, night sweats, abdominal fullness due to splenomegaly, skeletal pain, bleeding and anaemia.³ On clinical examination, splenomegaly and hepatomegaly may be present as well as purpura.³ In rare cases, hyperleukocytosis can give rise to leukostasis with symptoms such as dyspnoea/cyanosis, dizziness and confusion, visual disturbances and priapism due to decreased tissue perforation.⁴

Figure 1. The bone marrow in CML is hypercellular with granulopoiesis in different stages of maturation. Courtesy of Rose-Marie Amini.
Epidemiology and aetiology
The disease accounts for 15% of adult leukaemias and an incidence of 1-2/100,000 has been described worldwide. The incidence of CML in Sweden was 0.8-1.0/100 000 during 2002-2014 (approximately 90 cases per year) and the median age at diagnosis was 60 years (range 17-95), with 17% of patients being younger than 40 years of age and 29% being 70 or older. There is a slight male predominance in the incidence of CML.

The causes of CML are essentially unknown and the only established risk factor is exposure to ionising radiation. There is no evidence of hereditary causes of CML. However, in a recent report from the Swedish CML register, an increased prevalence of other malignancies and autoimmune diseases were found prior to CML diagnosis, indicating a hereditary or acquired increased predisposition to cancer and autoimmunity in CML patients.

Pathophysiology
CML is caused by the Philadelphia chromosome (Ph, Figure 1), which is a result of a reciprocal translocation between the long arms of chromosomes 9 and 22 ((t9;22)(q34;q11))\(^{11,12}\), rendering a shortened chromosome 22. It is found in up to 95% of CML patients and in 15-30% of patients with adult acute lymphocytic leukaemia (ALL)\(^{13}\). The Ph is a translocation of a 3’ segment of the Abelson (ABL) gene on chromosome 9q34 to the 5’ part of the Breakpoint Cluster Region (BCR) gene on chromosome 22q11, resulting in a fusion gene called BCR-ABL\(^1\). There are various breakpoints in the BCR gene and depending on which ones are involved in the translocation, different sized segments from BCR are fused with the ABL gene, rendering mRNA of different lengths that encode chimeric proteins with different molecular weights. The most common fusion variants in CML are b2a2 and b3a2, both encoding a protein of 210 kilo Dalton (kd) called p210\(^{BCR-ABL}\) or BCR-ABL major. In rare cases of CML, but in most cases of Ph positive ALL, the fusion protein is encoded by the e1a2 gene fusion, resulting in a smaller protein of 190 kd called p190\(^{BCR-ABL}\) or BCR-ABL minor. A third breakpoint variant, e19a2, gives rise to a protein of 230 kd called p230\(^{BCR-ABL}\) or BCR-ABL micro. This third fusion variant has been described in rare cases of CML.\(^5\) The fusion protein encoded by the BCR-ABL\(^1\) gene is a constitutively active tyrosine kinase.\(^{14}\) Under normal conditions, ABL has tyrosine kinase activity that is strictly regulated and has an important role in controlling cell proliferation and apoptosis as well as interactions between bone marrow stroma and hematopoietic progenitor cells. When fused with BCR, ABL is activated and as a consequence, malignantly transformed cells will proliferate more, have reduced apoptotic capacity and decreased adhesion to bone marrow stroma.\(^{15}\)

It is thought that the Ph arises due to genomic instability in haematopoietic progenitor cells, but the activity of the BCR-ABL kinase also further enhances
genomic instability, possibly through the generation of reactive oxygen species (ROS) that enhance oxidative DNA-damage. This genomic instability is thought to explain secondary drug resistance and transformation to AP and BC. Among the genes that have been found to be mutated in BC are several well-known oncogenes such as *p53*, *EVI-1*, *RB*, *MYC* and *p16*.17

![Image](image_url)

**Figure 2.** The Philadelphia chromosome results from a reciprocal translocation between the long arms of chromosomes 9 and 22.

**The leukaemic stem cell**

During recent years, CML research has been highly focused on the leukaemic stem cells (LSCs, defined as Ph+CD34+CD38- cells18) due to their resistance to tyrosine kinase inhibitors (TKIs). LSCs differ from normal haematopoietic stem cells due to BCR-ABL activity that modulates various signalling pathways, leading to survival advantages for LSCs and the ability to evade apoptosis during drug treatment.19 Presence of *BCR-ABL1* expressing LSCs in bone marrow has been described in patients with deep molecular response for several years and are certainly the origin of relapse after TKI cessation.20

**Phases of the disease**

CML in its natural course develops in three phases: the chronic phase (CP), the accelerated phase (AP) and blast crisis (BC). The CP can last for several years and is characterised by an excess of myeloid precursors and mature cells in the bone marrow, blood and extra-medullary sites such as the spleen. The cells in this phase still have a normal differentiation capacity and function. However, due to increased genomic instability as described above, the disease progresses to AP, which is characterised by increasing numbers of immature
cells and basophils in the blood and acquired secondary genetic alterations. The AP lasts several months and is followed by the BC in which the cells have lost their ability to differentiate, thus leading to accumulation of blast cells in bone marrow and blood resembling acute leukaemia with short survival. BC morphology is most often myeloid (60-80% of cases) but can also be lymphoid (20-30%) and is rarely also undifferentiated or biphenotypic, reflecting the fact that CML arises from a multipotent, early haematopoietic progenitor cell.

Diagnosis and definitions

The diagnosis of CML is suspected from the typical findings in blood and bone marrow as described above. However, definitive diagnosis requires demonstration of the Ph chromosome and/or BCR-ABL1 through cytogenetic analysis and RT-PCR or fluorescence in situ hybridisation (FISH), respectively. In about 5% of CML patients, the typical t(9;22) is not present, and in these cases FISH analysis can detect fusion variants that are not seen with conventional cytogenetics. Karyotyping is done on bone marrow samples whereas FISH and RT-PCR can be performed on both blood and bone marrow samples.

The definitions of CP, AP and BC are presented in Table 1. There are two sets of criteria currently applied: the World Health Organization (WHO) criteria, which were updated in 2016, and the European Leukaemia Net (ELN) criteria. They mainly differ in blast cell counts with the WHO criteria being slightly more “strict”. The WHO criteria are the most commonly applied in Sweden. In a study by Cortes et al., the WHO staging system was compared to the ELN criteria with regard to outcome in imatinib-treated patients. A total of 10% of patients were classified differently between the two staging systems and among patients who were classified as BC according to WHO criteria, those with a blast percentage of 20-29% (i.e., AP according to ELN criteria) had a significantly better response rate (21% vs 8%) and 3-year survival rate (42% vs 10%) than those with blasts ≥30%.

In the 2016 WHO update, some provisional “response to TKI” criteria have been added to the definition of AP. These include hematologic resistance to the first TKI, any (haematological, cytogenetic or molecular) indications of resistance to two sequential TKIs and occurrence of two or more BCR-ABL1 mutations during TKI therapy.
**Table 1. Definitions of CP, AP and BC.**

<table>
<thead>
<tr>
<th>Chronic phase</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELN criteria</td>
<td>Blasts in bone marrow &lt;15% and not fulfilling any criteria for AP/BC</td>
</tr>
<tr>
<td>WHO criteria</td>
<td>Blasts in bone marrow &lt;10% and not fulfilling any criteria for AP/BC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Accelerated phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELN criteria</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
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<tr>
<td>WHO criteria</td>
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<table>
<thead>
<tr>
<th>Blast crisis</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELN-criteria</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>WHO-criteria</td>
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<td></td>
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</tbody>
</table>

*Definition: Trisomy 8, 2nd Ph+ [+der(22)(t9;22)(q34;q11)], isochromosome 17 [i(17)(q10)], trisomy 19 and i der(22)(q10)t(9;22)(q34;q11). According to WHO complex karyotype and abnormalities of 3q26.2 are also included.
Prognostic factors and scores

There are several prognostic scores applied at diagnosis to stratify patients according to their risk of disease progression. Also, baseline factors not included in any of the scores and factors related to treatment and treatment response are of importance for prognosis.

The prognostic scores used are the Sokal, Hasford and EUTOS scores, and recently developed, the EUTOS long term survival (ELTS) score. The Sokal score from 1984 was originally developed for patients treated with busulfan and hydroxyurea and divides patients into three risk groups (low, intermediate and high) according to survival. It is dependent on patient age, spleen size, platelet count and percentage of blasts in blood. The Hasford score was developed in 1998 for patients treated with interferon (IFN) alpha and also divides patients into three risk groups according to survival. It utilises the variables age, spleen size, blast count, eosinophil count and basophil count. A score for imatinib-treated patients was developed in 2011. This so-called EUTOS score uses the percentage of basophils in blood and spleen size to discriminate between low and high risk patients with regard to the probability of reaching a complete cytogenetic response at 18 months. The ELTS score was published in January 2016 and is also developed for imatinib-treated patients and divides patients into three risk groups based on the probability of dying from CML related causes. It is based on age, spleen size, blast-counts and platelet-counts.

At diagnosis, the presence of so called additional chromosomal aberrations (ACAs; i.e., genetic alterations other than the Ph) are also known to have prognostic significance. Response related factors with prognostic significance include BCR-ABL1 >10% at 3 months, failure to achieve a complete cytogenetic response (CCyR) at 12 months and clonal cytogenetic evolution during treatment. ACAs appear in a non-random fashion and the most common changes are called “major route” ACAs. These include trisomy 8, additional Ph, isochromosome 17 and trisomy 19.

Treatment

History

The first cases of CML were described in the middle of the 19th century. Treatment with arsenic was tried and could control symptoms but not prolong life. In the beginning of the 20th century, radiation therapy was used for controlling symptoms. In the 1950s, orally administered busulfan was introduced and in the 1970s hydroxyurea became an alternative. Both of these drugs were efficient in restoring blood parameters to normal, but neither could prolong the time to transformation. In the beginning of the 1980s, allogeneic haematopoietic stem cell transplantation (allo-HSCT) became the treatment of
choice for younger patients with suitable donors\textsuperscript{40} and around the same time, IFN alpha was shown to induce cytogenetic responses in some patients.\textsuperscript{41} Subsequently, the combination of IFN alpha and cytarabine showed superior survival to IFN alpha alone.\textsuperscript{42} During the 1990s, autologous stem cell transplantation was also performed in patients not eligible for allo-HSCT.\textsuperscript{43} Research from the 1960s and until the late 1990s led to the discovery of BCR-ABL as an ideal target for CML therapy since it is expressed in all patients with CML and is the cause of the disease.\textsuperscript{22} In 1998, the first phase 1 study with a BCR-ABL inhibitor (imatinib, Glivec\textsuperset{\textregistered}) began.\textsuperscript{44} Imatinib was well tolerated and showed remarkable effect in IFN alpha pre-treated patients and the first randomised trial (the IRIS trial) began in June 2000.\textsuperscript{45}

Response

International and national guidelines have been developed for treatment with TKIs in CML. Monitoring of treatment results with cytogenetics and qRT-PCR is essential since this is the most important prognostic factor\textsuperscript{37}. The different definitions of treatment response are presented in table 2 and treatment goals for first line treatment with a TKI at different time points are presented in Table 3. Responses should be classified according to the treatment goals as optimal, treatment failure and warnings. Treatment failure should lead to change of therapy guided by BCR-ABL1 mutation analysis and warnings should prompt more frequent monitoring.\textsuperscript{25,37} The significance of deeper molecular responses other than major molecular response (MMR) has not yet been completely clarified, but since TKI cessation has been possible in a proportion of patients with sustained complete molecular response (CMR),\textsuperscript{46,47} these endpoints are becoming increasingly important.

The mechanisms behind poor response and drug resistance are not fully understood, but what is of clear importance is the emergence of kinase domain (KD) mutations in the BCR-ABL1 leading to selection of mutant clones that are insensitive to TKI treatment.\textsuperscript{17} Today, more than 50 BCR-ABL1 mutations have been described\textsuperscript{48} and to circumvent resistance, so-called second (dasatinib, nilotinib, bosutinib) and third (ponatinib) generation TKIs have been developed. The second generation TKIs (2G-TKIs) are effective against most imatinib-resistant mutations except for the T315I, for which only the third-generation TKI ponatinib is effective.
Table 2. Response definitions in CML.

<table>
<thead>
<tr>
<th>Sample modality</th>
<th>Response Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Haematology</strong></td>
<td>Complete Haematological Response (CHR)</td>
</tr>
<tr>
<td></td>
<td>Normal peripheral blood counts including differential count and normal spleen size</td>
</tr>
<tr>
<td><strong>Cytogenetics</strong></td>
<td>Minimal Cytogenetic Response (CyR)</td>
</tr>
<tr>
<td>Minor CyR</td>
<td>Ph+ 36-65%</td>
</tr>
<tr>
<td>Partial CyR</td>
<td>Ph+ 1-35%</td>
</tr>
<tr>
<td>Complete CyR</td>
<td>Ph+ 0%</td>
</tr>
<tr>
<td><strong>Molecular genetics</strong></td>
<td>Major molecular response (MMR)</td>
</tr>
<tr>
<td>MR4.0</td>
<td>( BCR-ABL IS \leq 0.01%</td>
</tr>
<tr>
<td>MR4.5</td>
<td>( BCR-ABL IS \leq 0.0032%</td>
</tr>
<tr>
<td>Complete molecular response (CMR)</td>
<td>( BCR-ABL ) not detectable</td>
</tr>
</tbody>
</table>

*The % of Ph+ metaphases with cytogenetic examination. At least 20 metaphases should be evaluated.

Table 3. Treatment goals for first line treatment with a TKI.

<table>
<thead>
<tr>
<th>Optimal response</th>
<th>Warning</th>
<th>Treatment failure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td>High risk or CCA/Ph+ (major route</td>
<td></td>
</tr>
<tr>
<td><strong>3 months</strong></td>
<td>( BCR-ABL1 \leq 10% and/or Ph+ \leq 35%</td>
<td>( BCR-ABL1 &gt;10% and/or Ph+ 36-95%</td>
</tr>
<tr>
<td><strong>6 months</strong></td>
<td>( BCR-ABL1 &lt;1% and/or Ph+ 0%</td>
<td>( BCR-ABL1 1-10% and/or Ph+ 1-35%</td>
</tr>
<tr>
<td><strong>12 months</strong></td>
<td>( BCR-ABL1 \leq 0.1%</td>
<td>( BCR-ABL1 &gt;0.1-1%</td>
</tr>
<tr>
<td><strong>At any time</strong></td>
<td>( BCR-ABL1 \leq 0.1%</td>
<td>CCA/Ph- (-7 or 7q-)</td>
</tr>
</tbody>
</table>

CCA/Ph+: clonal chromosomal abnormalities in Ph+ cells; CCA/Ph-: clonal chromosomal abnormalities in Ph- cells. Monitoring during first line treatment should be done by cytogenetic analysis at 3, 6 and 12 months or until achievement of a CCyR. RT-PCR for monitoring of \( BCR-ABL1 \) levels should be done every third month until achievement of MMR \( (BCR-ABL IS \leq 0.1\% ) \) and thereafter every sixth month.
Tyrosine kinase inhibitors used in CML

**Imatinib** was the first BCR-ABL inhibitor on the market and it was registered in Sweden in 2001. It binds to the inactive conformation of BCR-ABL and upon binding, blocks the ATP-binding site of the kinase, thereby inhibiting the phosphorylation of substrates. It also inhibits the activity of platelet-derived growth factor receptor (PDGFR) and Kit, but has no effect on Src-family kinases.22

The standard dose of imatinib is 400 mg once daily and it is approved for treatment of all disease phases. The IRIS-trial was the first randomised trial comparing imatinib to current standard treatment, which was then IFN alpha plus cytarabine for patients in CP.49 The six-year follow up of the IRIS trial was published in 2009 and showed an estimated event-free survival of 83%, freedom from progression to AP or BC of 93% and an overall survival of 88% for imatinib-treated patients.50 The recently published ten-year follow up showed a persisting effect over time with an estimated overall survival rate of 83.3% in the imatinib group.51 Studies of imatinib as single-agent therapy in BC show much more unsatisfactory figures with 12-month overall survival rates ranging between 22 and 36%.52

Common side effects of imatinib are (apart from hematological toxicity, which can be seen with all TKIs) oedema (peripheral and periorbital), gastrointestinal symptoms (nausea, abdominal pain and diarrhoea), muscle cramps, fatigue and rash. More severe side effects are rare.53

**Nilotinib** is closely related to imatinib and was developed to overcome imatinib resistance. It also binds to the inactive conformation of BCR-ABL, but has a more than 20-fold higher binding affinity. It also inhibits PDGFR (A and B) and Kit as well as Ab12. Nilotinib shows activity against most known *BCR-ABL1* mutations but the T315I mutation is highly resistant54 and some additional mutations with decreased sensitivity to nilotinib have also been described (Y253H, E255K/V, F359V/C/I).55

The standard dose of nilotinib is 300 mg twice daily for first line treatment and 400 mg twice daily for second line treatment of patients resistant to their previous TKI. Nilotinib is approved since 2007 for CML patients in CP or AP resistant or intolerant to imatinib and as first line treatment for CP patients. Data from the ENESTnd, comparing treatment with nilotinib and imatinib in newly diagnosed CP patients, showed significantly higher molecular response rates in nilotinib-treated patients and a lower risk of progression. Overall survival after three years was 95.1-97.0% for nilotinib-treated patients and 94.0% in the imatinib cohort.56 Also with nilotinib, treatment results in BC are much more dismal.52

Frequently occurring side effects of nilotinib comprise skin rashes, headaches, elevated liver enzymes and oedema. QT-prolongation can be seen. Also, metabolically relevant side effects are seen, including hypertension, elevated blood glucose levels and hypercholesterolemia.25,56 Among the serious
side effects described are pancreatitis, ischaemic heart disease and peripheral arterial disease. These observations have led to the recommendation not to use nilotinib as first line treatment in patients with cardiovascular disease or significant risk factors for such disease.

**Dasatinib** was originally synthesised as a Src-inhibitor, but was found to have potent activity against BCR-ABL. It binds to the active conformation of BCR-ABL and also has effects on Kit, PDGFR, Src-kinases and other kinases. It is much more potent than imatinib *in vitro* and has activity against most BCR-ABL1 mutations except the T315I which is highly resistant and the V299L, T315A, and F317L/V/I/C which have a decreased sensitivity.

The standard dose of dasatinib is 100 mg once daily. Dasatinib is approved since 2006 for second line treatment of CML (CP, AP and BC) patients resistant or intolerant to first line treatment and for first line treatment in CP. The DASISION trial comparing dasatinib with imatinib in newly diagnosed CP patients showed higher MMR rates in dasatinib treated patients (64% vs 46% at 24 months) and a lower progression rate to AP or BC (2.3% vs 5.0%). Dasatinib as a single agent in treatment of BC has shown 12-month survival rates between 22 and 49%.

Side effects include fatigue, gastrointestinal symptoms, headache and QTc prolongation. A more specific dasatinib-induced side effect is pleural effusion, which can be seen in around 20% of patients. Also, pulmonary hypertension has been described.

**Bosutinib** is a dual Src/Abl inhibitor which inhibits BCR-ABL more potently than imatinib *in vitro*. It has activity against all but two known imatinib-resistant BCR-ABL1 mutations (T315I and V299L).

The standard dose of bosutinib is 500 mg once daily. It is registered since 2013 for patients in CP, AP and BC who have tried at least one other TKI and for whom treatment with any of the above mentioned TKIs is not an option. Patients treated with bosutinib as second line therapy after imatinib resistance or intolerance had 2-year progression-free and overall survival of 81% and 91%, respectively while corresponding figures for bosutinib as third line treatment were 73% and 83%. Bosutinib has also been compared to imatinib as first line treatment of CP patients in the BELA trial. Bosutinib treated patients had a higher MMR rate at 12 months (41% versus 27%) but no difference in the CCyR rate was seen.

Gastrointestinal side effects are the most common for bosutinib, with diarrhoea being most pronounced. Elevation of liver enzymes is commonly seen and should be monitored.

**Ponatinib** is the only TKI with activity against the T315I BCR-ABL1 mutation. It also has activity against other clinically relevant mutants as well as off-target effects on Src, VEGF, PDGFR, Kit, Flt3 and other kinases.

The standard dose is not definitively determined, but doses between 15 and 45 mg once daily are being studied. Ponatinib has been approved since 2013 for use in CP for patients intolerant or resistant to second line treatment or...
with the T315I mutation. Ponatinib used in this population of patients induced complete cytogenetic responses in 46% of CP patients at 12 months and responses were also seen in AP and BC patients (CCyR 24% and 18% at 6 months, respectively).69

A concerning issue with ponatinib is the high prevalence of cardiovascular side effects described. In the phase 2 trial referred to above, 7.1% had cardiovascular events, 3.6% had cerebrovascular events and 4.9% had peripheral vascular events.69 Therefore, patients should be evaluated for cardiovascular diseases and risk factors before the start of treatment and patients with previous stroke or ischaemic heart disease should preferably not be treated with ponatinib.

TKI stopping trials

Until recently, TKI treatment has been considered lifelong. However, in recent years, several studies have emerged that demonstrate the feasibility and safety of TKI cessation in CML patients responding well to treatment. Although still considered experimental in most recommendations, TKI cessation is likely to become part of clinical routine care for a proportion of CML patients in the near future. TKI cessation has possible benefits both for individual patients with reduction of side effects and negative effects of long-term treatment such as cardiovascular events and for society with reduction of healthcare costs.

The TKI stopping trials published to date have used somewhat different criteria both for inclusion and for definition of molecular relapse. The first published stopping trial of a larger cohort of imatinib-treated patients was the French STIM study46 in which patients were eligible for TKI cessation if they had been treated with IFN alpha followed by imatinib ≥3 years and had CMR for ≥2 years. Relapse was defined as a positive qRT-PCR result (≥10^{-5}) in two successive assessments. The rate of treatment-free remission (TFR) was 39% after a median follow-up of 55 months. The According-to-STIM (A-STIM) trial70 used the same inclusion criteria as in the STIM study, but some occasional weakly PCR-positive samples were permitted. Relapse was defined as loss of MMR and the TFR rate was 64% at 23 months follow-up. In the Australian TWISTER study47, patients were included if they had been treated with imatinib for ≥3 years and had unmeasurable minimal residual disease for ≥2 years. Relapse was defined as loss of MMR at one occasion or any measurable levels of BCR-ABL1 in two consecutive samples. The TFR rate was 45% at 42 months. In the Italian ISAV study71, patients were eligible for inclusion if they had been treated with imatinib for ≥2 years and had been in CMR for ≥18 months. Relapse was defined as loss of MMR and 51.9% were in TFR after 36 months. Several 2G-TKI stopping trials are in progress, but only a few results have been presented so far. An interim analysis from the French STOP 2G-TKI study showed that patients treated with dasatinib or nilotinib for ≥3 years and in MR4.5 for ≥2 years had a TFR rate of 54% at 48 months.72 The
Japanese DADI trial included patients who had achieved deep molecular remission with dasatinib second line and showed a TFR rate of 49% at six months. The largest TKI stopping trial to date is the pan-European “Europe stops tyrosine kinase inhibitor trial” called “EURO-SKI” (ClinicalTrials.gov identifier: NCT01596114). EURO-SKI has included over 800 patients treated with imatinib, dasatinib or nilotinib for ≥3 years. The response requirement before inclusion is somewhat less stringent than in previous studies; MR<sub>4.0</sub> for ≥1 year, and relapse is defined as loss of MMR. Preliminary results from 717 patients showed molecular relapse-free survival of 56% at 12 months and 51% at 24 months.

In the EURO-SKI trial, the clinical factors that were of importance for a successful TKI stop were the duration of TKI treatment and MR<sub>4.0</sub> at stop. Treatment duration was also identified as an independent prognostic factor in the STIM study. Both the STIM and TWISTER studies identified Sokal risk group as a predictor, with the low risk group having a higher probability of remaining in TFR. Previous IFN-treatment was also of importance for a successful stop in the latter two studies.

In total, more than 2,000 patients have now stopped TKI treatment within different stopping studies. All patients who have relapsed have responded to TKI re-initiation and no <i>BCR-ABL1</i> mutations have been described. There has only been one reported progression to BC, which occurred in a patient who lost MMR and restarted imatinib treatment. The patient had regained MMR and lymphoid BC occurred 8.5 months after restarting TKI treatment.

**Treatment recommendations**

The most recent Swedish guidelines are based on clinical trials and data from the Swedish CML-register and is in accordance with European guidelines from the ELN. For CP, the recommended first line treatment is imatinib for patients who are Sokal low or intermediate risk and nilotinib for Sokal high risk (if no cardiovascular disease). In the case of treatment failure, second line treatment with a second or third generation TKI is recommended and the choice of substance should be guided by the analysis of KD-mutations. At diagnosis, hydroxyurea can be used as cytoreductive therapy in cases with high white blood cell counts (WBCs) and in patients with symptoms related to leukostasis, leukapheresis is used to quickly reduce leukocyte counts.

Since December 2016, generic imatinib is available in Sweden at a fraction of the cost of branded imatinib. Experiences from Poland show that the efficacy and safety of imatinib generics are not inferior to branded imatinib.

Treatment of patients in advanced disease phases is more complicated and the prognosis is much more dismal than in CP. Generally, patients in AP should be treated with second generation TKIs and should be considered for allo-HSCT if they do not respond optimally to TKI treatment. Patients in BC who have a suitable donor should be transplanted as soon as possible after disease stabilisation with second generation TKIs and chemotherapy.
choice of TKI should be guided by KD-mutation analysis in both AP and BC.²⁵

**Allogeneic stem cell transplantation**

Until the discovery of TKIs, allo-HSCT was the treatment of choice for younger patients with a suitable donor also in CP. Nowadays, allo-HSCT is performed in patients with BC who have a suitable donor and respond to TKI and/or chemotherapy and in cases of AP who do not respond optimally to TKI treatment. Also, allo-HSCT should be considered for patients responding poorly to second line treatment and for patients with the T315I mutation.

There are two recent publications studying the outcome of patients transplanted in the TKI era. The German CML study group prospectively randomised patients eligible for transplantation to allo-HSCT or the best available drug treatment depending on the availability of a matched related donor. Fifty-two percent of patients in the best available drug treatment group were treated with a TKI (imatinib was introduced during the study period) and 50% underwent allo-HSCT with a matched unrelated donor. The primary end point was long-term survival and did not differ between the two groups at 10 years (0.76 vs 0.69; patients in the drug treatment group who underwent allo-HSCT were censored at the day of transplant).⁷⁸ In a register report from the EBMT chronic malignancies working party⁷⁹, low risk transplants (i.e., transplants in first CP with HLA identical sibling donors and EBMT risk scores⁸⁰ of 0, 1 or 2) were studied with respect to survival. This group corresponded to 6% of all allo SCTs performed in Europe during the follow up period. Survival at 5 years was 85%, which could support choosing allo-HSCT over third generation TKI treatment in patients with low transplant risk and poor response to second line treatment.

Allo-HSCT was, until recently, considered the only potentially curing therapy option in CML, but studies are emerging showing that a proportion of patients are cured using TKI therapy only as described above. However, there is clinical evidence of a potent graft-versus-leukaemia (GvL) effect in CML such as a lower relapse rate after allo-HSCT in patients who develop graft-versus-host disease (GvHD), regression of leukaemia relapse after donor lymphocyte infusions (DLI), and higher relapse rates after allo-HSCT with T-cell depleted bone marrow.⁸¹ Thus, it is plausible that the immune system plays a role in controlling disease in CML and is therefore of interest to study further.

**Immunosurveillance**

The idea that the immune system can control cancer was formed in the late 19th century and the so-called cancer immunosurveillance theory was proposed by Burnet and Thomas in the 1950s.⁸²,⁸³ This theory postulates that
adaptive immunity is capable of preventing tumour development in immuno-
competent individuals. Further evidence for this theory came in the 1990s
when mice lacking IFNγ responsiveness or adaptive immunity were found to
be more susceptible to tumour formation.84

Central to the immunosurveillance theory is the fact that cancer cells ex-
press antigens that differentiate them from their normal, non-transformed,
counterparts and they can thus be recognised by the immune system as ‘for-
eign’. These so-called tumour antigens include differentiation antigens (re-
lated to the tumour’s tissue of origin), mutational antigens, overexpressed cel-
lar antigens, viral antigens and cancer/testis antigens.85 Several types of im-
mune cells are involved in anti-cancer immunity, but most central to the pro-
cess are the T-cells and natural killer (NK) cells. T-cell infiltration in tumours
is a prognostically favourable sign in several tumour types.86–88

T-cells

T-cells or T-lymphocytes, belong to the adaptive immune system. They are
central players in immune responses and function by secreting soluble medi-
ators or by cell-to-cell contact. There are two main types of T-cells: cytotoxic
T-lymphocytes that express CD8 glycoprotein on their surface (CTLs, CD8+)
and helper T-cells that express CD4 glycoprotein (Th cells, CD4+). CTLs
function by killing pathogen-infected cells and tumour cells (described in
more detail below) while Th cells are important for directing the immune re-
response. They differentiate into different subsets depending on which cyto-
kines are present and different subsets of Th cells are in turn characterised by
specific cytokine profiles. The first Th subsets to be described were Th1 and
Th2 subsets that form in the presence of interleukin (IL)12 and IFNγ and IL4,
respectively.89,90 Th1 subsets release pro-inflammatory cytokines IFNγ and tu-
mour necrosis factor (TNF) β and are important in immune responses against
intracellular pathogens and in tumour immunity by regulating the develop-
ment and persistence of CTLs and macrophages.91 Th2 subsets release IL4,
IL5, IL10 and IL13 and are important in the defence against extracellular path-
ogens through activation of eosinophils and promotion of antibody re-
sponses.92 Traditionally, Th2-mediated immunity has been considered to pro-
mote tumour growth by promotion of angiogenesis and inhibition of cell-me-
diated cytotoxicity. However, anti-tumorigenic properties of Th2-mediated
immunity have also been described, e.g., supporting tumour infiltrating eosin-
ophils.93 Other Th subsets that have been described are Th9, Th17, Th22, T-
folicular helper (fh) and T-regulatory cells (Tregs).94 Tregs are described in
more detail below.

After maturation in the thymus, T-cells enter the circulation and secondary
lymphoid organs as naïve, antigen-inexperienced, T-cells. Upon antigen en-
counter, naïve cells proliferate and differentiate into effector cells (CTLs and
Th cells), which take part in the inflammatory response in peripheral tissues. After resolution of the inflammatory reaction, a small fraction of the cells remain as long-lived memory T-cells which, upon antigen re-encounter, can undergo fast expansion resulting in rapid clearance of their targets such as virally infected cells.\textsuperscript{95} The two main subtypes of memory cells are central (T\textsubscript{CM}) and effector (T\textsubscript{EM}) memory cells. T\textsubscript{CM} are located mainly in the secondary lymphoid organs while T\textsubscript{EM} cells reside in the site of the recent inflammatory response. Effector and memory T-cells are both antigen experienced cells and can be differentiated from each other and from naïve cells by their expression of surface markers. CD45RA is expressed on both naïve and effector cells but is lost on T\textsubscript{CM} and T\textsubscript{EM} cells which instead gain expression of CD45RO. Further, C-C chemokine receptor 7 (CCR7) and CD62L (markers for lymph node homing) can be used to discriminate between T\textsubscript{CM} (CCR7\textsuperscript{+} and CD62L\textsuperscript{+}) and T\textsubscript{EM} (CCR7\textsuperscript{-} and CD62L\textsuperscript{-}) cells as well as naïve (CCR7\textsuperscript{+} and CD62L\textsuperscript{+}) and effector T-cells (CCR7\textsuperscript{-} and CD62L\textsuperscript{-}).\textsuperscript{96}

NK cells

NK cells are large granular lymphocytes that belong to the innate immune system. They express the surface marker CD56, but lack expression of the T-cell marker CD3. NK cells participate in immune surveillance by recognising and killing cells that lack MHC class I (the ‘missing self’ hypothesis\textsuperscript{97}). This is often the case in malignantly transformed cells as described below. However, NK cell function is not only dependent on missing MHC class I, but is rather dependent on both inhibitory and activating signals. NK cells carry inhibitory receptors such as killer immunoglobulin like receptors (KIRs) and activating receptors such as natural killer group 2D (NKG2D). Cytolysis requires a balance between the signalling systems. KIRs interact with classical MHC class Ia ligands (HLA-A, -B, -C) and NKG2D interacts with at least six different ligands with MHC class I homology that are induced by cellular stress such as malignant transformation.\textsuperscript{98} Thus, when MHC class I is down regulated and NKG2D is expressed as in some malignantly transformed cells, NK cell cytotoxicity can be activated and the malignant cell can be killed.

The ‘cancer-immunity cycle’

The ‘cancer-immunity cycle’, proposed by Chen at al. describes how specific anti-cancer T-cell immune responses are elicited (Figure 3).\textsuperscript{99} First, tumour antigens are released from dead cancer cells and captured by dendritic cells (DCs; a type of APC). DCs process the antigens and present them on MHC class I and II molecules to T-cells (class I and class II for CD8\textsuperscript{+} and CD4\textsuperscript{+} T-cells, respectively) in regional lymph nodes. For DCs to become efficient
APCs that stimulate CTL activation, maturation stimuli are required. These include pro-inflammatory cytokines (TNFα, IL1, IFNα), CD40 signalling, and signalling through toll-like receptors (TLRs). The interaction between co-stimulatory surface molecules CD80 and CD86 on DCs and CD28 on T-cells is also required for CTL activation. If an antigen is presented to T-cells without co-stimulation, antigen-presentation instead becomes tolerogenic to T-cells. Further, CTL activation requires the presence of IL2 and IL12 (secreted by Th cells and DCs, respectively). Activated CTLs are then directed to the tumour site by chemokines such as CXCL 9 and 10. The T-cells extravasate through the endothelium after interactions with intercellular adhesion molecule (ICAM)1 and selectins. After tumour infiltration, CTLs recognise and bind to cancer cells through the interaction between the T-cell receptor (TCR) and tumour antigen bound to MHC class I. This interaction results in release of cytokines such as IFNγ and cytotoxic granules as well as upregulation of death receptors including Fas ligand (FASL). Ultimately, the interaction leads to caspase activation and hence, induction of apoptosis of the tumour cells with subsequent release of additional tumour antigens and propagation of the cancer-immunity cycle.
Immunoediting and immune inhibitory mechanisms

How is it then possible for tumours to develop in immunocompetent individuals? The concept of cancer immunoediting describes the immune control of developing tumours, taking into account that the immune system can have both protective and tumour-promoting functions. The immunoediting process consists of three phases: elimination, equilibrium and escape. In the elimination phase, the innate and adaptive immune system recognises and kills cancer cells at an early stage so that no clinical tumour arises. If cancer cells manage to survive the elimination phase, they enter the equilibrium phase, in which the adaptive immune system keeps tumour cells in a functional state of dormancy. However, due to a combination of changes in the tumour cells and tumour immunity, cancer cells may propagate to the escape phase with outgrowth of clinically relevant tumours.

The changes that occur in tumour cells (Figure 3, step 1) leading to immune escape involve loss of tumour antigen expression, either through down-regulation of MHC class I or emergence of tumour cells that lack strong tumour antigens, and increased resistance to cytotoxic effects of the immune system.
through induction of anti-apoptotic mechanisms. These changes are caused by genetic instability, which is characteristic of tumour cells and through a type of natural selection where the least immunogenic cells escape recognition by the immune system and continue growing.

As a tumour develops, several different mechanisms are operating to suppress tumour-specific immunity and this leads to the escape phase in which the tumour can grow despite the immune surveillance. Many of these suppressive functions are part of the physiological regulatory system that hinders the immune system from being over-active, thus preventing autoimmunity and shutting down immune responses after inflammatory reactions to infection or injury. Inhibitory mechanisms can come into play in all steps of the cancer-immunity cycle, affecting the efficiency of tumour antigen presentation and activation of CTLs, T-cell tumour infiltration, recognition of tumour cells by T-cells, and cytotoxic killing of tumour cells.

Tumour antigen presentation and activation of cytotoxic T-cells (Figure 3, steps 2 and 3)

Defective antigen presentation caused by impaired DC function is one of the most important factors of tumour-induced immune suppression. The DC defects observed in cancer are systemic and probably caused by abnormal differentiation of myeloid cells that also lead to the accumulation of immature myeloid cells called myeloid-derived suppressor cells (MDSCs). Factors secreted by tumours and cells in the tumour microenvironment such as vascular endothelial growth factor (VEGF), macrophage colony-stimulating factor (M-CSF), IL6, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL10, transforming growth factor (TGF) β and gangliosides inhibit the differentiation of myeloid progenitors to DCs. Also, maturation of DCs is impaired and when immature DCs present tumour antigen on MHC class I to T-cells in the absence of, or with low levels of, co-stimulatory molecules and cytokines, T-cell tolerance to the presented tumour antigen develops with continued tumour growth as a result.

VEGF and IL10 induce programmed death ligand-1 (PD-L1) expression on DCs. The receptor for PD-L1, programmed death 1 (PD1), is expressed on activated CD4+ and CD8+ T-cells as well as on B-cells, NKT-cells and monocytes. PD1 binds to the two ligands PD-L1 and PD-L2, the former being more widely expressed on both hematopoetic and other cell types such as endothelial and epithelial cells. The main function of the PD1/PDL1-interaction is to mediate peripheral tolerance, but it is also used for immune evasion by cancer cells through inhibition of effector T-cells. Expression of PD-L1 has been found in numerous solid tumour types and haematological malignancies.
Cytotoxic T-lymphocyte-associated protein 4 (CTLA4) is also a negative regulator of T-cell effector function. It is expressed on activated T-cells and binds to the same surface molecules on DCs as CD28 (i.e., CD80 and 86) but instead of co-stimulation, conveys inhibitory signals to T-cells. CTLA4 plays an important role in tolerance, possibly through inducing T-cell anergy, costimulation of secretion of inhibitory cytokines and involvement in the function of Tregs.110

T-cell tumour infiltration (Figure 3, steps 4 and 5)
Chemokines, which are small, secreted proteins, coordinate homing of immune cells. Chemokines secreted at the tumour site can direct effector immune cells to the tumour, but can also shape the tumour microenvironment into a suppressive one by attraction of suppressive cell types such as MDSCs and Tregs.111 T-cell transmigration through the endothelium of blood and lymphatic vessels to reach the tumour is also impaired. Angiogenic growth factors such as VEGF secreted by the tumour block the expression of adhesion molecules on endothelial cells, thus inhibiting T-cell extravasation and infiltration. Also, endothelial cells can express factors that suppress effector T-cells, such as PD-L1, IL10 and TGFβ.104

Recognition of tumour cells by T-cells (Figure 3, step 6)
As described above, tumour cells can down-regulate their expression of MHC class I or fail to express strong tumour antigens thereby avoiding recognition by the immune system.

Cytotoxic killing of tumour cells (Figure 3, step 7)
A number of immunosuppressive mechanisms can inhibit cytotoxic T-cell function such as immune suppressive cells, surface molecules expressed on tumour cells and a toxic tumour environment containing soluble mediators such as TGFβ, IL10, hydrogen peroxide and other substances that inhibit CTLs.104 Some mechanisms will be discussed in more detail.

T-regulatory cells
T-regulatory cells (Tregs) are regulatory immune cells central to maintaining self-tolerance and preventing autoimmune disease. Tregs also control immune responses against pathogens and allergens as well as the foetus during pregnancy.112 However, Tregs are also enriched in the tumour microenvironment where they inhibit anti-tumour immune responses. Increased levels of Tregs have been described in many solid tumour types113 and also in haematological malignancies including Hodgkin lymphoma114, chronic lymphocytic leukaemia115, acute myeloid leukaemia116, myelodysplastic syndromes117 and
chronic myeloid leukaemia. In most solid tumours, increased Tregs are associated with a worse prognosis, but in haematological malignancies, a more diverse role for Tregs is described as some studies have demonstrated positive effects on survival by the presence of Tregs and yet other studies show quantitative and functional deficits of Tregs. This could be explained by the fact that the haematological malignancies themselves derive from immune cells, making it possible for Tregs to inhibit not only normal immune cells but also malignantly transformed cells.

Tregs can be both CD4+ and CD8+ but the most extensively studied are the CD4+ Tregs, which are defined as CD4+CD25+FOXP3+. FOXP3 is considered the most specific Treg-marker. However, expression of FOXP3 can also be transiently seen in both CD4+ and CD8+ effector cells upon stimulation. There are two main subsets of Tregs, the naturally occurring nTregs which are derived from the thymus and can suppress cells of both innate and adaptive immunity, and the inducible iTregs which develop from naive T-cells in secondary lymphoid organs under suppressive conditions such as tumour microenvironments with high levels of certain cytokines (IL2, IL10, TGFβ) and immature APCs.

Tregs exert their suppressive functions through different mechanisms. Secretion of the inhibitory cytokines IL10, IL35 and TGFβ is the most important mechanism for mediating inhibition of immune effector functions such as cytokine secretion and cytolysis. IL10 and TGFβ also inhibit DC maturation as described above. Another way for Tregs to interact with DC function is by expressing CTLA4 that binds to CD80 and CD86 on DCs. This interaction results in up-regulation of indoleamine 2, 3-deoxygenase (IDO), which has suppressive effects on T-cells. Tregs are dependent on IL2 for their normal function and have a high expression of CD25 that binds IL2. They can thereby deplete the local environment of IL2, leading to inhibition of effector T-cell activation. Further, Tregs also have the ability to directly kill effector T-cells by releasing cytolytic mediators (granzyme, perforin) or through induction of apoptotic pathways in target effector cells.

Myeloid-derived suppressor cells
Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells (myeloid progenitor cells and various immature myeloid cells) with immunosuppressive abilities, especially on T-cell responses. They expand under various pathological conditions such as infection, trauma and cancer due to induction of a block in the differentiation of immature myeloid cells. Expansion of MDSCs has been shown in patients with various solid tumours. Less is known about MDSC expansion in haematological malignancies, but some studies have shown elevated levels in patients with multiple myeloma, non-Hodgkin lymphoma, chronic lymphocytic leukaemia, acute myeloid leukaemia and CML. Also, experiments on
mouse models of haematological malignancies have demonstrated the presence of immature myeloid cells with immunosuppressive functions.\textsuperscript{128}

Human MDSCs have been differently defined by different investigators, but are most commonly defined as lineage-HLA-DR\textsuperscript{-}CD33\textsuperscript{+} or CD11b\textsuperscript{-}CD14\textsuperscript{-}CD33\textsuperscript{+}.\textsuperscript{103} Other markers including IL4R alpha, VEGFR, CD15 and CD66b have been found on some MDSC subsets. In mice, MDSCs are defined as CD11b\textsuperscript{+Gr1\textsuperscript{+}} and monocytic and granulocytic subgroups can be defined by expression of lymphocyte antigen (Ly) 6C and Ly6B, respectively. It has been suggested that monocytic and granulocytic subgroups of MDSCs also exist in humans, defined by their expression of CD14 and CD15, respectively.\textsuperscript{129} MDSC expansion and activation is caused by factors produced by tumour cells, tumour stroma cells and activated T-cells. Expansion is achieved by stimulation of myelopoiesis with subsequent inhibition of myeloid differentiation through persistent activation of the signal transducer and activation of transcription (STAT) 3 transcription factor. Triggering of STAT3 signalling can be caused by factors such as cyclooxygenase (COX)2, prostaglandins, stem cell factor (SCF), M-CSF, GM-CSF, IL6 and VEGF. Activation of MDSCs involves signalling through STAT1 and STAT6 leading to up-regulation of nuclear factor (NF)-kappaB. These signalling pathways can be activated by IFN\textgreek{y}, TLRs, IL4, IL13 and TGF\textbeta.\textsuperscript{103}

Activation of NF-kappaB leads to suppressive activity by MDSCs through up-regulation of the enzymes Arginase-1 and inducible nitric oxide synthase (iNOS). Both Arginase-1 and iNOS metabolise the conditionally essential amino acid L-arginine (L-arg) to produce urea and L-ornithine and NO, respectively. This leads to suppression of T-cells in different ways; depletion of L-arg leads to arrest in the G\textsubscript{0}-G\textsubscript{1} phase of the cell cycle in T-cells and to reduced expression of the CD3\textgreek{z} chain of the T-cell receptor, leading to T-cell dysfunction. The production of NO leads to suppression of T-cells through different mechanisms. Further, under conditions of low L-arg availability, iNOS can produce reactive nitrogen oxide species and reactive oxygen species (ROS) that have numerous suppressive functions such as nitration of proteins by peroxynitrates, leading to T-cell apoptosis.\textsuperscript{130,131} Activation of NF-kappaB also induces production of suppressive cytokines such as TGF\textbeta, which is involved in the induction of Tregs – another immunosuppressive mechanism utilised by MDSCs.\textsuperscript{132} MDSCs can also induce antigen-specific CD8\textsuperscript{+} T-cell tolerance which is an important mechanism for tumour-immune escape.\textsuperscript{133} Yet other mechanisms for immune suppression by MDSCs have been proposed, such as depletion of cysteine from T-cells and down-regulation of L-selectin on T-cells, both inhibiting cell activation.\textsuperscript{134}
CML, Tyrosine kinase inhibitors and the immune system

In newly diagnosed CML patients, signs of both immune activation and immune inhibitory mechanisms have been described. Higher amounts of NKT-like cells and effector memory T-cells have been found in the bone marrow of CML patients\textsuperscript{135} and leukaemia-reactive CD8\textsuperscript{+} T-cells have been found in peripheral blood of HU-treated CML patients before allo SCT.\textsuperscript{136} BCR-ABL junction-specific CD8\textsuperscript{+} T-cells have also been found in treated patients and were more likely to be found in patients with low tumour burden.\textsuperscript{137} However, immunosuppressive mechanisms must also be operating since patients develop clinical disease. Decreased proportions and impaired function of DCs resulting in impaired antigen presentation have been described\textsuperscript{135,138} as well as quantitative and functional defects in the NK cell compartment.\textsuperscript{139} Further, PD1 expression on CD8\textsuperscript{+} T-cells from CML patients as well as increased PD-L1 expression on myeloid cells have been found in peripheral blood of newly diagnosed CML patients.\textsuperscript{127,140} Also, we have previously found increased MDSC levels and Arginase\textsubscript{1} expression in Sokal high risk patients at diagnosis.\textsuperscript{127}

The effects of tyrosine kinase inhibitors on cells of the immune system and on anti-tumour immunity have been studied by many groups. Results so far are inconclusive, but most in vitro studies show immunosuppressive effects of TKIs in cell culture (some examples in \textsuperscript{141-144}). However, In vivo effects of TKI therapy indicate immunostimulatory effects such as large granular lymphocyte (LGL) expansion\textsuperscript{145}, enhanced anti-leukaemia T-cells responses\textsuperscript{146}, promotion of Th1-type immune responses\textsuperscript{147} and normalisation of immune cell profiles.\textsuperscript{148} A possible explanation for these discrepancies is that the continuous exposure to TKIs in culture might be more toxic to immune cells than the varying concentrations seen in vivo. Further, other immune cells might be affected in vivo, bringing about secondary activating immune effects. Chen at al\textsuperscript{146} proposed that imatinib therapy might give a ‘window’ during which anti-tumour immunity can be partially restored while apoptotic CML cells are present and can evoke anti-leukaemia T-cell responses. This was supported by the fact that the immune responses decreased over time, following the decrease in tumour burden.

Further support for the role of anti-leukaemia immunity in controlling disease in CML comes from the experience with allo-HSCT as described above.\textsuperscript{81} The fact that IFN alpha, which has immunological effects, could induce cytogenetic responses and even treatment-free remissions in a small proportion of patients\textsuperscript{149} also points to the importance of the patient’s immune status. Furthermore, in some patients who are stopping TKI treatment as described above, fluctuating low levels of $BCR-ABL$ can be measured\textsuperscript{150}, indicating that the disease is not completely eradicated, but rather being kept in control by
host immunity. Indeed, most potential biomarkers for successful TKI cessation reported so far are related to the patient’s immunological status. Ilander et al. explored the immune status of patients included in the EURO-SKI study and found that patients remaining in TFR after TKI discontinuation had a higher proportion of NK-cells than relapsing patients. Further, the NK cells in the non-relapsing group had a more mature phenotype and had a higher secretion of TNFα and IFNγ cytokines, which can enhance T-cell cytotoxicity. There are also other studies demonstrating the importance of NK cells for TFR after TKI discontinuation, indicating that NK cell-based immune surveillance is of importance for disease control after TKI cessation.

TKI effects on the immune system could have interesting implications other than a role in the therapeutic efficacy in CML. A TKI used for treatment of renal cell carcinoma (sunitinib) has been shown to reduce the presence of immunity-hampering cells through inhibition of STAT3 signalling. The TKIs used for treatment of CML might have similar mechanisms and could also be of potential interest to evaluate for clinical significance in the setting of immunotherapy conditioning.

Flow cytometry and multiplexing

Flow cytometry is used to identify different types of immune cells by their expression of cell surface markers, most often CD markers. Fluorescent-labelled antibodies against these surface markers are used to stain the cells before they are run through the flow cytometer in which laser beams excite the fluorophores that then emit light at different wavelengths. The light is detected in an optical system and translated into distribution patterns for the different surface markers, making it possible to distinguish different cell populations. The major advantage of flow cytometry is its possibility to detect different cell types in heterogeneous samples such as blood with high sensitivity. The major disadvantage is the lack of standardisation regarding both assay and instrument setup, at least in research laboratories, and how data is analysed and reported. Data analysis can be complicated and is done by manual gating, highlighting the importance of being transparent when reporting flow data.

To evaluate signalling in the immune system, measurement of soluble analytes in samples is necessary. Since signalling is dependent on complex networks of cytokines and chemokines, measurement of more than one analyte at a time is desirable. Proteomics is therefore an area of growing interest and the simultaneous measurement of many proteins in experimental samples is now possible using multiplex assays. Multiplexing is used for several purposes, such as surveys of changes in protein abundance, biomarker validation and clinical diagnostics. Many multiplex assays are immunoassays that use high affinity capture ligands and detection antibodies, but the techniques for arranging the ligands and detecting the antibodies vary between assays and
can be based on, for example, electrochemoluminiscence\textsuperscript{160}, flow cytometry\textsuperscript{161} or proximity ligation assay (PEA) techniques.\textsuperscript{162} The major advantage with multiplexing is the ability to analyse multiple proteins in one single sample, which might better reflect biological networks of, for example, cytokine signalling and also reduces the sample volumes needed. Multiplexing, on the other hand, is limited by difficulties of analysing multiple markers of different concentration ranges in a single sample at one dilution and the risk of cross-reactivity when mixing antibodies with different specificities.\textsuperscript{163}

The Swedish CML register

The Swedish CML register was founded in 2002 by the Swedish CML group and the Swedish Society of Haematology and covers a population of 9.5 million people. Clinicians report adult (\textgeq 18 years) patients with newly diagnosed CML to the register. In addition, new cases can be identified from the mandatory Swedish Cancer Register. This dual reporting system gives an estimated coverage in the CML register of \textgreater 95\% of all newly diagnosed adult patients with CML in Sweden, irrespective of disease phase.\textsuperscript{2} Reports are submitted electronically, and data are collected at baseline and follow-up (1, 2, 5 and 10 years after diagnosis). Register reports are made every other year and are available to clinicians.

The register contains an unselected patient material, and several population-based studies have been produced with register data. Two studies have focused on outcome in CML patients. The first study estimated relative survival for all CML patients diagnosed between 1973 and 2008, and demonstrated a major improvement in outcome of patients diagnosed after 2001, primarily due to the introduction of imatinib.\textsuperscript{77} The second focused on outcome in the TKI era (patients diagnosed 2002-2010) and demonstrated that the relative survival was close to 1.0 in patients younger than 60 years, but only 0.6 for those older than 80 years.\textsuperscript{2} Two other papers have explored the prevalence of prior and second malignancies and autoimmune diseases in CML patients. The first of these papers examined the development of second malignancies in patients diagnosed between 2002 and 2011 and found that, compared to expected rates in the general population, the risk of second malignancies was higher in the CML cohort (standardised incidence ratio of 1.52).\textsuperscript{164} In the second paper, CML patients were found to have a higher risk of both malignancies and autoimmune disease compared to the general population prior to their CML diagnosis (odds ratios of 1.47 and 1.55, respectively).\textsuperscript{10} These findings suggest that a hereditary or acquired susceptibility to cancer and autoimmunity might be involved in the pathogenesis of CML. The risk of developing cardiovascular disease during TKI treatment has also been assessed in a register-based study where patients diagnosed in CP between 2002 and 2012 and treated with a TKI were compared to the general population with regard to the
risk of arterial and venous vascular events. The relative risks for arterial and venous events in the CML cohort were 1.5 and 2.0, respectively, and the event rate for myocardial infarction was higher in patients treated with 2G-TKI than in those treated with imatinib. The impact on socioeconomic factors on survival and treatment choice in CML patients has also been explored in a recent paper. After adjusting for baseline factors, socioeconomic factors were not found to influence survival in CML patients diagnosed between 2002 and 2012. However, both education and income were linked to TKI treatment overall and to upfront treatment with 2G-TKIs.
Aims of the doctoral project

The overall aim of this doctoral project is to gain greater knowledge about factors that influence treatment outcome and survival in CML patients.

Specific aims:

Paper I
To evaluate the frequency of musculoskeletal symptoms in patients stopping TKI treatment and to explore the clinical characteristics of these patients.

Paper II
To study the presence of immune escape mechanisms in newly diagnosed CP-CML patients and the effects of TKI treatment on host immunity.

Paper III
To explore potential biomarkers in plasma at CML diagnosis and during TKI treatment which might be of biologic, prognostic or therapeutic significance.

Paper IV
To evaluate how monitoring of CML patients and treatment of advanced phase CML work in a population-based setting.

Paper V
To analyse plasma protein markers before and after TKI discontinuation with the purpose of identifying possible biomarkers for prediction of successful TKI discontinuation and to evaluate effects of long-term TKI treatment on plasma protein profiles.
Materials and methods

Paper I
In Paper I, which is a letter to the editor, we retrospectively evaluated the frequency of musculoskeletal symptoms in a cohort of patients included in the EURO-SKI trial (ClinicalTrials.gov identifier: NCT01596114) after a surprisingly high proportion of patients reported musculoskeletal pain after TKI discontinuation. The study cohort consisted of the first 50 patients included in the study in Sweden and were observed for at least six months (range 6-15 months) after stopping imatinib. Data were collected from patient records on patient and disease characteristics; presence, type and duration of musculoskeletal symptoms; laboratory findings and treatment. The study was approved by the regional ethical board in Lund, Sweden. Data were presented in a descriptive fashion.

Papers II and III
Patient samples
In Paper II and III, we studied patients from the randomised clinical trial NordCML006\textsuperscript{167}, comparing imatinib and dasatinib treatment in newly diagnosed CP-CML patients. In Paper II, samples from 32 patients (18 imatinib and 14 dasatinib treated) were analysed at baseline and after treatment initiation with flow cytometry and plasma analyses for the presence of immune escape mechanisms. In Paper III, which was conducted as a pilot study, plasma samples from a smaller cohort of 14 patients (9 imatinib and 5 dasatinib treated) were analysed with three different multiplex panels. A total of 124 different plasma proteins were measured at baseline and after three months of TKI treatment. Samples were obtained from multiple centres within the Nordic countries and all samples had been cryopreserved and shipped to Uppsala University prior to analyses. Samples from healthy control subjects were used in paper II and were obtained from the blood bank at Uppsala University Hospital. Peripheral blood mononuclear cells (PBMCs) were studied in the flow cytometry analyses and these were obtained by Ficoll gradient separation of peripheral heparinised blood. Citrate plasma was used for analyses.
of plasma proteins. The studies were approved by the medical products agencies and regional ethical boards in the participating countries.

Flow cytometry
Flow cytometry was performed with the LSRII flow cytometer (BD Bioscience). Before antibody staining, unspecific binding was blocked by adding an Fc-receptor blocking reagent. After blocking, cells were stained with specific antibodies (intracellular and extracellular staining) and, to exclude unspecific binding and define negative cell populations, isotype controls were used. The following cell populations were analysed: MDSCs (CD11b+CD14+CD33+; these cells were also stained with a CD40-antibody), NK cells (CD3-CD56/CD16+), CD8+ naïve T-cells (CD3+CD8+CD45RA+CCR7+), CD8+ memory T-cells (CD3+CD8+CD45RA-) and Tregs (CD3+CD4+CD127+FoxP3+). The results were analysed with the Flow Jo software (Tree star).

Plasma analyses
Plasma analyses were done with ELISA, Human pro-inflammatory 9-plex ultra-sensitive kit by Mesoscale, Custom Human Multi-analyte profiling (MAP) by Myriad RBM and Proseek Multiplex oncology 1 by Olink. The ELISA and Mesoscale analyses were performed on site according to the manufacturer’s instructions while samples were shipped to Myriad RBM and Olink for analysis.

All plasma analyses that were used are immunoassays based on antigen capture between a capture and a detection antibody, but the techniques for detecting the antibodies bound to antigens varies between the methods. In the ELISA, an enzyme is linked to the detection antibody and an enzymatic substrate is added that produces a coloured signal upon enzymatic conversion. The colour signal indicates the amount of antigen in the original sample. Mesoscale utilises electrochemoluminescence to quantify proteins in a sample. The detection antibodies are coupled to electrochemoluminscent labels that emit light after addition of a co-reactant followed by electric stimulation. The signal that is emitted corresponds to the amount of protein bound to the capture antibodies. Capture antibodies can be bound to different spots in the bottom of a microtiter plate, making multiplexing possible. Mesoscale utilises electrochemoluminiscence to quantify proteins in a sample. The detection antibodies are coupled to electrochemoluminscent labels that emit light after addition of a co-reactant followed by electric stimulation. The signal that is emitted corresponds to the amount of protein bound to the capture antibodies. Capture antibodies can be bound to different spots in the bottom of a microtiter plate, making multiplexing possible. The technique utilised for antibody detection by Myriad RBM is flow-based. Antigen is captured on fluorescent microspheres and detection antibodies are also fluorescently labelled. When run through a flow cell, both microspheres and antibody-bound fluorophores are excited by lasers and emit light. Each microsphere corresponds to a certain analyte and the amount of light emitted from the antibody-bound fluorophores corresponds to the amount of analyte in the sample. By including multiple microspheres, multiplexing is possible. The technique used by Olink is the proximity extension assay (PEA), in which
pairs of antibodies with a slight affinity for each other are linked to oligonucleotides (PEA probes). When the antibodies bind to their target proteins in a sample, the probes are brought together and extended by a DNA polymerase to form a sequence that acts as a surrogate marker for the target protein. The DNA sequence is quantified by qPCR and the product is proportional to the initial amount of protein in the sample. Also in this type of assay, multiplexing is possible at a high level due to the proximity requirement of the detection antibody-pairs to generate a signal and the use of specific primers in the qPCR.\textsuperscript{162}

Statistical analyses

Student’s $t$-test with Welsh correction for unequal variances was used to determine statistical differences between unpaired groups while Wilcoxon matched-pairs signed rank test was used to determine differences between pre- and post-treatment initiation samples. One-way ANOVA was used to calculate differences between three or more groups and post-testing was then performed with Wilcoxon for matched pairs. Correlation analyses were performed with the Spearman rank correlation test. Independent samples Mann-Whitney $U$ test was used to assess differences in the levels of analytes according to treatment response.

Paper IV

In Paper IV, we used the Swedish CML register to evaluate patients with advanced phase CML (AP or BC) in a population-based setting with regard to progression rates, adherence to guidelines for monitoring and treatment as well as outcome both in CML patients diagnosed in CP and progressing to AP or BC on treatment, and those already in an advanced stage at the time of diagnosis. Two populations were identified from the register: I) CML patients diagnosed in CP between Jan 1\textsuperscript{st} 2007 and Dec 31\textsuperscript{st} 2011 who progressed to AP or BC within 24 months from diagnosis and II) Patients diagnosed in CML AP or BC between Jan 1\textsuperscript{st} 2007 and Dec 31\textsuperscript{st} 2012. Patients diagnosed with CML in CP during the same period and who did not progress to AP or BC were selected as control group. Baseline data from the time of diagnosis were obtained from the CML register. More detailed information on cytogenetic and molecular monitoring during follow-up as well as on treatment and outcome in patients with AP or BC, was gathered by a systematic review of patient records.

Differences in baseline characteristics were tested using the chi-squared test or Fisher’s exact test for categorical data, or the Kruskal-Wallis test for continuous data. The cumulative probability of transformation to AP/BC within two years was estimated with competing risks analysis. Differences in
the probability of transformation were tested univariately using Gray’s test. Overall survival was estimated with the Kaplan-Meier method, and differences between groups were tested using the log-rank test.

Paper V

In Paper V, we used two proteomic panels from Olink (Proseek Multiplex Inflammation I and Oncology II) to study plasma protein expression in a Nordic patient cohort from the EURO-SKI trial (ClinicalTrials.gov identifier: NCT01596114). A total of 162 different proteins were studied in 56 patients before stopping TKIs, at 1 and 6 months after stopping TKIs and at molecular relapse (loss of MMR; $BCR-ABL \geq 0.1\%$). Protein levels were also evaluated in samples from 22 healthy control subjects obtained from the blood bank at Uppsala University Hospital. Frozen citrate plasma samples were used for analyses and the method has been described above. The study was approved by the regional ethics boards in the participating countries.

Statistical analyses were performed on log2 data which were approximately normally distributed. Welch’s unequal variances t-test was used to determine statistically significant differences between groups. Linear Mixed Effects Modelling (LMM) was used to investigate differences between groups over time and the effect of time itself (longitudinal analyses) during the first six months. The mean protein levels were estimated by a linear trend over time, and these linear trends were compared between the two groups. Analyses of the model residuals showed that the model fitted the data well for all the analytes.

All analyses were adjusted for multiple testing using the False Discovery Rate (FDR) method of correction. Since we have previously explored the effects of TKI treatment on plasma protein profiles (Paper III), we used a false discovery rate of 5% for analyses of TKI effects in treated patients. In the mixed effects model, a false discovery rate of 10% was applied since this was considered an exploratory setting and positive results would have to be confirmed in follow-up studies which would reveal any false positives.
Results and discussion

Musculoskeletal pain in patients with chronic myeloid leukaemia after discontinuation of imatinib: A tyrosine kinase inhibitor withdrawal syndrome? (Paper I)

Fifteen patients (30%) reported musculoskeletal pain starting from one to six weeks after TKI discontinuation. Of these, nine were women and six were men and the median age at TKI discontinuation was 62 years. Patients had been treated with imatinib for a median of 9 years. The severity of musculoskeletal pain was designated as 2 on the Common Terminology Criteria for Adverse Events scale (CTCAE, version 4.0) in eight patients and designated as 1 in seven patients. Only minor laboratory abnormalities were noted with marginally elevated C-reactive protein levels in two (out of ten investigated) patients and marginal inflammatory activity on serum protein electrophoresis in three (out of eight investigated) patients. The rate of molecular relapse within the first six months of imatinib discontinuation did not differ between patients with musculoskeletal symptoms and those without. Seven of the patients lost MMR and restarted imatinib therapy.

The symptoms were treated with paracetamol or non-steroidal anti-inflammatory drugs in most cases but in five of the more severely afflicted patients, corticosteroids were given with good effect. In all patients who restarted TKI therapy, symptoms resolved within three months. Among the eight patients remaining off treatment, symptoms fully resolved in two, partially resolved in four and persisted in two. One of the patients with persisting symptoms had persisting pain in the arms and legs after a follow-up time of ten months and the other still had hand arthralgia after a follow-up time of eight months.

No clear explanation for the observed symptoms could be found but we speculate that they might represent rebound phenomena, possibly linked to receptor signalling in off-target kinases.
The Tyrosine kinase inhibitors imatinib and dasatinib reduce myeloid suppressor cells and release effector lymphocyte responses (Paper II)

In Paper II, immune escape mechanisms were studied. The patients included in the study had a median (range) age of 55 (28-73) years and at 12 months, 59% had achieved MMR. We found that the MDSC population significantly decreased when comparing baseline values with values six months post treatment initiation. MDSCs were correlated to WBC at baseline (reflecting tumour burden). Furthermore, the level of Arginase1, an MDSC effector molecule, was also correlated with the level of MDSCs at baseline and decreased significantly after treatment. The expression of the co-stimulatory surface molecule CD40 increased on both MDCSs and monocytes after treatment. However, no correlation was found between the level of MDSCs at baseline and treatment response and there was no difference between imatinib and dasatinib treated patients.

Somewhat surprising, the level of Tregs was increased after six months of TKI treatment. Nevertheless, the level of Tregs at six months was similar to that of healthy controls and the Treg-associated cytokine IL10 was also decreased while Th1 effector cytokine IL12 was increased after treatment. Taken together, this points towards immune activation rather than suppression. The phenotype associated with Tregs can also transiently be seen on activated T-cells.

Further, we found that NK cells increased after one but not six months of treatment, and that naive CD8+ T-cells decreased while experienced effector and memory CD8+ T-cells increased after one month of treatment. Th1 effector cytokines IFNγ and monokine induced by gamma interferon (MIG) were also increased after three months of treatment. Hence, there may be an initial stimulatory effect on the immune system after TKI treatment initiation, as the numbers of experienced T-cells and NK cells increase, likely because of the reduction of suppressive factors. Nevertheless, this effect is lost with time, which may depend on the lack of incitements of immunity activation, as the tumour burden is decreased to undetectable levels in most patients.
Plasma proteomics in CML patients before and after initiation of tyrosine kinase inhibitor therapy reveals induced Th1 immunity and loss of angiogenic stimuli (Paper III)

In Paper III, three different multiplex platforms were used to study plasma protein expression before and after TKI initiation. Analytes that were detectable in fewer than 20% of samples were excluded from further analyses. The Human pro-inflammatory 9-plex ultra-sensitive kit by Mesoscale included nine different analytes and 11% were not detectable. The Custom Human Multi-analyte profiling (MAP) by Myriad RBM contained 44 proteins and 48% of these had measurable concentrations. On the Olink platform, 92 analytes were run, and 89% of these had measurable values. Several analytes were included in more than one kit and in most cases we found similar trends concerning the direction of change post treatment initiation (i.e. increase/decrease/unmeasurable). For a few analytes, however, results from different kits were contradicting. Concentrations of these analytes were generally low in plasma and results were close to the platforms’ limits of detection.

We found that multiple plasma proteins were differentially expressed before and at three months after initiation of TKI therapy. We noted a general pattern of decrease of pro-tumorigenic proteins (VEGF, TGFβ, IL10, CD31, MHC class I polypeptide-related sequence A (MICA)) while some analytes known to be of importance for Th1 and anti-cancer immunity were increased after TKI initiation (IL12, monokine induced by gamma-interferon (MIG), IFNγ). This likely reflects a restoration of normal immune functions after TKI initiation. We also noted a general decrease in angiogenic factors after treatment initiation that could reflect a normalisation of bone marrow angiogenesis. Both these patterns were in agreement with previous findings, supporting the accuracy of the multiplex assays.

We also identified some single plasma proteins that could be of potential interest to study further. E-selectin, which was decreased after treatment initiation, has been associated with the homing and engraftment of LSCs and should be evaluated for its biological and prognostic significance. Carbonic anhydrase IX (CAIX) was increased after treatment and is a tumour-associated antigen in AML. It has not, to our knowledge, been extensively studied in CML and may have prognostic and perhaps also therapeutic value. Urokinase plasminogen activator surface receptor (uPAR), which is expressed on myeloid precursors and has been connected to treatment response in AML, was decreased after treatment and might be of biological and/or prognostic significance. Finally, growth hormone (GH) was decreased after treatment initiation and could be of interest to study further for a possible connection to fatigue as a TKI-related side effect since adults with low GH levels have been shown to be less healthy than age-matched controls.
Advanced phase chronic myeloid leukaemia in the tyrosine kinase inhibitor era (Paper IV)

Population I: During the selected time period, 437 patients with a median age of 63 years were diagnosed in CP. Out of these, 18 patients, all treated with imatinib first line, progressed to AP or BC within 24 months of diagnosis (six within the first six months, seven between months six and twelve and five during the second year). This corresponded to a cumulative progression rate of 4.3% and is in line with findings from clinical trials with more selected patient material. At diagnosis, a higher proportion of progression patients were EUTOS high risk as compared with non-progression cases, supporting the validity of the EUTOS prognostic score for TKI-treated patients. Patients that were later progressing had higher blast cell counts at diagnosis as compared with those who remained in CP. No additional early warning signs were identified and four patients even reached CCyR or MMR prior to transformation. We observed that one third of patients later progressing to AP or BC had not been monitored according to guidelines, but in only four cases, no obvious cause for the inadequate monitoring could be found, suggesting that the decentralised care of CML patients in Sweden does not negatively influence adherence to guidelines for monitoring of CML patients. Rather, it seems like even though patients are carefully monitored and in some cases even achieve good treatment responses, progression cannot be avoided in a proportion of patients with current strategies for risk stratification, monitoring and treatment.

Patients were treated according to national guidelines with disease stabilisation with TKIs and/or chemotherapy and subsequent allo-HSCT in all patients under 65 years of age (except for one patient in AP who responded well to TKI treatment). Taken together, the median survival after transformation was 1.4 years for AP and BC patients.

Population II: 36 patients were diagnosed in AP or BC (20 AP and 16 BC) and 544 were diagnosed in CP during the same period. As expected, there were significant differences between the groups in all peripheral blood counts, spleen size and percentage of blast cells in bone marrow. Additional chromosomal aberrations (ACAs) at diagnosis were more common in AP and BC cases as compared to those in CP. Also in this population, patients were generally treated according to guidelines. In patients diagnosed in AP, allo-HSCT was performed in seven cases (median (range) age 32 (23-51) years). Seven patients aged 65 years or younger were not transplanted. Out of those diagnosed in BC, ten patients (median (range) age 39.5 (20-55) years) underwent allo-HSCT. Only two BC patients ≤65 years were not transplanted. Median survival in the BC group was poor (1.6 years) while survival in the AP group was better than expected with a 2-year survival rate of 80.0%.
Proximity extension assay-based plasma proteomics cannot predict relapse in chronic myeloid leukaemia patients stopping treatment with tyrosine kinase inhibitors (TKI) but reveal profound effects of long-term TKI treatment on plasma protein profiles (Paper V)

The patients included in the study (n=56) had a median (range) age of 62.1 (23.5-83.7) years at TKI discontinuation. The TKI treatment duration was 6.1 (3.1-12.3) years. At TKI discontinuation, 42 patients were treated with imatinib, 11 with dasatinib and three with nilotinib. After a follow-up of 22.9 (7.3-38.5) months, 32 relapses had occurred. Twenty-four of these occurred within the first six months (early) and eight thereafter (late). At relapse, all patients were re-started on TKI treatment. At the end of follow-up, all relapsing patients had regained MMR and all but two had regained MR^4.0.

At baseline, immediately prior to TKI discontinuation, no differences in protein levels could be found between patients who later relapsed and those who remained in TFR. However, when analysing data over time after TKI discontinuation with the LMM approach, some differences between groups could be seen. The analytes that differed between relapse and non-relapse cases were leukaemia inhibitory factor receptor (LIF-R), colony stimulating factor-1 (CSF1), transforming growth factor beta receptor type 2 (TGFR-2), stem cell factor (SCF) and folate receptor (FR) alpha. A difference between group means could be seen over time, with non-relapse cases having higher group means for all significant analytes. However, no clear cut-off value separating relapse and non-relapse cases could be found at one or six months.

Since most relapses after TKI discontinuation occur during the first six months, we were interested in finding possible biological differences between early and late relapses. Results from baseline and month 1 for patients who later relapsed were therefore compared between the two groups but no differences could be seen.

Results from healthy controls were compared to patients’ baseline values. In total, 87 proteins were significantly different between the two groups (adjusted p-values <0.05). A majority of the significantly different proteins were higher in patients than in controls. Since all patients at baseline were in deep molecular remission (MR^4.0 or better), observed differences could probably be attributed to effects of TKI treatment rather than disease. However, even though affecting partly different signalling pathways, only a few proteins were significantly different between imatinib- and dasatinib-treated patients. Analytes that differed between patients and controls were of several different protein classes such as immunological cytokines, chemokines and receptors, haematopoietic growth factors, angiogenesis factors, adhesion proteins, and proteins related to cell proliferation and apoptosis. This indicates that the off-
target kinase effects of TKI treatment have profound effects on many signalling pathways. Interestingly, after TKI discontinuation, a significant change over time during the first six months could only be seen for 37 proteins and the effects of TKI treatment thus seem to be long-lasting.
Conclusions

Paper I
In a Swedish cohort of CML patients stopping TKI treatment, 30% reported musculoskeletal pain starting within the first six weeks of TKI discontinuation. In 53% of affected patients, symptoms were severe enough to interfere with everyday activities. Although resolving spontaneously or with use of anti-inflammatory medication in most cases, symptoms prevailed in a small proportion of patients. Clinicians should be aware of the possible occurrence of side effects of TKI discontinuation and underlying mechanisms need to be investigated.

Paper II
Newly diagnosed CML patients have evidence of active immune escape mechanisms that decline with the course of TKI treatment. At the same time, effector lymphocyte responses are elicited, especially in the beginning of treatment, before the tumour burden has diminished. It is possible that these anti-leukaemia immune responses can help in the long term control of the disease, for example upon TKI discontinuation.

Paper III
Using multiplex techniques for the simultaneous measurement of many plasma proteins in one sample seems feasible in CML and might prove useful for finding potential biomarkers or patterns of markers with biologic or prognostic significance.

Paper IV
In a population-based setting and following first line imatinib therapy, approximately 4% of CP-CML patients transform to AP or BC within two years of diagnosis, which is comparable with what has been reported in clinical trials. Patients with either CML BC at diagnosis or during treatment with TKIs, as
well as those developing AP on TKI therapy, have a poor prognosis. Strict adherence to guidelines, with respect to cytogenetic/molecular monitoring of TKI treatment, is important for early identification of poor responders, but some progressions seem inevitable with current strategies of risk stratification, monitoring and treatment.

Paper V

Profound and long-lasting effects of TKI treatment were seen on plasma protein profiles in CML patients discontinuing TKI treatment but only a few differences in protein levels were seen between non-relapsing and relapsing cases. No predictive protein biomarkers for successful TKI discontinuation could be found. It could be of interest to confirm the differences between relapse and non-relapse cases in an experimental setting or in a bigger cohort of patients but it is likely that plasma protein markers alone are not sensitive enough to predict molecular relapse. It is plausible that several different clinical and biological factors will need to be combined for the best prediction of which patients will benefit most from TKI cessation attempts.
Future perspectives

Although the advent of TKIs have revolutionised the treatment of CML and drastically improved prognosis, some challenging matters still remain in the field of CML research. For patients responding well to TKI treatment, expected survival rates are now close to the general population, whilst patients not responding optimally to treatment or progressing to advanced disease phases, have a much more dismal outcome. To date, several risk factors for responding sub-optimally to treatment or progressing to AP/BC have been identified, but still some patients progress without any previous warning signs. With modern techniques for gene sequencing such as next generation sequencing (NGS), genetic aberrations not found on conventional karyotyping can be explored. This is currently being done on diagnostic samples from the patients in Paper IV in this thesis with the aim of finding genetic aberrations possibly contributing to progression to advanced disease stages.

By contrast, a proportion of CML patients treated with TKIs respond very well to treatment and TKI cessation with durable TFR has proven possible in a proportion of these patients. Both clinical and biological factors of importance for successful TKI stop have been identified, but still there is a lack of easily measurable, predictive biomarkers. In Paper V of this thesis, a few plasma analytes were found to be of potential significance for TFR. These results would need to be confirmed in a larger cohort of patients and in a mechanistic setting. Perhaps several different factors will have to be combined to be able to successfully predict successful TFR and it would be interesting to explore the possibility of creating an algorithm for this.

Clinically, the pursuit of reaching deeper treatment responses needs to continue. Deep molecular response is a prerequisite for successful TFR and prevents progression to AP/BC to a large extent. Clinical studies combining TKIs with other agents, such as immunotherapy or agents targeting LSCs, are warranted. A study combining the TKI bosutinib with IFN alpha is planned within the Nordic CML Study Group.

I hope to continue working with academic studies concerning CML within the Swedish and Nordic CML collaboration groups.
Svensk populärvetenskaplig sammanfattning

Varje år insjuknar omkring 90 personer i Sverige i kronisk myeloisk leukemi (KML). KML uppstår i primitiva blodbildande celler i benmärgen genom en genetisk translokation där genen ABL flyttas från kromosom 9 och hamnar bredvid genen BCR på kromosom 22 varvid en ny gen, BCR-ABL1, bildas. 

BCR-ABL1 kodar för ett tyrosinkinas som ger signaler till de blodbildande cellerna att delar sig oftare vilket leder till ansamling av vita blodkroppar i olika mognadstadiers i benmärg, blod och mjälte. Sjukdomen utvecklas relativt långsamt och i sitt naturalförlopp kan den indelas i tre sjukdomsfasers: kronisk fas, accelererad fas (AP) och blastkris (BC). Den kroniska fasen varar under flera år och karakteriseras av en ökad bildning av vita blodkroppar som fortfarande kan mogna ut normalt. De allra flesta patienter är i kronisk fas vid diagnos. Den accelererade fasen karakteriseras av en ökad genetisk instabilitet i de sjuka cellerna och en ökad andel omogna vita blodkroppar i benmärg och blod och varar ofta i några månader. 

Sjukdomen går sedan över i blastkris vilken liknar akut leukemi med ytterligare ökning av omogna vita blodkroppar som delar sig snabbt. Några få procent av patienterna är i AP eller BC vid diagnos. Med modern behandling är det ovanligt att sjukdomen går över i AP eller BC.

Sedan början av 2000-talet har prognosen vid KML förbättrats drastiskt i och med upptäckten av de så kallade tyrosinkinashämmarna (TKI). Överlevnaden, åtminstone bland yngre KML-patienter, är nu ungefär den samma som i normalbefolkningen. TKI har aktivitet mot det sjukdomsalstrande tyrosinkinaset BCR-ABL och gör så att cellerna slutar dela sig. Däremot kan behandlingen inte helt ta bort de allra mest primitiva sjukdomsalstrande cellerna i benmärgen och har fram till nyligen betraktats som livslång. Dock har studier på senare år ändå visat att en andel av patienter som behandlats under flera års tid och uppnått mycket gott behandlingssvar (mycket låga eller omätbara nivåer av BCR-ABL1 i blod) kunnat sluta med sin behandling utan att få tillbaka sjukdomen. Hos de som slutat med sin behandling och fått tillbaka sjukdomen, har behandlingen startats igen med god effekt. Idag vet vi en del om vad som påverkar sannolikheten för att man ska kunna sluta med behandling utan att sjukdomen kommer tillbaka men mer forskning kring detta behövs för att vi bättre ska kunna välja ut vilka patienter som har störst chans till varaktigt behandlingssvar efter utsättning av TKI.
På senare år har immunsystemets roll vid cancersjukdomar varit i rampljuset och ett flertal lyckade immunoterapier har tagit plats i klinisk rutinsjukvård. Immunsystemet kan spela dubbla roller vid cancer. Dels kan immunsystemets celler känna igen och döda cancerceller, dels kan cancercellerna forma miljön i en tumör så att den blir hämmande för immunförsvaret. Immunhämnning i tumörer uppnås genom frisättning av substanser med immundämpande egenskaper och genom ansamling av hämmande immunceller som vi har normalt i kroppen för att hindra att immunsystemet blir överaktivt. Exempel på hämmande celltyper är regulatoriska T-celler och myeloida suppressorceller. 

Vid KML har man visat att immunsystemet är aktivt vid diagnos men också att immunhämnande mekanismer förkommer hos obehandlade patienter. TKI-behandling har visats påverka immunsystemet hos patienter framför allt på ett stimulerande vis med bland annat förstärkning av immunsvaret mot KML-cellerna och ökning av immunstimulerande ämnena i blodet.

I den här avhandlingen har vi i fem olika delarbeten tittat närmare på faktorer som påverkar behandlingssvar och utgång för KML-patienter med fokus på immunförsvarets roll och den kliniska handläggningen. I delarbete I studerades patienter från en stor Europeisk studie där KML-patienter som behandlats med TKI under många år fick sluta med sin behandling under kontrollerade former (EURO-SKI-studien). Efter utsättning av behandlingen noterade vi något oväntat att många patienter klagade på värk i kroppen och vi gjorde därför en noggrann uppföljning av de 50 första svenska studiepatienterna avseende dessa symtom. Vi fann att 30% av patienterna hade upplevt värk i kroppen med debut 1-6 veckor efter att de slutat med sin medicinering. Värken var sällan förknippad med någon mätbar inflammatorisk aktivitet i blodprover och det var ingen skillnad i hur många som fick återfallet i sin sjukdom mellan dem som hade värk och dem som inte hade det. Hos de allra flesta gick värken över av sig själv, men en del behövde behandling med inflammationsdämpande läkemedel och två av patienterna hade fortfarande besvär vid studiens slut. Mekanismerna bakom värken är oklara men vi tror att det kan ha att göra med ett ”rebound”-fenomen då TKI påverkar olika signalvägar även i friska celler. I delarbete II och III studerade vi patienter från en nordisk studie som jämförde behandling med två olika TKI hos nydiagnostiserade patienter i kronisk fas. Vi tittade på förekomsten vid diagnos av immunhämnande mekanismer och vad som hände med dessa efter behandling. Vi tittade också på en stor mängd olika plasmaproteiner med koppling till bland annat immunförsvaret vid diagnos och efter behandling. Vi fann att TKI-behandling gav en initial immunstimulering med ökning av så kallade erfarna T-celler, NK-celler och immunstimulerande plasmaproteiner och samtidig minskning av immundämpande plasmaproteiner och myeloida suppressorceller. Effekten avtog med tid, sannolikt i takt med att sjukdomsbördan minskade. I delarbete IV studerade vi den kliniska handläggningen av patienter med AP och BC (avancerad sjukdom). Vi använde oss av det svenska KML-registret, i vilket
mer än 95% av alla KML-patienter i Sverige finns registrerade, för att identifiera dels patienter som diagnosticerats med avancerad sjukdom, dels gått över i AP/BC under pågående TKI-behandling. Vi gjorde en detaljerad genomgång av patientjournaler för de patienter som haft AP/BC med avseende på uppföljning och behandling. Vi fann att 7% av alla patienter under studieperioden diagnosticerades i AP eller BC. Av dem som diagnosticerats i kronisk fas var det 4% som gick över i avancerad sjukdom under pågående TKI-behandling. Hos en tredjedel av dessa fann vi bristande uppföljning av behandlingsresultat av olika skäl. Det är möjligt att noggrannare uppföljning i några av dessa fall hade kunnat förhindra övergång i avancerad sjukdom. Behandlingen av AP/BC skedde i enlighet med nationella riktlinjer men trots detta var medianöverlevnaden endast 1,4 år för patienter med AP/BC som utvecklats under pågående behandling och 1,6 år för patienter i BC redan vid diagnos. Den enda gruppen som hade en god prognos var de som diagnosticerats i AP där 2-årsöverlevnaden var 80%. Således är prognosen vid avancerad sjukdom mycket dyster trots modern behandling och det är därmed av yttersta vikt att i möjligaste mån förebygga övergång i avancerad sjukdom genom noggrann uppföljning och behandling enligt riktlinjer. I delarbete V analyserade vi patientprover från EURO-SKI-studien med en så kallad multiplex-panel där ett stort antal plasmaproteiner kan mätas på samma gång i ett prov. Vi analyserade totalt 162 olika plasmaproteiner hos 56 patienter före, 1 och 6 månader efter utsättning av TKI-behandling samt vid sjukdomsåterfall. Huvudsyftet var att undersöka om vi kunde hitta några skillnader i plasmaproteinnivåer mellan patienter som fick återfall efter att de slutat med sin behandling och de som inte fick återfall men vi ville också undersöka hur långvarig behandling med TKI påverkar proteinnivåerna i plasma. Vi fann inga plasmaproteiner som skilde sig mellan patientgrupperna innan utsättning av behandling och bara ett fåtal som skilde sig mellan grupperna när vi följde dem över tid efter utsättning av behandling. Däremot fann vi att det var stora skillnader i proteinnivåer mellan behandlade patienter och friska kontroller. Totalt 87 olika proteiner av flera olika typer (inflammationsmarkörer, tillväxtfaktorer, ämnen som stimulerar blodkärlsbildning och faktorer som reglerar celldelning och programmerad celldöd) var påverkade hos de behandlade patienterna och skillnaderna kvarstod för många av proteinerna även efter att behandlingen satts ut. Således verkar man inte kunna använda sig av plasmaproteinmarkörer för att förutsäga återfall vid utsättning av TKI-behandling men man skulle kunna använda sig av dem för att bättre förstå effekter på kroppen av långvarig TKI-behandling.
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